The Ly-4^+ T lymphocyte subset in the host immune response to the asexual stages of Plasmodium chabaudi chabaudi

Volume I

by

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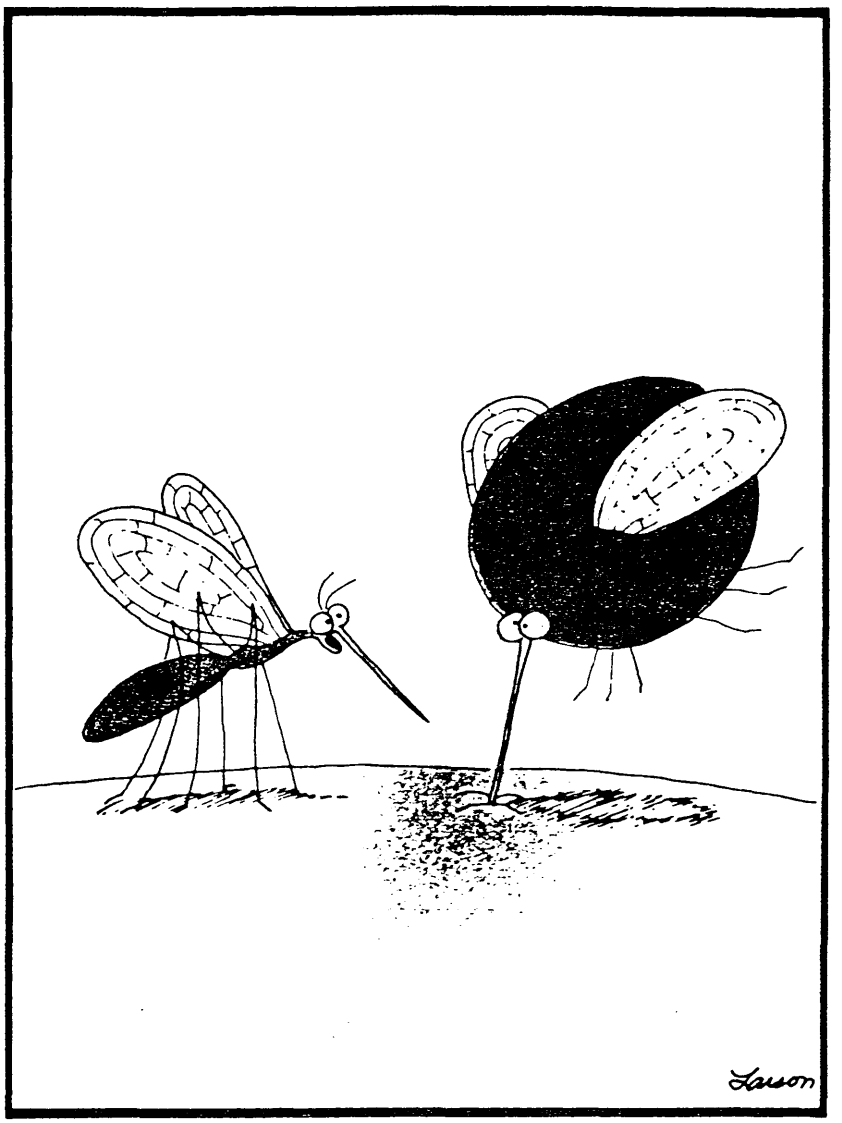
Dedication

To Lisa.

I love you.

I can't dance to a cold machine.



"Pull out! Pull out! . . . You've hit an artery!"

'Even where the milder zone afforded man
A seeming shelter, yet contagion there,
Blighting his being with unnumbered ills,
Spread like a quenchless fire; nor truth availed
Till late to arrest its progress, or create
That peace which first in bloodless victory waved
Her snowy standard o'er this favoured clime'

Shelley, The Daemon of the World

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Declaration

I declare that this thesis is my own composition and that the research described herein was performed entirely by myself except where expressly stated.

Andrew W. Taylor-Robinson

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Abbreviations

Ab Antibody; ADCC Antibody-dependent cellular cytotoxicity; Ag Antigen; ALS Anti-lymphocyte serum; APC Antigen-presenting cell; ATS Anti-thymocyte serum; Auto IL-2 Autocrine interleukin-2; B^+ & B^- P. falciparum binding phenotypes; B cell B lymphocyte; BCG Bacille Calmette Guérin; BP Blood passage; Bq Becquerel; BSA Bovine serum albumin; ^oC Degrees celsius; Ci Curie(s); CMI Cell-mediated immunity; ^{60}Co Cobalt 60; CO_2 Carbon dioxide; Con A Concanavalin A; cpm Counts per minute; d Day(s); d Dead/died; DTH Delated-type hypersensitivity; E. coli Escherichia coli; EBV Epstein Barr virus; EDTA Ethylenediamine tetraacetic acid; Fab Antigen-binding fragment of immunoglobulin; Fc Crystalline fragment of immunoglobulin; FCS Foetal calf serum; Fig. Figure; FITC Fluorescein isothiocyanate; g Acceleration in the earth's gravitational field; g Gramme(s); G Gauge; G6PD Glucose-6-phosphate dehydrogenase; GM-CSF Granulocyte-macrophage colony-stimulating factor; GUP Glasgow University Protozoology (prefix used to describe numbered batches of stabilate); H-2 Mouse major histocompatability complex; Hb Haemoglobin; HbA Adult haemoglobin; HBF Foetal haemoglobin; HbAS Heterozygous for the sickle cell gene; HbSS Homozygous for the sickle cell gene; HCH Hexachlorocyclohexane; HIV Human immunodeficiency virus; HLA Human histocompatability leucocyte antigens; H_2O_2 Hydrogen peroxide; hr Hour(s); HRP Histidine-rich protein; ICAM-1 Intercellular adhesion molecule-1; IFA Indirect fluorescent antibody; IFAT Indirect fluorescent antibody test; IFN Interferon; IFN-γ Interferon-gamma; Ig Immunoglobulin; IL Interleukin; IMDM Iscove's modified Dulbecco's medium; Ir Immune response; i.p. Intraperitoneally; i.u. International unit; i.v. Intravenously; K^+ Potassium cation; K^+ & K^- P. falciparum knobby and knobless phenotypes; KAHRP (HRP1) Knob-associated histadine-rich protein; kD Kilodalton(s); l Litre(s); LPS Lipopolysaccharide; M Molar; MAb Monoclonal antibody; MEM Minimal essential medium; MHC Major histocompatability complex; mg Milligramme(s); µg Microgramme(s); min Minute(s); ml Millilitre(s); µl Microlitre(s); mm Millimetre(s); m.o.i. Multiplicity of infection; MSP Merozoite surface protein; MW Molecular weight; N_2 Nitrogen; ng Nanogramme(s); nm Nanometre(s); NMS Normal mouse serum; NO Nitrogen/nitric oxide; NO_2^- Nitrite anion; nRBC Normal/uninfected red blood cell; NRS Normal rat serum; NS Normal serum; 0_2 Oxygen; 0_2^- Superoxide anion; $0H^\cdot$ Hydroxyl radical; PABA Para-aminobenzoic acid; PBS Phosphate buffered saline; PBMC Peripheral blood mononuclear cell; Pf EMP P. falciparum erythrocyte membrane protein; p.f.u. Plaque-forming unit; PHA Phytohaemagglutinin; p.i. Post infection; PMMSA Precursor of the major merozoite antigen; pRBC Parasitised/infected red blood cell;

RBC Red blood cell; RESA (Pf155) Ring-infected erythrocyte surface antigen; RNA Ribonucleic acid; RT Room temperature; s Second(s); S.D. Standard deviation; SICA Schizont-infected cell agglutination test; S/N Supernatant; T cell T lymphocyte; Tc T cytotoxic lymphocyte; TCA Trichloroacetic acid; Th T helper lymphocyte; TNF Tumour necrosis factor; TNP Trinitrophenol; TNS Tumour necrosis serum; Ts T suppressor lymphocyte; u.v. Ultra violet; v/v Volume per volume; WBC White blood cell; WEP Wellcome Experimental Parasitology (prefix used to describe numbered batches of stabilate); WLEP Wellcome Laboratories for Experimental Parasitology; WHO World Health Organisation; w/v Weight per volume; κ Kappa (Fc light chain); θ Macrophage; > Greater than; < Less than; % Percentage point(s).

Summary

T cells play a major role in acquired immunity to the asexual erythrocytic stages of malaria parasites. In different host/parasite combinations there is evidence that human $CD4^+$ lymphocytes or their murine equivalent, $Ly\text{-}4^+$, can act as helper cells in the production of protective Ab and also mediate cellular protective functions. The details of how the effector mechanisms operate *in vivo*, however, are not understood clearly. There is indirect evidence supporting an important role for Ly-4-bearing lymphocytes in the protective immune response to *Plasmodium chabaudi chabaudi* AS strain, a good animal model of *P. falciparum* infection. Experiments were performed to examine the nature of the $Ly\text{-}4^+$ response to this parasite both *in vivo* and *in vitro* in order to characterise the cells responsible for the mediation of protective activity.

Initial studies showed that during the course of a primary infection of *P. c. chabaudi* AS, there was a marked transient lymphocytosis in the peripheral blood, which occurred at a time just after peak parasitaemia (d 12-13 p.i.). The adoptive transfer to syngeneic NIH recipients of either peripheral blood or splenic lymphocytes taken from donors at this early stage of infection conferred protection against homologous challenge. This was manifested in both competent and sublethally irradiated recipients as a reduced level and quickened remission of primary parasitaemia, and as a more rapid clearance of pRBC from the blood stream, compared to control mice receiving unprimed lymphocytes. Although it was possible to transfer immunity with preparations enriched for either T or B cells, optimal protection was conferred by an unfractionated population containing both lymphocyte phenotypes, suggesting that there was a degree of synergistic activity between parasite-primed T and B cells in the control of malarial infection. This concept was supported further by examination of serum Ab titres for recipients of semi-immune T, B or T & B spleen cells. In each instance, the level of specific anti-*P. c. chabaudi* AS Ig reached a peak between d 31-33 p.i., at or just prior to recrudescence, but the highest titres were recorded for recipients of a mixed splenic population. Since serum Ig levels were quite low during the first wave of patent parasitaemia, it suggested that resolution of acute infection was achieved largely through Ab-independent mechanisms of immunity. This correlated well with a significantly quicker remission of primary parasitaemia observed in sublethally irradiated recipients of semi-immune T cells, compared to similarly treated mice receiving the same inoculum size of either B or T & B cells.

To dissect further the protective immune response in this model, splenic T lymphocytes were taken from P. c. chabaudi AS strain-infected NIH mice on d 16 and d 20 of primary infection and after resolution of secondary and tertiary infections, and each of these preparations established as cell lines in vitro using a lysed extract of pRBC as the source of antigenic stimulation. All four lines were maintained in long term culture and all proliferated specifically in response to P. c. chabaudi AS Ag processed and presented by syngeneic APC. It was shown that recognition of the APC/Ag complex by T cells was an MHC class II-restricted phenomenon, each cell line requiring APC of compatible H-2 haplotype for an in vitro proliferative response. By using surface immunofluorescence and the complement-mediated cytotoxicity assay, each line was characterised phenotypically as Ly-4$^+$, i.e. belonging to the helper/inducer T cell subset.

In vivo, adoptive transfer of each Ly-4$^+$ line was effective in conferring protective immunity to naive and to immunocompromised mice. This was demonstrable, compared to controls given naive T and/or B cells, as both a reduced level and shortened duration of primary parasitaemia, and as a quicker parasite elimination. Inoculation of the P. c. chabaudi AS-reactive lines into non-immune mice challenged with genotypic or phenotypic variant pRBC indicated that there was a strain-specific element of the immunity transferred. Although mice were able to control infection with heterologous parasites, the greatest protection was conferred against challenge with the homologous pRBC to which the lines had been raised. For the two Ly-4$^+$ lines taken from reinfected mice, the protective activity against P. c. chabaudi AS challenge upon adoptive transfer into adult-thymectomised, irradiated and bone marrow-reconstituted mice was improved significantly by the cotransfer of additional naive B cells. This suggested that these Ly-4-bearing lymphocytes act by Ab-mediated mechanisms in vivo. In contrast, the immune response of the two lines collected from primary P. c. chabaudi AS infection was similar in the presence or absence of B and/or other non-reactive T cells. It appeared that these lines may be B cell-independent in their reactivity in vivo. The divergent patterns of behaviour attained in vivo suggested a functional heterogeneity between the Ly-4$^+$ lines.

Cloning by limiting dilution was performed for one line of each pair and the monoclonal populations shown to have a similar protective activity in vivo as the parent lines. The availability of P. c. chabaudi AS-specific Ly-4-bearing lymphocyte clones enabled investigation at the clonal level of the cellular properties underlying the varying immune responses observed in vivo. Assays were performed in vitro for lymphokine

secretion and for helper T cell function. The results demonstrated that the Ly-4$^+$ lymphocyte response to P. c. chabaudi AS pRBC is heterogeneous, in that distinct functions can be performed by different responding T cells. The reactivity in vitro of the lines and clones generally supported the concept of two functionally distinct Ly-4$^+$ subsets. The populations shown to be B cell-independent in vivo secreted high levels of IL-2 and IFN-γ upon antigenic stimulation in vitro, whilst the B cell-dependent cells produced IL-4 and acted as effective helper cells for Ab production in vitro. This suggests that these Ly-4$^+$ lymphocytes fall into the two proposed helper T cell subsets, T_H1 and T_H2, respectively. The differential synthesis of certain lymphokines appeared to correlate with the functional dichotomy between the various lines and clones to initiate humoral or cell-mediated immunity. It was noted that it was those lines derived at an early stage of primary P. c. chabaudi AS challenge that had the effector repertoire of the inflammatory-type T_H1 Ly-4$^+$ subset. Moreover, those lines taken after resolution of further infections displayed the helper activity for specific Ab production characteristic of T_H2 cells of the Ly-4$^+$ phenotype. This sequential appearance during the course of infection of T_H1, then T_H2 lymphocytes specific for P. c. chabaudi AS-infected RBC supported the hypothesis that for this malaria parasite, early resolution of parasitaemia may be Ab-independent but that the mechanism of final clearance coincides with the appearance of helper cells and specific Ig.

Using the same P. c. chabaudi AS/NIH mouse system, selective depletion of T cells using rat MAbs to murine Ly-2 and Ly-4 determinants revealed that the Ly-4-bearing population of lymphocytes is critically required for protection against erythrocytic infection. Mice lacking a Ly-4$^+$ cell compartment suffered very high primary parasitaemias which they could not reduce below 18% for at least 60 d p.i.. In contrast, the removal of Ly-2-bearing lymphocytes from mice challenged with P. c. chabaudi AS did not affect parasitaemias significantly and they were able to clear infection with similar kinetics as immunocompetent control animals. Mice rendered Ly-4$^+$ cell-deficient by adult thymectomy and anti-Ly-4 MAb treatment could have their ability to control blood stage infection restored by adoptive transfer, at the time of challenge, of the protective cell lines, but not of a similar number of naive splenic T cells. This demonstrated unequivocally the crucial role played by P. c. chabaudi AS-specific Ly-4$^+$ lymphocytes in the protective immune response to the asexual erythrocytic stages of this malaria parasite.

CHAPTER ONE

INTRODUCTION

1.1 Historical perspective

Malaria is an ancient human affliction, with references in Egyptian hieroglyphics and in the Hindi vedic literature. Although Hippocrates was the first to describe the clinical disease, it was not until just over a century ago that the causative agent was discovered. Fortunately, discovery of a treatment was not so long delayed; quinine, a specific cure for the recognisable syndrome of clinical malaria, as opposed to other fevers, has been available for centuries. Quinine is a basic alkaloid isolated from the bark of the tree Cinchona legenaria. The cinchona bark was so named due to the spu rious association with the malarious wife of the Spanish Viceroy of Peru, the Countess of Chincon, who, in 1629, was cured of the disease by treatment with powdered bark (Haggis 1941). On her return to Spain, knowledge of the beneficial properties of the bark went with her, and it was subsequently imported into Europe by Jesuits as Cardinal's powder (Gramiccia 1987).

From early times, malaria, or 'ague' (blight) as it was originally called, was associated with marshes, the breeding ground for the mosquito vector. This association is reflected in the old names, marsh fever or paludism (from the French word for 'marsh'), whereas malaria is a relatively recent name, its first use being attributed to Horace Walpole in 1740. Malaria is a corruption of the Italian 'mal aria' meaning 'bad air' and reflects a traditional view that the agent responsible for the disease wafted in the noxious effluvia emanating from these wet regions.

At its zenith, malaria transmission occurred over most of the inhabited world. With such a long human association and wide geographical distribution, malaria has affected most civilisations and human populations, causing incalculable morbidity and mortality. The agricultural and economic development of many nations has been greatly influenced by the disease, as has world history as a whole, especially at times of large population movements and during warfare (documented for Europe by Bruce-Chwatt & De Zulueta 1980).

Another consequence of the long established and potentially fatal host-parasite interaction is the effect malaria has had on the human genome. Several RBC phenotypes have been described in which the heterozygous condition appears to confer some protection against early death and is balanced against a lethal homozygous phenotype (reviewed by Pasvol & Wilson 1982, Weatherall 1987, Nagel & Roth 1989). The concept of balanced polymorphism arose from Haldane's consideration of the probable impact that malaria has had in maintaining sickle-cell anaemia in Africa (Haldane

1949). In the Western World, however, the debilitating impact of these common and widespread genetic diseases is far more important than malaria itself.

Throughout time, malaria has had a role to play in war, most notably during World War II. For the U.S. Army campaigning on the Pacific front, there were as many losses to this one disease as there were casualties of battle, an event repeated in Vietnam a generation later. Such was the crippling effect of malaria on the U.S. war effort that it was to determine anti-malarial strategy for almost two decades. After the remarkable initial success of the spraying of the residual insecticides dichloro diphenyl trichloroethane (DDT) and hexachlorocyclohexane (HCH) and of the newly available safe blood schizonticidal drugs in dramatically curbing the transmission of the disease, in 1955 the WHO initiated a policy of global malaria eradication. Large scale spraying of insecticides, the mainstay of the programme, contributed to the subsequent elimination of malaria for an estimated 400 million people and eradicated the disease in most temperate regions (Nogeur et al 1978). Unfortunately, in the last 15 years there has been an alarming resurgence of malaria, with a 2.3-fold increase in prevalence, and it now appears that the potential for transmission may be close to its original level (Bruce-Chwatt 1979). Today, malaria is still the most important infectious disease in the world, remaining endemic in 102 countries, placing over half the world's population at risk (Tropical Diseases Report 1989). There are an estimated 110 million malaria infections each year (WHO 1990) and one or two million deaths, mostly of infants and children (Anonymous 1975, WHO 1990).

The overly optimistic notion that malaria could be eradicated primarily by DDT spraying limited enthusiasm for basic biomedical research in malaria during the 1950s and 1960s. The apparent imminent demise of a once important disease removed the necessity for training scientists in malariology. It took 10 more years and the Vietnam war to halt this trend. The renaissance of malariological research occurred at a time when there was a gradual extension of resistance in mosquitoes to insecticides and in parasites to drugs. Attention in the 1970s was paid primarily to detecting and testing new chemical formulations for insecticidal or parasiticidal properties. The intensification of research demanded ever increasing supplies of plasmodia, particularly those that infect man, emphasising the need for reliable in vitro cultivation techniques. In 1976, the asexual erythrocytic cycle of Plasmodium falciparum was successfully cultured (Trager & Jensen 1976, Haynes et al 1976), representing a major breakthrough in facilitating the study of the parasite under laboratory conditions.

Various recent technological achievements have revolutionised malaria research. They include hybridoma fusions, which have provided powerful reagents in the form of MAbs; and new techniques in protein chemistry and recombinant DNA technology. Basic research aimed at an improved understanding of the biology and immunology of malaria was intensified in the 1980s in the hope that potent malaria vaccines would soon be developed. At present, Ags from different stages of the parasites are being identified and isolated with the aim of determining immunogenic epitopes for multivalent synthetic vaccine development. The Plasmodium genome is being probed, not only in an attempt to understand the regulation of parasite macromolecular synthesis, but also with the aim of applying recombinant DNA methods in vaccine production.

1.2 The discovery of malaria parasites

The last 150 years has seen an explosion of knowledge about malaria as medical science has advanced. The first step towards identifying the aetiological agent of the disease is attributed to Meckel, a German pathologist, who, in 1847, described black granules (now known to be haemozoin, an insoluble waste product of malarial metabolism) in the blood, spleen and liver of cadavers of malaria victims (Harrison 1978). The critical finding that not only pigment but also the parasite itself was present in the blood of infected individuals was made by Laveran, a doctor in the French Army, when posted to Algeria. In 1880, Laveran first described the crescent-shaped bodies now known to be the gametocytes of Plasmodium falciparum in the blood of a malarious patient, and this finding was subsequently confirmed by Marchiafava & Celli (1883). The next development was made by Ross in implicating the female mosquito in malarial transmission. Whilst working in India, Ross first described malarial stages in the stomach of mosquitoes recently fed on infected blood (1897) and the next year he showed mosquito transmission of P. relictum, a malaria parasite of sparrows (Manson 1898). The same year, Grassi confirmed this finding for human malaria (reviewed by Garnham 1966, Harrison 1978). The avian species continued to make a substantial contribution to malaria research, including the first demonstration of a cycle of development outside the RBC; James & Tate (1937) observed exo-erythrocytic schizogony in P. gallinaceum of chickens in the reticulo-endothelial cells of the bone marrow. However, it was not until after World War II that a tissue phase was documented for mammalian malaria. In 1948, Shortt & Garnham described the pre-erythrocytic forms of P. cynomolgi in the liver of a rhesus monkey (Shortt & Garnham 1948 a), and similar forms were soon

found in the livers of human volunteers infected with P. vivax and P. falciparum (Shortt & Garnham 1948 b, Shortt et al 1951). The recurrence of clinical P. vivax malaria a considerable time after initial infection led to the postulation of a resting stage of the parasite (Shute et al 1976). Moreover, Krotoski et al (1980, 1982 a) discovered dormant stages (hypnozoites), developed from a subpopulation of sporozoites in the liver of rhesus monkeys infected with P. cynomolgi bastianelli. With this finding of relapse parasites, the last intracacy of the life cycle of the malaria parasite may have been revealed.

1.3 Classification

Since the first discovery of the causative agents of malaria, more than 100 species of Plasmodium have been described in a wide range of vertebrate hosts such as primates, rodents, birds and reptiles, each parasite exhibiting a narrowly defined host specificity. The vertebrate represents the intermediate host of the parasite, the sexual stages of the life cycle taking place in the body of female mosquitoes, of the genus Anopheles and Culex for mammalian or avian and reptilian infections, respectively. Four species of Plasmodium commonly infect man: P. falciparum, P. vivax, P. malariae and P. ovale. Of these, the first is the most important as it is responsible for malignant tertian or falciparum malaria, which causes the most fatalities and morbidity. In addition, a naturally acquired infection of P. knowlesi has been recorded (Chin et al 1965) and other zoonotic infections are likely to have occurred unnoticed.

Malaria parasites belonging to the genus Plasmodium are classified in the Phylum Sporozoa (parasites having a resistant spore, or stage derived from the spore, which contains sporozoites), Class Telosporea (sexual reproduction and sporozoites), Subclass Coccidia (mature trophozoites small and intracellular), Order Eucoccidia (schizogony, asexual and sexual reproduction) and Suborder Haemosporina (two hosts, with asexual development in a vertebrate and sexual reproduction completed in a dipterous insect) (Honigberg et al 1964).

1.4 Life cycle

Infection of the mammalian host is initiated by the bite of an infected female anopheline mosquito and the inoculation into the bloodstream of motile sporozoites contained in the insect's saliva during the taking of a blood meal. The inoculum size is small, averaging 15 sporozoites in one study (Rosenberg et al 1990). The sporozoites remain in the

peripheral blood for between 15-60 min (Fairley 1947, Sinden & Smith 1982) before sequestering to the liver, where they enter hepatocytes, either directly (Shortt 1948, Shin et al 1982), or indirectly after uptake by phagocytic Kupffer cells (Smith et al 1981). Once within the hepatocyte, the parasites develop into exo-erythrocytic schizonts (Garnham et al 1966) by asexual multiplication. When mature, each schizont and its host cell rupture, discharging around 30,000 haploid merozoites in the case of Plasmodium falciparum. Mammalian malaria parasites are thought to undergo only one cycle of exo-erythrocytic multiplication, this taking between 5.5 and 15 d for human malarias, depending on the species. In P. falciparum and P. malariae infections, tissue schizogony follows directly sporozoite invasion. However, for P. vivax and probably P. ovale infections, a proportion of the sporozoites first develop into the latent hypnozoite form which is responsible for producing relapses (Krotoski et al 1982 a & b). The stimulus for resumption of growth of hypnozoites is unknown but is thought to be predetermined (Bray & Garnham 1982).

The released merozoites enter the bloodstream, where they rapidly invade RBC and begin an asexual cycle of parasite multiplication. The merozoite attaches to an RBC and orientates itself so that the apical complex comes into contact with the RBC membrane, probably via a species-specific receptor. For the human malarias P. vivax and P. falciparum, these receptors are known to be associated with the Duffy blood group Ags (Miller et al 1975 b) and glycophorin (Miller et al 1977, Perkins 1981), respectively. After attachment of the merozoite, the RBC membrane thickens and forms a junction with the merozoite's plasma membrane (Aikawa et al 1978). The parasite releases material from its rhoptries and micronemes, causing the RBC membrane to invaginate, when the junction moves over the parasite which enters the invagination until it lies completely enclosed within the parasitophorous vacuole (Dvorak et al 1975, Aikawa et al 1978). During the entry process (reviewed by Mitchell & Bannister 1988), the surface coat of the merozoite is sloughed off (Bannister et al 1975, Miller et al 1975 a). After uptake into the RBC, the parasite develops a vacuole and becomes a ring stage, so called because of the signet ring-like morphology upon examination of Giemsa's stained bloodsmears. The ring stage feeds on host cell cytoplasm (mostly haemoglobin), the vacuole disappears and the parasite enlarges, producing malarious pigment, to become an amoeboid trophozoite. Asexual multiplication (schizogony) begins with the repeated division of the parasite nucleus within the intact nuclear membrane. The parasite then segments to form a schizont containing between 8-32 merozoites,

depending on the species. In all cases, as many merozoites are formed as there were nuclei, each new merozoite regaining its surface coat (Bannister 1977). After vesiculation and swelling of the pRBC membrane, the erythrocytic schizont bursts, releasing the merozoites which invade further RBC (Dvorak et al 1975). The length of the asexual erythrocytic cycle depends on the species of malaria parasite: one cycle takes 24 hr in P. chabaudi chabaudi, 48 hr in P. falciparum, P. vivax and P. ovale, and 72 hr in P. malariae. The asexual process is relatively synchronous in the natural host, and for some parasite species in laboratory-adapted hosts; in such synchronous infections, rupture of infected cells and merozoite release gives rise to the clinical manifestations of alternating fever and chills characteristic of malaria (Hawking et al 1968).

The pRBC membrane is often altered during the later stages of parasite growth, as shown by electron microscopy studies (Brown & Hockley 1966). The observation of electron-dense plaques beneath the membrane during parasite growth (Aikawa 1971, 1977, Wunderlich et al 1982) has been shown in P. knowlesi to correlate with the appearance of parasite specific Ags on the pRBC surface (Wunderlich et al 1982). Also, the membrane of P. falciparum-infected RBC may develop knob-like protrusions during the late trophozoite and schizont stages of the asexual erythrocytic cycle (Trager et al 1966). These knobs are thought to be implicated in the sequestration of pRBC in the capillary beds of vascular tissue which protects these stages from passage through the potentially hostile environments of the spleen and liver (Luse & Miller 1971). Loss of knobs during in vitro culture of P. falciparum is associated with a loss of antigenicity (Langreth & Reese 1979).

Upon RBC reinvasion, rather than undergoing asexual replication, a proportion of merozoites differentiate, without cell division, into the sexual stages or gametocytes within the host RBC (gametocytogenesis). The stimulus which directs a merozoite into a sexual rather than an asexual cycle is not known, but both micro- and macrogametocytes can be found in an infection initiated with a single parasite (Carter & Walliker 1975). The time for a newly invaded merozoite to mature into a ripe gametocyte is usually 6-12 hr longer than that for erythrocytic multiplication, P. falciparum (10 d) proving an exception. Hawking et al (1968) showed that, in synchronous infections of several species of Plasmodium, gametocytes matured and had a peak of infectivity for the anopheline mosquito during the night. They hypothesised that this represented a mechanism whereby their presence in the peripheral circulation coincided with the feeding behaviour of the nocturnal mosquitoes, so facilitating transmission. The sexual

cycle is completed when a female mosquito (of a suitable species) ingests circulating gametocytes when taking a blood meal from an infected individual. When the mature gametocytes are taken into the mid gut of the vector, they lose the RBC membranes and undergo gametogenesis. Within 10 min, the male microgametocyte divides mitotically three times (Sinden 1981) and exflagellates, releasing eight long, thin , flagellated microgametes. These swim towards, and ultimately fertilise, the macrogametes, so forming diploid zygotes. In each instance, the spherical zygote transforms into a motile ookinete which, within 24 hr, crosses the gut wall, usually passing intracellularly through the mid gut epithelium, and develops into a haploid oocyst between the gut epithelial layer and the basal lamina of the mosquito mid gut wall (Sinden & Strong 1978). The point at which meiosis takes place is unknown, but it is probably in the zygote soon after fertilisation (Phillips 1983), the parasites being otherwise haploid throughout the life cycle. Over the next 10-16 d (depending on external environmental conditions), the oocyst divides many times so that when it ruptures up to 10,000 sporozoites are liberated into the haemocoel (reviewed by Russel et al 1963). The motile sporozoites migrate and penetrate into the lumen of the mosquito's salivary glands, in so doing becoming infective to the vertebrate host (Vanderberg 1975) and remaining viable indefinitely until discharge during one of frequent blood meals. Figure 1.1 illustrates schematically the life cycle of a mammalian malaria parasite.

Although there is no suitable experimental evidence, it seems that P. malariae is able to evade complete elimination by the host's protective immunity since its life span in man is known to reach decades. The life span of P. vivax is commonly estimated to be 3-4 years. Since the hypnozoites in vivax malaria are sheltered against immune attack, the host's immune response probably only affects the level of parasitaemia and the clinical manifestations of infection and not the survival of the parasite itself. The same can be expected to apply to P. ovale malaria. In contrast, P. falciparum infections tend to become shorter as the immune response of the host increases. This is not surprising since, as the plasmodia are confined to the blood after completing the exo-erythrocytic tissue phase, immune factors exert a major impact on the survival of the parasites (WHO 1987). It is generally considered that P. falciparum has a longevity of no longer than 12 months in the human host, although infections of up to 4 years have been reported from Mauritius (Verdrager 1964). Observations of Bekessy et al (1976) in Nigeria have shown that high immunity in areas with intensive malaria transmission increases the natural rate of recovery from infection by a factor of 10, and that it

reduces the period of patent gametocytaemia. Thus, under natural conditions, it is impossible to consider the life span of P. falciparum in man separately from immune factors.

1.5 The clinical disease

Despite being such a widespread and prevalent infection which causes enormous morbidity and mortality, the clinical course of malaria is relatively straightforward. Most illness and death is associated with acute infection, though some fatalities result from unusual chronic immunologically based sequelae. Plasmodium vivax and P. ovale usually cause only temporary morbidity, whilst P. malariae infections result in nephrosis and splenomegaly. Host mortality is almost entirely produced by P. falciparum. Cerebral malaria is the most important and well recognised manifestation of such severe disease (Spitz 1946), which may also involve anaemia, jaundice, pulmonary oedema, hypoglycaemia and acute renal failure. Secondary infections, especially Gram negative septicaemia, are not uncommon (WHO 1990) and are probably the usual cause of 'algid' malaria.

In synchronous infections, most of the pathogenesis (fever and acute symptoms) is caused by the multiplication and subsequent release of merozoites from pRBC. After a variable incubation period (8-14 d) for P. falciparum, the patient develops headaches, general body pains, nausea and gastrointestinal disturbances. None of these clinical features are themselves diagnostic of malaria as opposed to acute febrile illnesses. However, the presence of parasites in the blood causes fevers which occur with a distinctively regular periodicity. P. falciparum has a 48 hr asexual cycle in the peripheral circulation and, correspondingly, in many patients a regular tertian fever may occur. The bouts of paroxysms characteristic of malaria fever are associated with the synchronous bursting of schizont-infected pRBC, which releases much antigenic parasite and altered host material into the blood stream. This, in turn, causes the secretion of endogenous pyrogens (such as cachectin or TNF) from monocytes into the peripheral blood. The feverish period typically lasts less than half a day, and is characterised by a high temperature, when the patient feels cold with intense shivering and rigors, and a hot phase of marked sweating. During this period, the affected individual feels acutely unwell, but between paroxysms the patient may feel quite normal (Kitchen 1949). It has recently been suggested that malaria fever may be of mutual benefit for parasite and host (Kwiatkowski & Greenwood 1989). This is based on

the observation that unconstrained plasmodial growth in the acute phase of infection would be fatal for both host and parasite, since the transmissible gametocyte forms take some time to mature. Therefore, both the infective Plasmodium and its mammalian host have growth constraints which do not require immunological pre-exposure; such constraints may include fever and the non-specific host defence mechanisms (e.g. cachectin/TNF release) with which it is associated. Malarial synchronisation, which occurs at febrile temperatures (Kwiatkowski 1989), enables the fever to show classical periodicity, and is seen as an escape mechanism if the host response becomes too vigorous.

If the parasitaemia is allowed to rise unchecked by natural or acquired resistance or by drug treatment, the patient will become severely ill. The severity of the disease is usually proportional to the number of pRBC (Field & Niven 1937). The most obvious effect on body function is on the blood, since RBC are destroyed directly by the parasite. The development of anaemia is due to the phagocytosis of both pRBC and nRBC (Rosenberg et al 1973, Seed & Kreier 1988), particularly in the liver and spleen, which, in turn, leads to enlargement and tenderness of both these organs. If RBC destruction is intense, the released intracellular debris may form immune complexes which affect renal function and can lead to complete renal failure. Free haemoglobin may be liberated into the plasma, which is then excreted, a condition known as haemoglobinuria.

As P. falciparum reaches the late trophozoite stage, it induces changes in the pRBC surface membrane, causing it to become more adhesive to the endothelial cells of the deep tissue vasculature. When the capillaries become blocked with pRBC, it prevents normal blood flow; the resulting haemorrhaging contributes further to blood loss through anaemia. The occlusion of blood vessels increases the anoxia already resulting from a decreased blood flow (the haematocrits of anaemic individuals may fall below 20%; Phillips et al 1986), and is partly responsible for the pathogenicity of P. falciparum. In the brain, blockage of capillaries causes cerebral dysfunction, the most severe complication of P. falciparum infection in man. Cerebral malaria may progress slowly after initial symptoms, or develop rapidly resulting in convulsions, nervous disorders and eventually coma. As only a small proportion of examined cadavers of patients presenting with cerebral malaria show evidence of high peripheral parasitaemia or severe anaemia, there is some dispute over both the pathophysiology and actual aetiology of cerebral malaria (reviewed by Phillips & Warrell 1986, Warrell 1987).

Malaria infection has also been implicated in the incidence of tropical splenomegaly

syndrome (reviewed by Greenwood 1979), Burkitt's lymphoma (Deldorf et al 1964, Burkitt 1969), spontaneous abortion (Herd & Jordan 1981) and the incomplete response to vaccination shown by malarious children (McGregor & Barr 1962) (immunopathology reviewed by Marsh & Greenwood 1986). However, there is as yet no evidence that immunosuppression caused by Human Immunodeficiency Virus (HIV) leads to major complications or reactivations (Fleming 1990, Lucas 1990). Unfortunately, by being a potent cause of anaemia, malaria enhances transmission of HIV to children through blood transfusion (Greenburg et al 1988).

Over several weeks, an untreated attack which has not proved lethal will cause less noticeable symptoms until it becomes clinically inapparent. Symptomatic recurrences can occur for several months or years after the acute episode; either as a recrudescence from a subpatent, asymptomatic blood stream infection, or as a relapse originating from dormant liver hypnozoites (reviewed by Krotoski 1989). This pattern of disease is well documented in non-immune adults living in areas with unstable, seasonal or epidemic malaria transmission. However, in holoendemic areas with high levels of transmission, heightened acquired immunity makes this relationship between parasitaemia and clinical disease less clear-cut. In such situations, the main impact of malaria is on young children (Edington 1967). In the Gambia, death commonly occurs under the age of five, and children experience about one clinical episode per annum till at least seven years of age (Greenwood et al 1987). In adolescence and adulthood, parasites are still present in the blood but these people mostly remain asymptomatic.

1.6 Chemotherapy

None of the available anti-malarial drugs are effective against all stages of the malaria parasite found in the vertebrate host, but have a selective action on different phases of the Plasmodium life cycle. Tissue schizonticides eliminate developing exo-erythrocytic schizonts and some of these are effective against hypnozoites, whilst blood schizonticides act on asexual erythrocytic stages. Drugs with gametocytocidal activity destroy the sexual forms of the parasite, and sporontocidal treatments prevent sporozoite production by inhibiting the development of the oocyst in the mosquito, thereby blocking parasite transmission. The choice of anti-malarial drug for use in man will depend on whether it is for prophylaxis (i.e. to prevent or suppress clinical malaria; causal prophylactics) or for curative purposes (i.e. to treat acute clinical disease and/or to eliminate tissue stages). For this latter case, the treatment priorities in severe and uncomplicated cases

have to be distinguished. In life-threatening infections (usually only P. falciparum), the objective of treatment is to save life; considerations such as prevention of late recrudescent patencies or minor toxicity would be irrelevant in this context. In uncomplicated malaria, where death is most unlikely, these considerations assume greater importance, and may well influence the choice of therapy. Causal prophylactic drugs kill early but not late liver stages and prevent the establishment of the parasite in the bloodstream. The type of infection will also affect the selection of chemotherapy; P. vivax or P. ovale infections require the use of a drug effective against hypnozoites, while P. falciparum or P. malariae do not. The addition of gametocytocidal or sporontocidal drugs to a standard therapeutic regimen may help to decrease the spread of drug resistant plasmodia. No anti-malarials currently available are effective against sporozoites.

After its introduction into Europe in the mid 1600s, the cinchona alkaloid quinine remained the standard remedy for malaria till well into this century. During the 1930s and 1940s, it was largely replaced by chloroquine and other synthetic compounds that are more efficacious and less toxic (Geary & Jensen 1983). However, with increasing resistance of some malaria parasites to these compounds, quinine, a fast acting blood schizonticide, has returned to occupy an extremely important niche in malaria chemotherapy. Resistance to quinine, although still rare, is a potentially devastating problem.

Chloroquine is the prototype member of the 4-aminoquinoline class (reviewed by McChesney & Fitch 1984), which, like quinine, acts by inhibiting parasite DNA and RNA synthesis by intercalating with DNA. Introduced in 1945, chloroquine was the mainstay of global malaria chemotherapy for nearly two decades. The drug's low expense, low toxicity and high efficacy in combination with the use of vector control programs led to impressive gains against malaria during the 1950s and 1960s. The development and spread of chloroquine resistance greatly eroded this achievement and was partially responsible for the return of P. falciparum to cleared areas. Chloroquine is a rapid blood schizonticide and can be used as both a preventative and as a curative drug. It remains the drug of choice for the treatment of P. malariae infection, and, with primaquine, of ovale and vivax malaria. For falciparum malaria, it is still the best drug for infections that retain sensitivity to it, in which case, it is curative.

Primaquine is an 8-aminoquinoline, which, in vivo, is broken down to the active agent diquinone, an analogue of ubiquinone, which is found in the respiratory chain, and hence

may disrupt malarial energy metabolism. It affects all mammalian stages of malaria parasites. Killing of exo-erythrocytic stages and gametocytes are the most important, and these actions provide the clinical rationale for use. Although use of primaquine has been restricted since it became commercially available in 1951 due to toxicity to the host, it is used to give a radical cure of P. vivax and P. ovale infections, as it destroys persistant liver stages.

Proguanil (introduced in 1948), a biguanide, and pyrimethamine (introduced in 1952), a diaminopyrimidine, share a common mechanism of action, inhibition of the dihydrofolate reductase-catalysed conversion of dihydrofolate to tetrahydrofolate, an important cofactor in parasite metabolism. For this reason, they are known as anti-folate drugs. They kill the early tissue stages, especially of P. falciparum, and hence are used for prophylaxis. Both drugs have a blood schizonticidal action, but often too slow to be useful in treating acute malaria attacks.

Sulphonamides and sulphones were initially developed in the 1940s as anti-bacterial agents, but have been found to possess useful anti-malarial properties. This is because they, or compounds derived from them in vivo, act as analogues of the essential growth factor para-amino benzoic acid (PABA), thereby blocking the synthesis of dihydrofolate and ultimately of tetrahydrofolate. The most important so-called PABA antagonists are dapsone and sulphadoxine, which have proved useful as blood schizonticides in treating infections of chloroquine resistant P. falciparum, especially when used in combination with pyrimethamine (Fansidar^{R}).

The arylaminoalcohol compounds mefloquine and halofantrine were the first new anti-malarials to be clinically tested in over 30 years, the former being first marketed in 1984 (Rinehart et al 1986, Cosgriff et al 1982). They are both potent blood schizonticides which are highly effective in the treatment of acute malaria and have demonstrated efficacy against multi-drug resistant strains of P. falciparum. Since mefloquine has a very similar chemical structure to quinine, resistant strains of P. falciparum are already appearing (Boudreau et al 1982, Bygbjerg et al 1983). It may be, therefore, that halofantrine, still in clinical development, may take a leading role in malaria chemotherapy in the next decade.

Artesiminine (qinghaosu), the active principle of the Chinese medicinal herb Artemisia annua, is an emerging anti-malarial which is structurally novel and is thus anticipated not to have any cross-resistance with other available drugs. The parent compound, a water soluble succinate derivative (artesunate) has proved a dramatically effective

anti-malarial (China cooperative research group on qinghaosu and its derivatives as antimalarials 1980, 1982). Their rapidity of action in the treatment of P. vivax and chloroquine resistant strains of P. falciparum (White 1988) makes them attractive prospects for the therapy of severe and complicated malaria, but detailed pharmacological and clinical studies are needed before wider use.

1.7 Drug resistance

Malarial drug resistance has been defined as the 'ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in a dose equal to or higher than those usually recommended but within the limits of tolerance of the subject' (WHO 1965). Drug resistance in the field is usually in relation to the asexual erythrocytic stage of Plasmodium falciparum. Whereas 30 years ago sensitivity of infecting parasites was the norm, the resistance of P. falciparum to drug treatment has probably become the most important threat to effective control of malaria today. It has largely arisen through a combination of massive anti-malarial drug deployment and a failure to combat transmission of the disease.

Chloroquine and other 4-aminoquinoline compounds have been the mainstay in the treatment of symptomatic cases of falciparum malaria, and have been particularly important in regions where insecticide application inside residences has been impractical or ineffective. Unfortunately, the report in 1961 by Moore & Lanier of two cases of chloroquine resistant P. falciparum malaria from Colombia heralded what has become a rapidly expanding problem in many countries where the disease is endemic. The other plasmodia that infect man have remained sensitive to chloroquine and it continues to be the drug of choice in the treatment of these infections. Recent case reports of chloroquine resistant falciparum malaria from East Africa (Fogh et al 1979, Weniger et al 1982) forbode a potential major problem on that continent, where most strains apparently continue to be sensitive to chloroquine, but resistance to which is becoming a problem, even in West Africa (Phillips, R.S., personal communication). It is now known that chloroquine resistant strains frequently also develop resistance to the closely related amodiquine and to mepacrine (now obselete), and, in some instances, become less sensitive to quinine (Peters 1987 a). Multi-drug resistant parasites are now widespread. As there is no fully adequate alternative drug to quinine, if resistance were to spread, procedures now in their infancy, such as exchange transfusion or immunotherapy, may have to be evaluated (Phillips, R.S., personal communication).

The second string treatment for chloroquine resistant falciparum malaria which is being used extensively is a combination of antagonists of parasite folate metabolism, sulphadoxine and pyrimethamine, marketed under the name Fansidar^{R}. It has been an effective chemoprophylactic and therapeutic regimen but already has been identified as ineffective in some regions; indeed, in Thailand, Fansidar^{R} has lost most of its efficacy after 10-15 years of use (Peters 1987 a). This was probably precipitated by its being freely available on the black market in the Far East (Hurwitz et al 1981). Nevertheless, there are some areas, notably East Africa, where the drug is effective and the simplicity of single dose therapy makes it an attractive alternative to quinine.

When clinical trials for mefloquine took place, it was hoped it would become an established anti-malarial effective against multiple resistance infections; initially it proved so (Trenholme et al 1975), but unfortunately, resistance had already been reported well before widespread use. In order to 'protect' mefloquine against the development of resistance, it has been recommended that it be used in combination with other drugs (WHO 1984).

The resistance problem has encouraged research into new compounds, spearheaded by the clinical development of halofantrine. In a recent study, Robinson & Peters (1985) demonstrated the resistance to halofantrine of the RC line of the murine malaria P. berghei. This parasite line has a similar resistance to mefloquine (Peters et al 1977). Although it has been proposed that failure of a compound to cure infection with P. berghei RC parasites does not indicate that it will be of no value against chloroquine resistant P. falciparum, it does suggest that there is a serious risk that the latter will become rapidly resistant to the new compound (Peters et al 1975). Experience to date with mefloquine supports this hypothesis (Peters 1987 b). The grim conclusion drawn is that a high risk exists that multiple resistant P. falciparum could rapidly become resistant to halofantrine also. Since current clinical trials seem to confirm that halofantrine may find a place in the therapy of malaria infection, steps should be taken to find an appropriate partner compound that would be suitable for deployment with halofantrine, so preventing the widescale use of this new anti-malarial on its own.

The other three malaria parasites of man are much simpler to treat, or to protect against, as they are never life-threatening and drug resistance is not such a problem. The only difficulty is that most blood schizonticides, such as chloroquine, have no effect on the hypnozoites of P. vivax and P. ovale. Primaquine has to be used to produce radical cure but can cause significant haemolysis in people with glucose-6-phosphate

dehydrogenase (G6PD) deficiency; it is no coincidence that the distribution of malaria correlates with that of this genetic defect, probably conferring a protective benefit on the heterozygous condition (reviewed by Weatherall 1987).

1.8 Laboratory models for malaria research

Research into human malarias involving the natural host is sanctioned only in the final stages of vaccine or drug trials. Although several species of non-human primates are partially susceptible to infection with human plasmodia, for practical and ethical reasons the use of such models is difficult to justify on a regular basis. For this reason, various species of rodent, avian and non-human primate plasmodia are used as laboratory models, offering the opportunity to study the biology of the parasite at the cellular and molecular levels in different hosts.

The similarity between human and non-human primate malarias in theory makes the latter the preferred models for laboratory use. For the plasmodia of the higher apes (chimpanzees, gorillas, orangutans and gibbons), the considerable expense involved in obtaining and maintaining these rare hosts precludes their general use for malaria research. Of the malaria parasite species that develop in the more readily available monkey hosts, Plasmodium knowlesi and P. cynomolgi have been widely used. The natural host for both is the Old World monkey, the common Kra (Macaca fascicularis), found in the jungles of South East Asia. Nearly all laboratory investigations with these parasites have employed the rhesus monkey, Macaca mulatta. The New World monkeys Aotus trivirgatus and Saimiri sciureus infected with P. brasilianum and P. simium have also been used (reviewed by WHO 1987).

Much recent work, mainly by Collins and his colleagues (eg. Collins et al 1983), has examined the suitability of models for vaccine studies in monkeys. P. falciparum and P. vivax have been adapted to develop in new and more susceptible (sub)species of Aotus and Saimiri. Unlike the simian parasites in macaques, which infect their natural hosts on a predictable basis, the human malarias require long periods of adaptation if predictable results are to be obtained. Certain isolates have become highly virulent after several blood passages, and have lost their ability to infect mosquitoes. Infection from mosquitoes is rarely obtained by feeding on intact monkeys, successful infections following sporozoite inoculation being greatly enhanced by splenectomy (Garnham 1966). In splenectomised simians, the host is altered immunologically and this makes vaccine studies extremely difficult to interpret.

There has been recent interest in a Plasmodium parasite able to infect the common marmoset, Callithrix jacchus; this was first thought to be P. vivax (Mitchell et al 1988), but is now thought to be P. malariae (Mons & Sinden 1990). This model would be a step forward in malarial research as it represents the successful adaptation of a human malaria parasite to an intact, commonly available primate. For the most part, however, the use of primates in the laboratory is severely restricted.

The difficulties of working with primate malaria parasites and human malarias adapted to other primates have meant that numerous species of Plasmodium infective to birds and rodents have been used widely to study the biology of plasmodia. In the 1920s, P. cathemerium from the American house sparrow and P. relictum from a variety of passerines were found to infect canaries. These two species provided the basis of experimental work on avian malarias, including the screening of compounds for anti-malarial activity (Richards 1984). However, with the availability of P. gallinaceum and P. lophurae as experimental parasites, young chickens and ducks became the hosts of choice in the laboratory. The major drawback of these model organisms is their uncertain phylogenetic relationship with human plasmodia, which undermines their relevance as biochemical or molecular models. Despite certain similarities between P. falciparum and avian plasmodia (Sinden 1978), differences in their life cycles, vectors, and in the immune systems of their vertebrate hosts limit the usefulness of avian malarias as a model, hence stressing the importance of rodent malarias in fundamental studies.

In 1948, Vincke and Lips captured two infected tree rats (Thamnomys surdaster) in the Congo (now Zaire) and succeeded in transmitting P. berghei from them to laboratory rats and mice by inoculation of blood. Three years later, P. berghei was transmitted successfully through Anopheles mosquitoes (Yoeli & Wall 1951). The availability of P. berghei , and of several other species of rodent malaria isolated subsequently, opened up the field of mammalian malaria research and made it possible to carry out work on the genetics of (Beale et al 1978), and chemotherapy and host immunity to, the parasite. Four species of rodent malarias are now recognised: P. yoelii and P. chabaudi from Thamnomys rutilans; P. berghei from T. surdaster; and P. vinckei which infects both natural hosts. All except P. berghei contain two or more sub-species. Established strains can be maintained for years by syringe passage of infected blood in mice and rats, and can be mosquito-transmitted, so that all aspects of the Plasmodium lifecycle can be studied. Several species of anopheline mosquito can be used to transmit rodent malaria,

though the single proven natural vector, Anopheles dureni millecampsi, has not been colonised. In most laboratories, A. stephensi is employed exclusively as the mosquito vector.

Mouse malaria parasites are very convenient experimentally. In terms of their behaviour, they are divided into two groups: P. berghei and P. yoelii ; and P. vinckei and P. chabaudi. The similarities within, and distinctions between, the two groups are manifest in the structure and behaviour of blood stage parasites, serology, isoenzyme types and patterns of cross protection (Carter & Diggs 1977). Within each group, it is possible to obtain infections from which most strains of mice recover and are fully immune, so that after a second homologous challenge negligible parasitaemias are detectable (e.g. P. y. yoelii 17XNL, P. c. chabaudi and P. vinckei petteri), or strains that are lethal in naive mice (e.g. P. y. yoelii 17XL or P. v. vinckei). Immunisation against the asexual erythrocytic stages of the latter can be achieved by infection and drug cure (Cox 1964), by infection with a non lethal parasite in the same group (e.g. P. y. yoelii 17XNL protecting against P. y. yoelii 17XL, or P. c. chabaudi protecting against P. v. vinckei), or by immunologically priming with homologous crude lysates (Playfair et al 1977) or purified pRBC Ags (Holder & Freeman 1981). The availability of cross-reacting virulent and avirulent strains of plasmodia affords experimental analysis of the mechanisms of immunity. Protective immunity to P. chabaudi adami is thought to be Ab independent, whereas recovery from primary P. yoelii infections is Ab dependent (Allison & Eugui 1983). Thus, various mouse malarias can be used to illustrate different mechanisms of acquired immunity, although care must be taken when attempting to extrapolate from these models to human malarias.

1.9 In vitro culture of malaria parasites

The first asexual erythrocytic stages of Plasmodium to be maintained in continuous culture in vitro were from P. falciparum (Trager & Jensen 1976, Haynes et al 1976). These studies, in particular those of Trager & Jensen, have revolutionised research on human malaria, making it possible to study the clinically most important malaria parasite. The candle jar method (Jensen & Trager 1977) is now used routinely for in vitro cultivation of P. falciparum, although several modifications of this method are available. There is no evidence for loss of infectivity after prolonged cultivation in vitro; indeed, several lines have been used to infect Aotus trivirgatus monkeys after more than three years continuous culture in vitro (Trager 1982).

The asexual stages of P. falciparum appear morphologically normal after continuous in vitro growth, although they may change characteristics, e.g. loss of RBC membrane knobs (Langreth et al 1979), and all developmental stages are generally seen in culture at any one time, since the parasite tends to lose its in vivo synchrony. These observations, together with the fact that certain isolates need time to adapt to the conditions of in vitro cultivation, suggests that parasite selection may occur in vitro, so changing the in vitro-maintained parasites in both phenotype and genotype from the original isolate.

Although in vitro culture of asexual bloodforms is now possible for most laboratory parasites, it remains problematic (Mons et al 1988), and only P. berghei (Mons et al 1980) is widely cultured on a routine basis. Virtually all isolates of P. falciparum maintained in vitro have proved capable of producing gametocytes under suitable conditions, but in highly variable numbers (Sinden 1983). Pure sexual stages of P. berghei can also be produced (Mons & Sinden 1990), but for all other malaria parasites cultured in vitro, the production of infective gametocytes is readily achieved for most malaria parasites, but the subsequent production of viable ookinetes has proved more difficult. Using P.berghei, Weiss & Vanderberg (1977) were the first to obtain such transformation, and the technique, improved by Janse et al (1985) and by Sinden et al (1985), is now used routinely. However, propagation of P. falciparum ookinetes has been more difficult, only Carter et al (1987) reporting any success.

Although the exo-erythrocytic stages of avian malaria parasites have been in continuous tissue culture for over 20 years, it is only recently that these stages of mammalian malarias have been cultured in vitro successfully. The first demonstration of the complete in vitro development of the exo-erythrocytic stages of P. berghei was by Hollingdale et al (1981) and it is only with this parasite that functional maturation to merozoite release has been obtained reproducibly in vitro (Suhrbier et al (1987). Sporozoites from other species of plasmodia readily invade hepatocytes or hepatoma cells from a variety of vertebrates, and show complete morphological maturation of exo-erythrocytic schizonts, but this is yet to be achieved for P. falciparum.

At present, only for P. berghei can the vertebrate host be totally replaced by culture in continuo from sporozoite to infective gametocyte. A significant advance in culture techniques would be the culture of the late sporogonic stages from ookinete to sporozoite for any Plasmodium species, thereby circumventing the mosquito host and enabling in vitro culture of the complete malaria life cycle. Clearly, there is no single Plasmodium

species, or method of maintenance, that can satisfy all the needs for malaria research, and there is no reliable laboratory model of human malaria under endemic conditions. It is considered, however, that the fundamental biology of the malaria parasite can be studied equally well, and with greater convenience, in rodent models (Mons & Sinden 1990). This is because they offer the combination of in vivo and in vitro methods of maintenance. Results obtained in rodent systems have proved to be of significance in the development of vaccination candidates and in the detailed examination of parasite antigenic variation, as well as in the areas of immunology, genetics and chemotherapy.

1.10 Host resistance to malaria

The ability of an individual to control a malaria infection takes two forms, innate and acquired resistance. Innate resistance, which can be parasite specific, is expressed regardless of any previous exposure, and has no immunological specificity. Of the protective mechanisms involved, some are genetically controlled whilst others may simply be incidental, reflecting changing environmental factors such as food availability. Conversely, under certain circumstances, these factors may act to exacerbate malaria infections. Acquired resistance requires previous contact with the parasite and is immunological in nature. Between these two extremes is non-specific resistance, which is itself immunological but necessitates exposure to an organism or substance (e.g. Propionibacterium acnes or Con A) which is unrelated to Plasmodium, but which nevertheless stimulates suppression of the erythrocytic infection. Non-specific immune and non-immune mechanisms of resistance benefit the host by moderating the severity of infection during the vulnerable period between RBC invasion and the mounting of an effective acquired immune response.

1.10.1 Innate resistance

In human populations, genetically and phenotypically very diverse, some aspects of innate resistance can be observed which play a role in host specificity. In populations with a high exposure to malaria, genetic alterations which lessen the severity of disease or enable potential hosts to be completely refractile to infection would increase an individual's chances of survival and reproduction. Therefore, such beneficial genetic traits tend to spread through the population (Haldane 1949). In theory, this could affect all stages of the life cycle, but to date only the RBC stages of the parasite have been studied. During the phase of asexual multiplication, the parasite requires specific RBC

receptors for recognition and attachment, RBC deformability for invasion, and a suitable internal environment for supplying essential nutrients (reviewed by Pasvol & Wilson 1982). Several inherited characteristics of RBC lead to resistance at the erythrocytic stage, and the existence of such RBC genetic defects at high frequency is believed to reflect the positive selective pressure of malaria. In regions where malaria is, or was, endemic, widespread genetic RBC abnormalities are thought to occur because the heterozygote condition confers protection to malaria, outweighing any debility or lethal effects to the individual or population, respectively, of the homozygote. Evidence for this hypothesis comes from field studies showing that those persons carrying the abnormal gene have a lower incidence of, or are less susceptible to malaria, and from *in vitro* studies (which may or may not mimic the environment *in vivo*).

(a) Factors affecting the parasite's ability to invade RBC

A significant proportion of African Americans are entirely resistant to *Plasmodium vivax* malaria (Young *et al* 1958). Many such individuals are negative for the Duffy blood group determinants (Welch 1977), a trait which is rare elsewhere. The Duffy negative characteristic corresponds to lack of expression of two Mendelian alleles Fy^a and Fy^b ($Fy(a^- b^-)$). Miller *et al* (1975 a & b) first demonstrated that Fy ($a^- b^-$) RBC are resistant to invasion by *P. knowlesi* merozoites *in vitro*. This has not been repeated with *P. vivax* due to the inability to propagate this species *in vitro*. Resistance of Duffy negative individuals to infection when exposed to mosquitoes infected with *P. vivax* was soon established (Miller *et al* 1976, 1977, Spencer *et al* 1978). These findings explain the very high frequency of the Duffy negative phenotype in West Africa where *P. vivax* is now absent. As *P. vivax* is not a lethal parasite, nor is it thought to affect greatly reproductive capacity, this fixation has probably arisen through spread of a fortuitous mutation rather than selection (Pasvol & Wilson 1982). It is possible that in the past it was more virulent, but then absence of Duffy Ag would be more widespread if selection were operating.

As individuals lacking the Duffy Ag are refractile to infection, it is thought that it is probably the receptor for parasite attachment, as although *P. knowlesi* attaches *in vitro* to Duffy negative RBC in the normal way, junction formation and parasite invasion do not take place (Miller *et al* 1979). As Duffy negative RBC are susceptible to *P. falciparum*, it is probable that the receptors on the human RBC for *P. vivax* and *P. falciparum* are different.

Ovalocytosis, a morphological RBC variant due to a cytoskeletal abnormality, is present in ~30% of the Melanesian population of Papua New Guinea and other aboriginal populations of South East Asia (Amato & Booth 1977). Epidemiological evidence suggests that individuals with ovalocytosis have a lower parasitaemia than normal when infected with P. falciparum, P. vivax, or P. malariae (Serjeantson et al 1977).

Experimentally, ovalocytes are highly resistant to invasion by both P.falciparum and P. knowlesi merozoites in vitro (Kidson et al 1981, Hadley et al 1985). These parasites do not bind to the same receptor, suggesting that resistance may be due to a major difference in the cytoskeletal structure of the ovalocyte membrane.

Eliptocytosis is another RBC cytoskeletal defect which has been reported to promote resistance to invasion by both P. knowlesi and P. falciparum (Hadley & Miller 1988). Decreased invasion by two different plasmodia suggests that this defect alters a later stage of invasion than RBC membrane receptor recognition.

The susceptibility of RBC to invasion by malaria parasites is dependent upon their age (reviewed by Bray & Garnham 1982). P. berghei, the rodent parasite, has a preference for reticulocytes (very young RBC), and thus causes severe infections in immature rats, where reticulocytes comprise 20% of the RBC population, but in adult rats, where only 4% of RBC are reticulocytes, erythrocytic infections are mild. P. vivax and P. ovale are predominantly found in either reticulocytes or slightly older normocytes. P. falciparum is now thought to have a preference for metabolically young RBC (Phillips 1983). This preferred RBC type may explain the low incidence of severe cases of falciparum malaria in babies: the rate of erythropoiesis slows dramatically soon after birth for a period of several months, thereby restricting parasite multiplication. During extremely severe P. falciparum infections in children and adults alike, erythropoiesis may again be reduced, thus constraining parasite growth through lack of young RBC.

The reason for the inability of P. falciparum to invade older RBC is unclear, but it may be either due to the lack of deformability of older RBC, so preventing parasite entry, or it may be that mature RBC are not sufficiently metabolically active to meet the needs of the invasion process.

(b) Factors affecting the intraerythrocytic environment

The gene controlling a deficiency of the RBC enzyme glucose-6-phosphate dehydrogenase (G6PD) is sex-linked and the geographical distribution of the G6PD deficient mutant suggests that it gives some protection in the presence of endemic falciparum malaria

(Luzzatto 1979). The selection pressure against G6PD deficiency is not strong because in the male heterozygote or the female homozygote, the predisposition to suffer haemolytic anaemia occurs rarely, and thus only a marginal advantage with respect to malaria may be required to maintain the G6PD deficiency gene.

Epidemiological evidence favours protection of female heterozygotes rather than totally deficient individuals (Bienzle et al 1979). To explain this, Eckman & Eaton (1979) proposed that, since G6PD-deficient RBC are sensitive to oxidant stress, parasitisation makes them susceptible to premature haemolysis, which would probably kill the parasite. This argument was based upon the assumption that plasmodia are dependent on host cell NADPH as a cofactor in parasite glutathione reduction. As a distinctive G6PD of parasite origin has now been identified at low levels in P. falciparum infected RBC (Hempelmann & Wilson 1981, Usanga & Luzzatto 1985), the assumption that the parasite deprives G6PD-deficient cells of NADPH and renders them more liable to oxidative stress is questionable. Nevertheless, oxidative stress remains a major hypothesis for G6PD deficiency-dependent mechanisms of malaria resistance (Nagel & Roth 1989).

There is considered to be a link between favism, the eating of fava beans in the Mediterranean and Near East, and the G6PD deficiency (Friedman 1979). Isouramil, an oxidant isolated from fava beans, was found to be more damaging to parasites in G6PD-deficient RBC than to parasites in normal RBC (Golenser et al 1983). This suggests a pharmacological approach to malaria therapy, but may not be relevant to the interacting evolution of P. falciparum and RBC G6PD deficiency.

Haemoglobin abnormalities prevalent in areas endemic for malaria have also been implicated in protection against malaria. Sickle haemoglobin results from a mutation in the gene locus controlling the synthesis of the tetra-polypeptide chain of adult haemoglobin (HbA). This results in a single amino acid substitution, valine for glutamic acid, in each of the two polypeptide chains (reviewed by Motulsky 1964). Individuals who are homozygous for HbS (genotype HbSS) have ≥ 80% HbS haemoglobin, the rest comprising mainly foetal haemoglobin (HbF). The majority of persons homozygous for the sickle gene die during childhood, usually from sickle cell anaemia (due to distortion of RBC,' sickling', at quite high oxygen tensions), and thus do not reach sexual maturity. In contrast, sickle cell heterozygotes (HbAS), in whom about half the haemoglobin is Hbs, and the rest mainly HbA, are unlikely to suffer any obvious disability unless they are subjected to low partial pressures of oxygen. When this happens, for example, at

high altitude, carriers of the sickle cell trait suffer RBC sickling.

Although the sickle cell gene is rarely passed on to the next generation by homozygous individuals, in parts of tropical Africa and around the Mediterranean, 1% of the population may be HbSS and as much as 40% may be HbAS. It is highly improbable that the mutation rate at the locus controlling the polypeptide of the haemoglobin molecule could account for the persistant presence of the sickle gene in the human genome. This apparent anomaly may be explained by the considerable selection pressure conferring resistance to P. falciparum.

Early epidemiological and clinical studies suggested that sickle trait individuals become infected with falciparum malaria, but fewer died of the infection as compared with those with normal haemoglobin (Allison 1954). The high incidence of the sickle cell trait in P. falciparum-endemic areas suggested that a survival advantage was associated with it, i.e. relative protection against the malaria parasite (Allison 1957). Where the sickle gene is no longer placed under the selective pressure of malaria, as happened to the HbAS heterozygotes and their descendants when the carriers were transported from West Africa to the United States, its frequency diminishes.

The cellular mechanism, whereby HbS gives protection, is slowly becoming unravelled. At 50% oxygen tension, accelerated morphological sickling occurs in sickle trait cells containing parasite ring forms compared to non-parasitised sickle trait RBC (Roth et al 1978). Hence, the accelerated destruction of pRBC is probably one of the mechanisms by which HbS carriers are afforded protection against P. falciparum (Friedman 1979). Other mechanisms may also be involved. Under reduced oxygen tensions there is a significant retardation of development of parasites invading RBC (Pasvol et al 1978). It is thought that sickle trait pRBC that survive despite enhanced sickling during the ring stage may be compromised during deep vascular schizogony. During this period in the parasite's life cycle, pRBC adhere to endothelial surfaces of venules because of trophozoite-induced knob formation on the RBC surface (Luse & Miller 1971, Raventos-Suarez et al 1985). Venules filled with adherent pRBC become partially or totally obstructed, leading to hypoxia and low blood pH. Such conditions favour sickling and compromise parasite nutrition (Friedman et al1979 a). The mechanism of parasite death in sickled HbAS and HbSS cells is not fully understood, but it may be due to low intracellular K^+ (Friedman et al 1979 a), or, alternatively, enhanced HbS polymerisation caused by an increase in the RBC internal haemoglobin concentration accompanying loss of water during parasite metabolism (Olson & Nagel 1986, Ginsburg

et al 1986).

In the developing human foetus, initially all the haemoglobin synthesised is of the foetal type, HbF. HbA production starts around mid term of pregnancy, and at birth, when HbF production ceases, the proportions of HbA and HbF are 20% and 80% respectively. By four months, however, 90% of the haemoglobin is HbA. In the newborn child, there is temporary resistance to P. falciparum infection, due in part to passive transfer of anti-parasitic Abs across the placenta, but also due to the fact that HbF is less supportive of parasite growth. Evidence in support of the latter hypothesis comes from cases of thalassaemia, a group of genetic disorders of globin synthesis, in which there is a significant retardation in the foetal to adult haemoglobin switch during the first five years of life (Beaven et al 1961). Haldane (1949) first proposed that the small, under-haemoglobinised cells found in thalassaemia were protective against malaria and this has been subsequently supported by several epidemiological studies (summarised by Weatherall & Clegg 1981), which indicate a selective advantage for β-thalassaemia genes in the Mediterranean region. Children who are homozygous for β-thalassaemia rarely survive to adulthood and reproduce. Despite this selection pressure, there is a high frequency of various forms of thalassaemia throughout the Mediterranean basin, in Africa and in Southeast Asia, all malarious or formerly malarious areas. Indeed, a recent study by Flint et al (1986) showed that the frequency of α-thalassaemia exhibited an altitude and latitude-dependent distribution that correlated with the presence of malaria endemicity throughout Melanesia. Thus, the thalassaemias appear to confer some resistance to malaria, and this is responsible for the high frequency of the genes carrying them. In turn, the effect of HbF may account for the protection afforded those individuals with abnormal globin genes before immune-mediated resistance becomes effective.

Development of the malaria culture system allowed study of thalassaemic RBC in vitro. Pasvol et al (1977) showed that in β-thalassaemic trait, RBC HbF retards parasite growth but not invasion. Friedman (1979) reported normal growth in thalassaemic RBC, but the parasites were more susceptible to oxidant stress than were parasites growing in normal RBC. Findings such as this have led to several theories concerning the manner in which deficient globin production in thalassaemic RBC may retard parasite growth; at present, the inhibitory mechanism is not known (reviewed by Nagel & Roth 1989).

Haemoglobin C (HbC) is another β-chain variant and is common in Central West Africa

(Bodmer & Cavalli-Sforza 1976, Labie et al 1984), a finding interpreted as implying that the production of HbC confers some protection against malaria. However, field studies on infected individuals carrying the HbC gene have failed so far to support this; since homozygotes (HbCC) are relatively healthy, less selection pressure would be required to maintain the HbC gene frequency, which in some areas is as high as 28%. Thus, the deleterious effect of HbC-containing RBC is difficult to demonstrate in vivo. This has not been so in vitro, where Friedman et al (1979 b) first described severely decreased growth of P. falciparum in HbCC RBC when compared to HbAC and HbAA cells. This group showed that the selective pressure to increase HbC gene frequency may operate in double heterozygotes for HbS and HbC. Although oxygenated RBC containing HbC and HbC from the HbSC double heterozygotic condition are indistinguishable from normal RBC as hosts for plasmodia, parasites die rapidly under conditions of low oxygen, when HbSC cells behave like HbSS cells (Labie et al 1984).

HbC coexists with HbS in nearly all populations in which the HbS gene is common, with double heterozygotes sometimes surviving to reproductive age. It appears, therefore, that for the HbSC genotype, HbC increases the resistance conferred by HbS. This model could explain the expanded frequency of a second advantageous gene in a population in which one or more genes already provide resistance to malaria. Almost all populations exposed to endemic malaria exhibit more than one RBC defect that provides protection.

The gene for haemoglobin E (HbE) confers another β-chain mutation and is very frequent in South East Asia. Epidemiological studies have long suggested a causal connection between the high frequency of this abnormal haemoglobin and malaria (Flatz 1967). Nagel et al (1981) demonstrated a moderate decrease in growth of P. falciparum in RBC from homozygotes (HbEE) but normal growth in heterozygous HbAE RBC. It is thought that HbE, a somewhat unstable haemoglobin (Frischer & Bowman 1975), may enduce oxidative damage to parasites by generating free radicals. More recently, significantly higher levels of anti-malarial Abs and lower parasitaemias have been found in carriers of HbE as compared to HbAA individuals from the same endemic regions (Vernes et al 1986). This may be due to an increased rate of phagocytosis of HbEE and HbAE RBC by human monocytes than for infected normal RBC (Bunyaratvej et al 1986), but this putative mechanism of action has yet to be substantiated.

(c) Predisposition to infection

In at least one rodent model of malaria, P. chabaudi in inbred mice, which is used in this

study, susceptibility to infection seems to be under genetic control by a single , dominant, autosomal, non H-2-linked gene (Stevenson et al 1982,1988). Susceptible mouse strains, such as A/J, develop a fulminating parasitaemia, with most animals dying within 10 d. Resistant hosts, such as C57 BL/6 and NIH mice, develop a moderate level of peak parasitaemia, eliminate the acute infection in five weeks, and are immune to reinfection. The gene product or mechanism involved in conferring this protection has not been established, but it is related to the development of splenomegaly, and is more effective in the female sex (Stevenson et al 1982). The factors underlying severe disease and death due to malaria are poorly understood in man, and host polymorphisms related to this, apart from in the RBC, are not recognised. Possible sites where this may be expected are the recently reported endothelial ligands, e.g. ICAM-1 (Berendt et al 1989), for the sequestering cytoadherence Ag of the Plasmodium-infected RBC.

The effect of the host's diet, or nutritional state, on malaria infections is not fully understood, and little studied. Most of what is known comes from rodent malaria models, in which diet changes have been shown to be a variable in the host-parasite system (Gilks et al 1988). If rodents are kept on a diet of milk only, an otherwise severe malaria infection is markedly inhibited (Maegraith et al 1952), but this suppression of parasitaemia can be abolished by supplementation with PABA (Hawking 1954)(milk is deficient in PABA). This has also been shown for P. knowlesi infections in rhesus monkeys. These results are consistent with the anti-malarial effects of sulphonamides, whose mode of action in organisms unable to utilise exogenous folic acid is competition with PABA for the enzyme dihydropteroate synthetase (Peters & Howells 1978). The inhibitory effect of a PABA-deficient diet is certainly operative against the asexual erythrocytic stages and perhaps the exo-erythrocytic stages as well. To overcome diet-related suppression of malaria in experimentally infected mice and rats, where a patent infection is desired, some laboratories routinely add PABA to the drinking water.

In humans, the inhibitory effect of an exclusively milk diet provides another plausible explanation of why the malaria infection rate in infants under 12 months in malarious areas is lower than would be expected (Phillips 1983). The overall nutritional status of an infected individual may affect the course and outcome of clinical disease. Refeeding malnourished children during famine relief has been shown to result in outbreaks of clinical malaria within a few days of individuals starting on the nutritional programme -so called 'feeding malaria' (Murray et al 1981).

As it is known that protein-deficient diets can depress P. berghei infections in rats

(Gilks et al 1989), there may be a correlation between protein intake and parasitaemia, which would in part explain why famine relief in humans is sometimes accompanied by outbreaks of malaria.

In areas where malaria is endemic, pregnancy, even in immune women, is accompanied by increased parasite prevalence and greater parasite densities (Gray & Anderson 1979). Similarly, mice immune to P. berghei tend to relapse during pregnancy (Van Zon & Eling 1980). Pregnant women (especially primagravida) apparently lose anti-malarial immunity, since they contract acute falciparum malaria at rates 4-12 times those of their non-pregnant counterparts (Gilles et al 1969). Such women can become severely anaemic, a complication that can be prevented with anti-malarial therapy. Since P. falciparum infected RBC sequester in the placenta, and can induce cellular hyperplasia in the intervillous spaces, foetal health may also be compromised. Indeed, spontaneous abortion and low birth weight may occur, both characteristic of maternal malaria (McGregor & Avery 1974).

1.10.2 Non-specific immunity

Prior administration of agents unrelated to Plasmodium has been shown to confer non-specific protection against malaria. Most of the experiments showing acquisition of non-specific immunity have been on murine malarias, and the protection conferred is not absolute; in many cases it is manifested as an increased prepatent period of the malaria infection, and the survival of animals from an otherwise lethal infection.

Inoculation with killed Propionibacterium acnes (formerly Corynebacterium parvum) confers a degree of protection against subsequent challenge with P. berghei (Nussenzweig 1967, Murphy 1981), P. vinckei (Lucia & Nussenzweig 1969, Cottrell et al 1977), or P. chabaudi (Clark et al 1977). Previous exposure to Mycobacterium bovis (BCG) can also provide resistance to murine malarias (Clark et al 1976, Murphy 1981), although it can lessen the protection conferred by vaccination with P. berghei killed by formalin treatment (Smrkovski 1981).

The agent of rat infectious anaemia also protects rats against P. chabaudi (Thoongsuwan et al 1978). A range of substances has been shown to affect the course of murine malaria infections. These include Con A, lipopolysaccharide, and diethylstilboestrol (Cottrell et al 1977), endotoxin (Martin et al 1967), magnesium silicate in calcium phosphate gel (Michel et al 1982), Coxiella burnetti extract (Clark 1979), and freeze-thawed Toxoplasma gondii tachyzoites (Omata et al 1981).

The mechanism by which an unrelated Ag can confer protection against malaria infection may involve the activation of macrophages, either to increase phagocytosis (Nussenzweig 1967), or to release the macrophage autocrine factor, tumour necrosis factor (Clark et al 1981, Taverne et al 1981, Clark & Hunt 1983, Dockrell & Playfair 1983). This latter protective factor has been reported to mediate intraerythrocytic either through the release of superoxide ions (Allison & Eugui 1982), or nitric oxide (Green et al 1990). The mechanisms of intraerythrocytic destruction of malaria parasites are discussed in 1.11.

Some evidence that the growth of P. falciparum in vitro can be retarded by a non-Ab, non-dialysible factor has been observed by Jensen et al (1983). As pRBC death was caused during the resolution or crisis period of an acute, primary malarial infection in laboratory models (when parasites appear stunted in their intracellular development), this factor was called 'crisis form factor'. This has since been identified in serum samples from immune Sudanese adults by Jensen et al (1982,1983), who found a stronger association between clinical immunity and in vitro serum inhibition with this crisis form factor than with IgG (although some Ig fractions from immune individuals could induce crisis form pRBC in vitro).

1.11 Acquired immunity

The immune response and subsequent resistance to various species of Plasmodium has been studied extensively. Acquired immunity is a general feature of the host response to malaria, and is more complex than innate immunity. It is largely species-specific and has clear strain differences; in addition, it is stage-specific, affecting the sporozoite, which stays in the peripheral circulation for a brief time only, the exo-erythrocytic stage, the asexual erythrocytic stage (against which immunity is mainly directed), and gametocytes (immunity to which would interupt transmission).

Several aspects of the host's immune response to malaria have been observed and their relative importance in parasite clearance and subsequent resistance to reinfection investigated. Techniques available to dissect the immune response include selective depletion of part of the host's immune system; transfer of cells, serum or other factors from immune to non-immune hosts; and in vitro studies.

Evidence of acquired immunity to malaria infection in humans starts to appear at the beginning of the second week of patent parasitaemia and is manifested as a reduction in the reproduction rate and in the number of pRBC in the blood. After a variable period,

the immunity decreases the parasitaemia to undetectable levels (McGregor 1956). There is not a direct correlation between parasitaemia and symptomatology, however, and the immune response can occasionally diminish the clinical manifectations of infection even in the presence of considerable blood-borne parasite burden. In malaria holoendemic areas, acute malaria is fundamentally a disease of children, who suffer repeated and severe attacks that become increasingly mild with time until turning into frequent but low parasitaemias, with benign or no symptoms in the immune adult (Wilson et al 1950, McGregor 1960). For a long time, it was believed that acquired resistance to malaria was exclusively of the premunition type and that , as such, it waned after plasmodia were eliminated from the host. Nevertheless, the presence of sterilising immunity has been verified in rodents, where, for example, mice protected against lethal P. berghei by either chemotherapy (Cox 1964), or vaccination (Playfair 1977) are likewise solidly immune. The presence of sterile immunity in humans has not been investigated experimentally yet, but epidemiological studies do not indicate that it exists.

(a) Host genetic factors and the immune response

It is well known that some genetic traits can influence the innate resistance of certain populations to malaria, as exemplified by the genes controlling the expression of constituents of human RBC which affect the penetration of merozoites into, or their growth in, RBC (see 1.9). However, the role played by the genes controlling the host immune response in regulating host susceptibility to malaria infections is as yet ill-defined. Limited data link the human lymphocyte antigen (HLA) haplotype to malaria endemicity, but this is a poorly researched area. In a study carried out in Sardinia, Piazza et al (1972) found that the HLA haplotype AZ-BW17 was more frequent in lowland villages exposed to malaria than in highland villages never exposed to malaria. In another study in north east Tanzania, the same AZ-B17 (together with the AZ-AW30) haplotype was found to be more frequent in individuals with high titres of Abs against P. falciparum pRBC (Osoba et al 1979). The significance of this possible linkage between resistance to malaria and HLA class I Ags is not known.

(b) The immune response to sporozoites

For many years, there was no evidence of acquired immunity to the natural inoculation of sporozoites in humans under normal conditions. Viable sporozoites are now known in

both animals and man to induce a detectable Ab response. Nardin et al (1979) detected rising levels of anti-sporozoite Abs with increasing age in serum samples from Gambian communities. They found that > 90% of the adult population had Abs to sporozoites of P. falciparum in their blood. Less than half the children had this Ab, indicating that repeated exposure to sporozoites over many years is necessary to induce its formation. There is also indirect evidence that during natural infection, the anti-sporozoite Ab response is suppressed by acute blood stage infection (Orjih & Nussenzweig 1979).

Attenuated sporozoites have been shown to be strongly immunogenic. The induction of immunity by sporozoites inactivated by u.v. light or formalin, or mechanically disrupted, was first demonstrated in avian malaria (Mulligan et al 1941). The most successful form of vaccination has involved the use of irradiated sporozoites (Vanderberg et al 1970), and has been investigated extensively in P. berghei malaria, which is uniformly lethal in mice. More than 90% of animals were immune to unattenuated sporozoite challenge after three or more immunisations with irradiated sporozoites (Nussenzweig et al 1969 a); protection was maintained for almost two months and then declined progressively. Vaccination with irradiated sporozoites has proved relatively ineffective against simian malaria (Collins & Contacos 1972, Gwadz et al 1979), but protection by repeated injection of irradiated sporozoites has been observed in human malaria (Rieckmann et al 1974, Clyde et al 1975). Immunity was species-specific and lasted for about three months after exposure to P. falciparum sporozoites and up to six months after P. vivax immunisation.

Protection induced by sporozoite vaccination is strictly stage-specific. Thus, immune, vaccinated mice challenged with homologous strain P. berghei pRBC suffer fatal infections indistinguishable from those in control animals (Nussenzweig et al 1969 b). In addition, rodents immunised effectively against sporozoites were found by Foley & Vanderberg (1977) to remain susceptible to infections with exo-erythrocytic schizonts. In this regard, cross reactivity has been reported between some species of murine malaria (Nussenzweig et al 1969 b, 1972 a), but for the primate malarias only interstrain specificity has been observed (Chen et al 1976).

Mice immunised with repeated inoculations of irradiated sporozoites clear challenge sporozoites from their blood more quickly (Nussenzweig et al 1972 b). Similarly, in recipients of serum collected from immunised mice, there was an increased rate of clearance, and the prepatent period of the challenge infection was prolonged considerably (Nussenzweig et al 1972 b), implying a role for Ab.

Protective MAbs directed against the surface coat sporozoite Ag of P. berghei have been isolated (Yoshida et al 1980) and the Fab fragment shown to be active in protection experiments (Potocnjak et al 1980), indicating that the Ab functions by blocking sporozoite attachment to the hepatocyte receptor cells (Hollingdale et al 1982). MAbs recognising analogous cell surface determinants in P. knowlesi infections have been isolated and are protective (Cochrane et al 1982). Nardin et al (1982) used MAb treatment to reduce the infectivity of P. falciparum sporozoites to splenectomised chimpanzees, since when other MAbs have been shown to block transmission of the disease (Hollingdale et al 1984, Miller et al 1986).

A correlation between clinical immunity and the levels of anti-sporozoite Ab is often observed but is not invariable. Thus, mice may show resistance in the absence of detectable Ab during the early stages of immunisation (Spitalny & Nussenzweig 1973) or when splenectomised before vaccination (Spitalny et al 1976). Such findings are suggestive of a role for cell-mediated immunity in acquired resistance to sporozoite infection. This and other studies by the same workers (Spitalny et al 1977) implied a role for the thymus in immunity. They found that thymectomised, irradiated bone marrow-reconstituted mice or nude (congenically athymic) mice did not develop sporozoite neutralising Ab or clinical immunity after inoculation with irradiated sporozoites. However, adoptive transfer of thymus cells restored the capacity of immunosuppressed animals to synthesise Ab and become immunised; from this it can be concluded that T cells have at least a helper function in anti-sporozoite immunity. That there may be additional cell-mediated effector mechanisms was suggested by the finding that B-cell-suppressed mice became clinically immune after sporozoite vaccination (Chen et al 1977), and the adoptive transfer of immunity by T cells if accompanied by further boosting (Verhave et al 1978). The mechanisms underlying such acquired resistance to the sporozoite stages of plasmodia are as yet undefined.

(c) The immune response to exo-erythrocytic stages

Until recently, there was little known about any protective immune response to the exo-erythrocytic stages of malaria and it was held that only after exo-erythrocytic schizont rupture and merozoite release that this stage of the parasite provokes a cellular response. Phagocytic infiltration of P. cynomolgi infected livers and engulfment of large numbers of merozoites was observed to support this view (Shortt & Garnham 1948 a, Garnham & Bray 1956, Lupascu et al 1967). However, evidence in support of some

clinical immunity was reported by Beaudoin et al (1975). Rats treated with chloroquine to suppress blood stage parasites and simultaneously administered viable P. berghei sporozoites showed fewer exo-erythrocytic forms on challenge than did control animals. Whether this phenomenon of interference occurs in man is unclear, as people living in holoendemic areas and receiving chemotherapy directed against asexual erythrocytic stage parasites are exposed to repeated liver stage infections, yet remain susceptible to malaria when prophylaxis is suspended (Cohen & Lambert 1982, Hollingdale 1985).

That exo-erythrocytic malaria parasites could be killed by non-specific immune mediators was demonstrated by Jahiel et al (1968 a & b). As it became clear that such mediators were released from lymphocytes following antigenic stimulation, the issue of lymphokine-mediated immunity to this stage of the malaria life cycle was readdressed. Recombinant IFN was shown to inhibit the in vitro growth of liver stage parasites of P. berghei, P. cynomolgi and P. vivax (Ferreira et al 1986, Maheshwari et al 1986), relatively small quantities of IFN-γ being required. Schofield et al (1987 a) showed the lymphokine to be acting hormonally, by binding to specific hepatocyte receptors and inducing intracellular death. To test the hypothesis that IFN-γ secretion was required for exo-erythrocytic immunity, immunised mice and rats were injected with a neutralising MAb to rodent IFN-γ after challenge with live sporozoites. This treatment abrogated immunity, as determined by growth of liver stages, leading to the production of parasitaemia (Schofield et al 1987 b). Moreover, immunity was abolished by depleting immunised mice of their Ly-2^+ cells (Schofield et al 1987 b, Weiss et al 1988). It is considered that upon parasite challenge, the Ly-2^+ T cell subset releases IFN-γ which then inhibits the malarial exo-erythrocytic development. Ly-4^+ T cells cannot be the source of IFN-γ as their depletion does not affect host immunity to this stage of the parasite. These findings do not, however, exclude the possibility that Ly-2^+ T cells are also directly cytotoxic for liver stage parasites (reviewed by Schofield 1989).

Recently, the results of immunising mice with irradiated sporozoites of P. berghei and P. yoelii have revealed that the host control of protective immunity to the resultant liver stage parasites is different in the two rodent malarias, and suggest the presence of another, Ly-2^+-independent, mechanism in cellular immunity to the exo-erythrocytic stages of malaria (Weiss 1990). Initially, it appeared that all mice inoculated with sporozoites required Ly-2^+ T cells for protection, as depletion of the Ly-2^+ T cell

subset by injection of anti-Ly-2 MAbs eliminated protective immunity to P. berghei (Schofield et al 1987 b) and P. yoelii (Weiss et al 1988). Now there is evidence that there is genetic control of immunity, and , depending on the mouse strain, Ly-2^+ T cells may or may not be critical effectors, and a second, undefined, immune effector arm may protect infected animals (Weiss et al 1989). This was discovered when mice of BALB/c, B10.BR and B10.Q strains were simultaneously immunised with P. yoelii sporozoites and depleted of Ly-2^+ T cells, whereupon the BALB/c mice died but the B10.BR and B10.Q mice remained protected. This implied the activation of an effector mechanism independent of Ly-2^+ cells which is sufficient to confer protection to P. yoelii in certain strains of mice.

Similar evidence that this newly discovered cellular effector arm is limited to certain different mouse strains has now been seen in P. berghei (Weiss 1990). It appears that every pairing of mouse strain and Plasmodium species generates its own pattern of protective immune responses, dependent on the mouse genetic background. Although there is a degree of Ir gene control, congenic strains of mice may show high or low degrees of protection, depending on the make-up of background genes. Such variety should be expected in the acquired immunity to human malarias, and may complicate the development of universally applicable vaccines.

(d) The immune response to asexual erythrocytic stages

The immune response of mammalian hosts to the asexual erythrocytic forms of Plasmodium and consequently the course of blood stage infection depends both on the species of parasite involved and on the host species. In some host-parasite combinations, e.g. P. knowlesi in the rhesus monkey, there is no effective immune response and the disease is rapidly fatal. In other experimental models, such as P. berghei in the rat, a sterilising immunity is induced, characterised by a transient parasitaemia followed by parasite elimination and long lasting resistance to further challenge. In most natural infections, including human malaria, the immune response is characterised by the acquisition of partial immunity which controls but does not eliminate the infection, which persists at low density over long periods. Much information is available on different aspects of the host immune response to asexual erythrocytic stages of malaria. However, the roles that each plays in the resolution of, and subsequent protection from, reinfection are debatable. Only some immune responses may be protective, whilst others may help the parasite to evade a protective immune

response (Anders 1986), or may give rise to immunopathological reactions, harmful to the host (Grau et al 1987). Due to the complexity of the host response, the relative importance of various immune mechanisms very probably varies widely between different host-parasite models, and at different times during the course of infection.

Acquired immunity to blood stage malaria is predominantly species- and stage-specific. Clinical immunity of humans to malaria shows species specificity and to some extent strain specificity (Jeffrey 1966). The immune response to the asexual erythrocytic forms is specific to that stage and does not prevent exo-erythrocytic development of malaria parasites in primates, but suppresses the subsequent phase of erythrocytic multiplication (Garnham 1970, Richards et al 1977). This stage and species specificity implies that acquired immunity to the asexual blood parasites is mediated predominantly by mechanisms involving specific effector processes.

(i) The role of the humoral response

The asexual erythrocytic stages of malaria can stimulate a strong Ab response. Malarial infection produces markedly increased levels of serum Ig (Cohen et al 1961), and although the production of specific Abs appears to contribute to the clearance of at least some species of Plasmodium from their hosts (e.g. Freeman et al 1980), most of the Ig synthesised (up to 95%) has no apparent reactivity with plasmodial Ags (Targett & Voller 1965, Abele et al 1965, Cohen & Butcher 1969). The Ags recognised by this non-specific Ig may include Ags of lymphocytes and RBC, complement, rheumatoid factor, and nuclear components (Deans & Cohen 1983). The frequent presence of autoantibodies is related to the immunopathology of malaria infection (1.12). In general, the correlation between total anti-malarial Abs and protective immunity and/or clinical status is poor, indicating that many of the Abs formed have no protective effect (Brown 1969). There is, however, conclusive evidence that specific Abs play a major role in controlling the asexual erythrocytic development of plasmodia (Cohen 1979). Specific anti-malarial Ab titres rise with repeated P. falciparum blood stage infection and correlate to some degree with the clinical immune status of the host (McGregor & Williams 1978). More specific evidence comes from passive transfer experiments.

The passive transfer of protection with immune sera has been demonstrated, firstly by Coggeshall & Kumm (1937), who transferred protection in serum collected from Aotus monkeys which had been infected with P. knowlesi and then drug-cured. Subsequently, protective activity in serum has been demonstrated in the human system (Cohen et al

1961, Cohen & McGregor 1963), with protective activity largely being confined to the IgG-rich fraction, although anti-malarial Abs are also found in IgM and IgA classes. Immune IgG mediated protection has also been shown in various animal models (Diggs & Osler 1969, Diggs et al 1972 a, Phillips & Jones 1972, Green & Kreier 1978, Reese & Motyl 1979). Both the decrease in parasitaemia following passive Ig transfer, and the observation that protective Abs did not destroy intracellular parasites indicated that merozoites and mature schizonts are likely to be the targets of the protective IgG response (Cohen & McGregor 1963). Treatment of serum with rabbit anti-rat IgG removes the protective activity of rat hyperimmune serum (Diggs & Osler 1969).

Considerable variation is seen in passive transfer experiments, especially in rodent malarias, and appears to be related to the timing of the collection of serum from donor animals and to the dose of immune serum given to recipient challenge animals. Total protection against homologous parasite challenge has been reported in recipients of hyperimmune serum (Jayawardena et al 1975 a, Golenser et al 1975), but it is more usual to observe the protective activity of immune serum as a delay in the onset of a patent parasitaemia of recipients (Briggs et al 1968, Diggs & Osler 1969, Brown & Phillips 1974, Jayawardena et al 1978). In some host-parasite systems, recipients of immune serum are able to clear an otherwise lethal infection (Diggs & Osler 1969, Brown & Phillips 1974), whilst in others passive transfer enhanced the later stage of parasitaemia (Jerusalem et al 1971, Jayawardena et al 1978), probably by depressing the protective immune response of the immunocompetent recipient animals at the start of infection. Using P. berghei, several studies have shown that prior incubation of hyperimmune serum with the parasite only protects challenged rats if a small volume of serum is inoculated with the parasites simultaneously (Brown & Phillips 1974, Hamburger & Kreier 1975, Diggs & Osler 1975, Golenser et al 1975). Washing of pRBC after serum incubation may reduce the parasite-specific Abs to subcritical levels, or the Ab may be effective only against limited stages of the parasite. Indeed, subinoculations from hyperimmune serum recipients infected with 10^3 P. berghei pRBC have revealed that such serum is effective against only one stage of the asexual erythrocytic cycle (Golenser et al 1975). Quinn & Wyler (1979 a) reported that in large challenges of immune serum recipients, P. berghei developed normally until the late trophozoite stage when it sequestered. However, after RBC invasion, the number of early ring stages was reduced compared to that seen in recipients of normal serum.

The passive transfer of serum collected from host animals at different times during or

after infection has shown that the protective activity of serum is highest at the time of parasite elimination (Phillips & Jones 1972, Murphy 1979), and that protective activity diminishes rapidly after parasite clearance (Hamburger & Kreier 1976, Murphy 1979).

A role for B lymphocytes has been shown in some host-parasite models, e.g. P. yoelii in mice, where recovery from primary infection is Ab-dependent. Congenitally B cell-deficient mice have higher parasitaemias and more prolonged infections than do normal controls (Jayawardena et al 1979), and in some instances, fail to control infection resulting in fatality (Hunter et al 1979 a). Similarly, normal mice selectively depleted of B cell function by treatment with goat anti-mouse μ-chain Ig die from a usually self-limiting infection with a non-lethal strain of P. yoelii (Weinbaum et al 1976 b, Roberts et al 1977). After drug cure and rechallenge, B cell-deficient mice can survive, but suffer from a chronic low level parasitaemia (Roberts & Weidanz 1979).

The role of specific Abs in immunity to malaria has also been indicated by B cell transfer experiments in which B cell enriched populations of immune spleen cells have been shown to be effective in adoptively transferring protection. This has been observed for P. chabaudi and P. berghei infections of mice (McDonald & Phillips 1978, Ferraroni & Speer 1982), and for P. berghei infected rats (Gravely & Kreier 1976).

Ultrastructural studies indicate that the later stages of P. knowlesi (Brown & Hockley 1966) and P. falciparum (Langreth & Reese 1979) disrupt the host RBC membrane. Thus, late trophozoites, schizonts, and possibly also merozoites prior to RBC rupture, may be available to the host immune system for Ab recognition and attachment (Diggs & Osler 1975). Although several possible roles for anti-malarial Abs in the clearance of plasmodia have been investigated, the precise mechanism by which Abs confer protection has not been clearly established.

The in vitro growth of P. knowlesi (Cohen & Butcher 1970) and P. falciparum (Reese & Motyl1979) in the presence of immune serum or its extracted Ig is slowed or blocked at the time of schizogony, thereby preventing invasion of new RBC by merozoites which would otherwise be released from infected cells (Phillips et al 1972, Miller et al 1975 a, Mitchell et al 1976, Cohen et al 1977). In simian and human malaria, more recent work has shown that MAbs directed against specific merozoite and schizont-derived Ags inhibit the in vitro growth of the parasite in culture (Epstein et al 1981, Perrin et al 1981, Deans et al 1982, Miller et al 1984, Saul et al 1984, 1985, Banyal & Inselburg 1985, Schmidt-Ullrich et al 1986, Udomsangpetch et al 1986). All of these in vitro

studies suggest, but do not prove (Fandeur et al 1984) that Abs have a protective role in immunity to malaria. Inhibition of merozoite invasion generally occurs by agglutinating merozoites (Butcher & Cohen 1970) before (Cohen et al 1969) or after (Butcher & Cohen 1972, Miller et al 1975 a) release from the ruptured cell. This inhibition is species-specific, complement independent, and mediated by IgM and IgG and its $(Fab')_2$ fragment, but not by monovalent Fab (Cohen & Butcher 1970). Due to this latter property and also the ineffectiveness of these Abs against the intracellular stage of the parasite, they have been likened to viral neutralising Igs (Cohen & Butcher 1970). The site of binding of such invasion-blocking Abs is thought to be one or more merozoite surface determinants (Epstein et al 1981, Deans et al 1982). However, as the activity of anti-P. berghei hyperimmune serum is not adsorbed out by nRBC, it has been concluded that inhibitory Ab activity is not directed against the parasite receptor on the pRBC (Golenser et al 1975). In this study, free P. knowlesi merozoites were observed invading RBC in the usual manner in the presence of immune serum inhibiting growth of the parasite.

In vitro agglutination of P. knowlesi merozoites has been shown to correlate well with specific sterilising immunity induced by merozoite vaccination (Butcher et al 1978), but not always with functional immunity (Miller et al 1977). That anti-merozoite Abs can control blood stage malaria has been confirmed by passive transfer studies using MAbs raised against P. yoelii (Freeman et al 1980). Ascitic fluids from mice bearing two different hybridomas secreting MAbs reacting with a single merozoite component, both protected mice from a lethal P. yoelii infection, although they did not prevent the initial rise in parasitaemia. In contrast to its protective activity during primary infection, the presence of Ab capable of inhibiting growth in vitro in the serum of a P. knowlesi infected monkey does not always correlate with immunity to reinfection (Cohen 1977, Chulay et al 1981).

Apart from the agglutinating activity of inhibitory Ab, other serum Abs offer protection against asexual erythrocytic malaria parasites. Opsonising Abs, which promote the phagocytosis of pRBC, have also been detected in vitro (Zuckerman 1945, Khusmith et al 1982). Using P. berghei, Hunter et al (1979 b) showed that the opsonic activity in immune rat serum is mainly associated with the IgG fraction, and only weakly with the IgM fraction. Opsonins against P. knowlesi are variant specific (Brown et al 1970 a). Opsonisation may be important in vivo in clearing parasites early in infection, but later, in P. berghei infections at least, phagocytosis is inhibited by serum factors,

possibly circulating immune complexes (Shear et al 1979, Brown & Kreier 1982). Quinn & Wyler (1979 a) found that the protection afforded by passively transferring hyperimmune anti-P. berghei serum was not opsonin-associated, and suggested that serum Ab inhibitory to merozoite invasion confers the protection observed in passive transfers. This may be linked to the fact that opsonins promote the destruction not of merozoites, but of the more mature asexual blood stages, the late trophozoites and schizonts, and that the early growth of the intraerythrocytic parasites seems to proceed normally in the presence of these Abs.

Cytophilic Abs have been detected in hyperimmune rat serum raised against P. berghei (Chow & Kreier 1972). On passive transfer, the fraction of hyperimmune serum containing cytophilic Ab has no discernible effect, but did have a strongly synergistic effect when combined with the opsonising Ab fraction (Green & Kreier 1978). This is little surprising since both types of Ab induce specific phagocytosis of Ab-coated pRBC in vitro by macrophages, monocytes and polymorphonuclear leukocytes (Chow & Kreier 1972, Tosta & Wedderburn 1980, Hunter et al 1979 b, Shear et al 1979, Celada et al 1982,1983, Khusmith et al 1982). Khusmith & Druilhe (1983) have also observed cytophilic Abs in sera from P. falciparum immune individuals. In an in vitro test, IgG was bound to peripheral blood monocytes, which were then able to attach P. falciparum schizonts and merozoites, but to phagocytose only merozoites (Khusmith et al 1982). The level of phagocytosis observed correlated with the immune status of the patients whose sera were tested.

Studies on Ab-dependent cellular cytotoxicity (ADCC) have implicated K cells, monocytes and polymorphonuclear leucocytes in destruction of Ab-coated pRBC or parasites. For example, Brown & Smalley (1980) demonstrated ADCC against P. falciparum infected RBC (reviewed by Deans & Cohen 1983). The part played in protective immunity in vivo of ADCC or opsonisation (Ab-dependent phagocytosis) has not been established. From conflicting reports on the importance of opsonisation in vivo with murine and primate malarias (Deans & Cohen 1983), it would appear that the effector mechanisms that operate in naturally acquired anti-malarial immunity may vary in different host-parasite combinations.

Serological tests such as complement fixation, precipitation, agglutination and IFAT (reviewed by Fife 1972) have been used to detect specific anti-malarial Abs. These tests have been used as tools in immunodiagnosis for sero-epidemiological studies to examine the effect of control measures on the immune status of human populations

(Brögger et al 1978) and to study the kinetics of Ab synthesis during experimental malaria infections. Serological cross reactions between species of Plasmodium revealed by such tests (Voller et al 1966, Cox & Turner 1970) cannot always be correlated with cross immunity in vivo (Voller et al 1966), as much of the specific Ab produced during infection may have non-protective function. During malaria infections, serological examination has revealed that Ab levels are raised initially and remain elevated for an extended period before diminishing. This has been observed in experimental rodent malaria infections using IFAT (Cox et al 1969, Cox & Turner 1970), indirect haemagglutination (Weinbaum et al 1978), gel double diffusion (Zuckerman et al 1969), and radial immmunodiffusion (Hunter et al 1979 a). Ab titres have also been measured in human volunteers infected with malaria, when a similar pattern of Ab production was observed using IFAT (Kuvin et al 1962, Tobie & Coatney 1964, Collins et al 1971).

A survey of a human population living in a P. falciparum endemic area, using double diffusion in gel, was undertaken by McGregor & Wilson (1971). Anti-malarial precipitins were present in the serum of virtually all newborn children, yet their prevalence had fallen rapidly after the first three months of life (presumably indicating a decay in passively acquired maternal Ab). In the second year of life, precipitin levels rose rapidly and by the 5-6 year age band, 95% of children had demonstrable precipitins in their serum.

(ii) The role of complement

In both experimental P. falciparum and P. vivax infections (Dulaney et al 1948, Neva et al 1974) and naturally occurring P. falciparum infections in man (Greenwood & Brueton 1974, Srichaikul et al 1975), depressed complement levels are observed. Fogel et al (1966) found that the reduction of complement levels in P. knowlesi, P. berghei and P. gallinaceum infections was associated with schizont rupture, and Cooper & Fogel (1966) suggested that this may be due to the fixing of complement by circulating Ab as it comes into contact with free merozoites.

In experimental P. vivax infections in man, a decrease in the levels of complement was observed during relapse. It was correlated directly with the degree of parasitaemia and the presence of complement fixing Ab, with the lowest detectable complement titres within a few hours of schizont rupture and with peak fever (Neva et al 1974). Glew et al (1975) showed that in P. coatneyi infections of rhesus monkeys, reduced complement levels were limited to C1, 2, & 4, whilst depletion of C3-9 by treatment of monkeys

with cobra venom factor did not alter either the degree or course of parasitaemia (Atkinson et al 1975). In addition, cobra venom treatment of rats had no discernible effect on the course of P. berghei infection (Diggs et al 1972 b), nor did infection of mice congenitally deficient in C5 (Williams et al 1975).

The weight of evidence outlined indicates that complement has a negligible role in the immune response to malaria and may not contribute to any extent to parasite clearance. The depletion of early complement components shortly after schizont rupture is thought to be due to complement fixation by Ag-Ab complexes (Greenwood & Brueton 1974) and may contribute immunopathologically to the vascular damage observed in P. falciparum infections (Greenwood & Brueton 1974, Srichaikul et al 1975).

(iii) The role of the cell-mediated response

Although most investigations agree that both cellular and humoral factors are involved in the slow development of immunity to malaria, the role of cell-mediated responses is incompletely understood and has been explored mainly in murine malarias. There has been an accumulating weight of evidence, however, that suggests that anti-malarial immunity is mediated by additional mechanisms which can act together with, or independently of protective Abs.

The presence of a functional thymus plays a major part in tha development of immunity to malaria. Nude mice (nu/nu) which are congenitally athymic (and therefore deprived of mature T lymphocytes) are unable to clear an infection with a strain of P. yoelii from which intact mice recover (Clark & Allison 1974, Weinbaum et al 1976 b, Roberts et al 1977). The parasitaemia in nude mice escalated to 76-80%, when all animals died. Moreover, it was shown that after termination of acute disease by prolonged clindamycin treatment, parasitaemia recrudesced in nude mice to give an ultimately fatal infection; however, recrudescence was not observed in nude mice which had been grafted with thymic tissue or received a passive transfer of hyperimmune serum (Roberts et al 1977). This consistent aggravation of non-lethal malaria infection by deprivation of T cells was also demonstrated with P. chabaudi, where nude mice on a CBA background developed lethal infections (Eugui & Allison 1980, Cavacini et al 1986).

In most studies, malaria infections of thymectomised hosts have resulted in higher, more persistent parasitaemias, with increased host mortality and anaemia in comparison to sham thymectomised controls. The first observations showing the importance of T lymphocytes in recovery from malaria infections were made by Brown et al (1968 a). Rats inoculated with P. berghei when 13 weeks of age recovered from infection, but in

neonatally thymectomised rats, there was a higher and more prolonged parasitaemia and an appreciable mortality; these observations were independently confirmed by Stechschulte (1969). Similar results have been attained in P. berghei infections of thymectomised mice (Cottrell et al 1978) and hamsters (Chapman & Hanson 1971); P. yoelii infections of thymectomised, lethally irradiated and B cell-reconstituted mice (Jayawardena et al 1977), and P. chabaudi chabaudi and P. c. adami infections of thymectomised mice (McDonald & Phillips 1978, Cavacini et al 1986). Resolution of acute infection in recipient adult-thymectomised mice following adoptive transfer of immune spleen cells was dose-dependent (Cavacini et al 1986). P. chabaudi infection was chronic but not lethal in thymus-depleted mice. A conflicting finding was reported by Wright (1968) who showed that neonatal thymectomy of golden hamsters increased their survival time of infection with P. berghei. This model system is unusual, however, in that normal golden hamsters succumb to P. berghei infection when the parasitaemia is still very low, and post mortem revealed cerebral haemorrhages. In contrast, thymectomised animals died when the parasitaemia was much higher, and showed no cerebral sequelae. Prolonged survival in fulminating infections was also observed in hamsters depleted of T cells by treatment with anti-thymocyte serum (ATS) (Wright et al 1971) and adult thymectomised and anti-lymphocyte serum (ALS) treated mice (Sheagren & Monaco 1969). Thus, these early observations implicated T cells in the occurrence of cerebral malaria, now attributed to the toxicity of a key mediator of inflammation, TNF (Clark 1987).

T cell depletion of potential host animals has also been achieved through treatment with ATS. Spira et al (1970) showed that ATS treatment of rats prior to P. berghei infection suppressed both natural and age resistance to the parasite, whilst ATS administration after clearance of infection failed to enhance rat susceptibility to reinfection. In contrast, Brown & Phillips (1971) found that rats chronically infected with P. berghei had a marked recrudescence upon ATS treatment. T cell deprivation has been shown by Eling (1979) to have a maximal effect when brought about six hr after challenge of P. berghei infected, drug cured mice, who implied from this that T cells play an important role in resistance to malaria early in primary challenges. ALS treatment of infected mice has given similar suppression of immunity as that shown for ATS, i.e. increased mortality (Bruce-Chwatt et al 1972) or lengthened duration of patent infection and heightened parasitaemia (Barker & Powers 1971, Bruce-Chwatt et al 1972), especially recrudescences (McDonald & Sherman 1980).

The role of T cells in the immune response to malaria has been studied by evaluating differences in the T cell response to lethal and non-lethal infection. Jayawardena et al (1975 b) reported massive T cell mitosis and proliferation in spleens of mice infected with a non-lethal strain of P. yoelii, but not in lethal P. berghei infections. In addition, the in vitro response of splenic T cells to malaria Ag was considerably reduced early in lethal P. yoelii strain infections. Again, this indicates that T cell activation may occur early in the course of infection in the natural host. Further evidence for a role of T cells in immunity to blood stages of murine malaria parasites came from observations of Finerty & Krehl (1976) that infection with the lethal 17XL strain of P. yoelii could be converted into a non-lethal infection by pretreatment with cyclophosphamide. This drug, administered 2 d before infection, increased delayed-type hypersensitivity to parasite Ags (presumably from the elimination of suppressor cells), but Abs against asexual erythrocytic stages were not detectable during the recovery period.

It has long been recognised that immunisation of rhesus monkeys against P. knowlesi requires complete Freund's adjuvant (Freund et al 1945, 1948, Targett & Fulton 1965, Brown 1971, Butcher et al 1978), which elicits cell-mediated immunity, and resistance to challenge is not well correlated with the presence of Abs inhibiting parasite reinvasion and replication in vitro (Langhorne et al 1979).

Adoptive transfer experiments using rodent models have indicated that animals receiving T cell-enriched spleen preparations are protected against subsequent challenge (e.g. McDonald & Phillips 1978). However, reconstitutions with cell preparations deficient of the T and/or B cell complement suggest that although some immunity to the asexual erythrocytic stages of malaria can be transferred to irradiated recipient mice by immune T cells alone, better protection is obtained when both T and B cells are transferred (Brown et al 1976, Gravely & Kreier 1976, McDonald & Phillips 1978, Jayawardena et al 1979, 1982, Brinkmann et al 1985, Fahey & Spitalny 1986), which is thought to be due to a synergistic effect (Jayawardena et al 1982). Such experiments have established the helper role for T cells in the synthesis of anti-malarial Ab (Brown & Phillips 1971, Brown et al 1976 a & b). This was confirmed by Jayawardena et al (1977), who found that intact mice produce high levels of specific anti-plasmodial Abs and a marked proliferative response in germinal centres; in T cell-deprived animals, however, IgG_1 synthesis is negligible, IgG_2 and IgM levels considerably reduced and the cortical cellular response severely impaired. The normal pattern of functional immunity was restored by reconstitution with syngeneic thymus cells.

Additional support for the role of T cell-mediated mechanisms in malaria has come from adoptive transfer studies in athymic nude mice. Recipients of immune T cells resolved their infections more rapidly and demonstrated lower peak parasitaemias than recipients of non-immune T cells. Protection was best achieved with the helper/inducer T cell subsets expressing the Ly-4^+ phenotype (McDonald & Phillips 1980, Cavacini et al 1986, Vinetz et al 1990), or with in vitro-propagated IL-2-dependent and P. chabaudi adami-specific T cell lines or clones of the Ly-4^+ phenotypes (Brake et al 1986, 1988). Moreover, recent studies have shown that depletion of the Ly-4^+ T cell subset in vivo through treatment with MAbs renders mice incapable of clearing the acute phase of infection (Süss et al 1988, Kumar et al 1989, Langhorne et al 1990).

Chickens rendered agammaglobulinaemic by combined immunological and chemical bursectomy (Rank & Weidanz 1976, Robert & Weidanz 1979, Grun & Weidanz 1981) displayed an Ab-independent immunity which was also T cell-dependent. Similarly, studies of the murine malaria P. c. adami demonstrated that B cell-deficient mice resolved acute primary infection with the same kinetics as normal mice and were immune to subsequent challenge with homologous parasites (Grun & Weidanz 1981, 1983).

Expression in this parasite system of Ab-independent immunity was suppressed by treatment with ATS and could not be achieved by transfer of hyperimmune serum (Grun et al 1985). In addition, it was claimed that to confer protection required the presence of an architecturally intact spleen. This is in contrast to all other published adoptive transfer experiments where reconstitution of immunosuppressed mice with dispersed immune spleen cells gave effective protection. In all such studies, patent parasitaemias developed in all recipient animals, inferring that the grafted cells did not limit parasite growth directly, suppression of infection being achieved by activation of other effector mechanisms.

B cell-deficient mice are usually susceptible to infection with P. yoelii (Weinbaum et al 1976 b), but when immunised by live infection and drug cure, can control infection (Roberts & Weidanz 1979). CBA/N mice, which are defective in producing high affinity Abs as well as those of the IgG_3 isotype, could still clear a P. yoelii challenge, and these mice were subsequently immune to reinfection (Jayawardena et al 1979). The mechanism for resisting a secondary challenge with the homologous parasite is considered to reside primarily in the primed T cell rather than the B cell compartment of the immune animal. In contrast to the observations with P. yoelii, B cell-deprived

mice recover from primary infections with P. c. adami and are immune to reinfection, so that Ab-independent mechanisms of immunity appear to be of major importance with this parasite (Grun & Weidanz 1981). A reconciliation of these apparently contradictory findings would be that cell-mediated immunity plays a significant role in protecting the host against acute infection caused by certain plasmodia but is essential for premunition, immunity to reinfection malaria, regardless of parasite aetiology.

These results underline the importance of effective priming of the T cell compartment for the development of immunity against malaria blood stage parasites (Jayawardena 1981). They also indicate that the mechanisms by which T cells mediate clearance and provide protection are not identical in different rodent models. In general, however, the consensus of adoptive transfer studies is that the T cells which confer protection against the asexual stages of rodent malaria parasites are of the helper/inducer phenotype. i.e. Ly-4^+ (Jayawardena et al 1982, Brinkmann et al 1985, Brake et al 1986, 1988, Cavacini et al 1986) and thus may provide help for Ab production (Weinbaum et al 1976 b, Jayawardena et al 1977, Roberts et al 1977, McDonald & Phillips 1978, Playfair 1982). It has recently been suggested that the Ly-4^+ cells that act as helper cells for Ab production belong exclusively to the recently proposed T_H2 subset (Langhorne et al 1989 b). These cells are not prevalent soon after challenge, but their increased presence later in infection correlates well with detection of an anti-plasmodial humoral response.

Ab may therefore be necessary for the host to survive acute infection and to clear the blood of parasites during chronic infection. However, cells of the helper/inducer T cell phenotype also include the subsets mediating Ab-independent cell-mediated immunity. Attempts to demonstrate involvement of the cytotoxic phenotype (Ly-2^+) in immunity against blood stage malaria parasites, unlike the anti-sporozoite immune response, have been unsuccessful (e.g. Jayawardena et al 1982, Süss et al 1988). This is not surprising in view of the fact that Ly-2^+ cells can only recognise Ag in association with class I Ags of the MHC (HLA-A, -B, -C in man; H-2-K, -D, in mice), Ags which are absent from mature human RBC or are present only at low concentration (reticulocytes, mouse RBC) (Jayawardena et al 1983). In the P. c. chabaudi-mouse system, H-2 restriction operates in T cell recognition of plasmodial Ags (Chemtai et al 1984 a & b), but presumably occurs in vivo when malarial Ags released from pRBC are taken up by presenting macrophages. Tc cells may yet be shown to play a part in immunity to asexual erythrocytic stages, but cytotoxicity would most likely be directed against target

cells not appreciably MHC restricted. This is possibly because immature RBC, which do express MHC Ags, are preferentially infected by some plasmodial species. However, even where T cell cytotoxicity has a role to play in normal immune function, one of the pathological consequences of patent parasitaemia is the depression of specific immune responses to non-malarial Ags, e.g. cytotoxic T cell responses against , for instance, viruses (Nickell et al 1987). Additional evidence against a role of cytotoxic T cells in blood stage malaria is the observation that the adoptive transfer of immune T cells failed to delay the patency of challenge infection (e.g. McDonald & Phillips 1978, Cavacini et al 1986). If the cells were directly cytotoxic for intraerythrocytic parasites, the inoculum size should have been reduced at the time of infection and the prepatent period extended. Thus, Ab-independent, T cell-mediated immunity to the plasmodial blood stages proceeds by a different mechanism(s). Available evidence indicates that the release of soluble mediators such as immune IFN-γ from Ag-stimulated T cells may be of importance, resulting in activation of macrophages with enhanced parasiticidal activity.

(iv) The role of the reticulo-endothelial system

Phagocytosis is a prominant feature of malaria. Early investigations noted free merozoites, pRBC, nRBC, malaria pigment and RBC debris in the macrophages of the spleen, liver, and bone marrow of malarious hosts (Taliaferro & Mulligan 1937, Brown 1969). The malarious host responds to circulating pRBC by a sharp increase in blood monocytes and the accumulation of macrophages in the spleen and liver (Jayawardena et al 1977, Lee et al 1986). Peripheral blood monocytes and macrophages from these organs marked changes in surface phenotype and secretory activity (Playfair et al 1979, Shear et al 1979, Lee et al 1986). Macrophages may contribute to the control of malaria infections by phagocytosis and/or release of extracellular mediators. For a long time it was believed that phagocytosis of infected cells or free parasites was the principal mechanism by which immunity was effected (Taliaferro 1929). Increased phagocytic activity has been reported during malaria infection. For instance, an increased carbon clearance rate was observed during P. vinckei and P. chabaudi infections in mice (Lucia & Nussenzweig 1969) and at the beginning of P. berghei infection in rats (Cantrell & Elko 1966, Cantrell et al 1970, Kitchen & Di Luzio 1971). Hyperphagocytosis of several other particles has been observed during malaria infections; these include ^{51}Cr-labelled sheep RBC in P. berghei infections of mice (Loose & Di Luzio 1976) and ^{125}I-labelled microaggregated human serum albumin in human malaria (Sheagren et al 1970).

Macrophages of mice with P. berghei infections showed enhanced phagocytic activity towards pRBC and nRBC in culture (Shear et al 1979) and in millipore chambers implanted in the peritoneal cavity (Criswell et al 1971). The former study revealed that splenic macrophages from P. berghei infected mice ingest pRBC more efficiently than do those from normal mice in vitro, the ingestion apparently being mediated by disease-associated Igs which bind to the surface of pRBC (Lustig et al 1977).

Activated macrophages may also mediate pRBC destruction by the release of factors which can kill the intracellular parasite (Clark et al 1981, Allison & Eugui 1982). The mechanisms by which macrophage secretion products destroy blood stage parasites are discussed in 1.11(a) (v, vi, & vii). The recruitment of macrophages and monocytes and their activation are mediated by lymphokines such as IFN-γ, macrophage chemotactic factor and IL-2, and possibly IL-3, secreted by T cells, which are themselves activated by plasmodial mitogens as well as specific malarial Ags (Wyler & Gallin 1977, Allison & Eugui 1983, Ockenhouse & Shear 1983).

In human malaria infections, both pRBC and nRBC have been observed within splenic macrophages in vivo (Pongponratn et al 1987). pRBC (Cranston et al1984), and to a lesser extent, nRBC (Gupta et al 1982) from infected animals have been shown to be less deformable than nRBC from normal controls. Phagocytosis may be enhanced through increased trapping of these rheologically altered RBC, as has been shown in P. berghei (Wyler et al 1981) and P. yoelii (Smith et al 1982) infections of rodents and more recently in patients with acute falciparum malaria (Looareesuwan et al 1987). Splenic filtration is increased by splenomegaly. Both enhanced phagocytosis and splenomegaly have been found to be thymus-dependent responses to malaria in mice (Roberts & Weidanz 1978). This is little surprising in light of the role of soluble factors secreted by T cells in macrophage activation.

The part played by immune phagocytosis in the clearance of P. falciparum is controversial. In vitro, immune serum has been shown to facilitate the phagocytosis of merozoites by normal macrophages, but not of pRBC (Khusmith & Druilhe 1983), and increased clearance of pRBC cannot be demonstrated in normal animals given hyperimmune serum (Quinn & Wyler 1979 a & b). These studies, using either resting peripheral blood monocytes or uninfected animals, are difficult to interpret, since immune clearance depends not only on opsonisation of RBC but also the state of activation of the monocyte/macrophage population. In Thai patients with falciparum malaria, Ward et al (1984) measured Fc receptor expression in vitro, and showed that the

activity of monocytes from cases of uncomplicated malaria was significantly increased compared to healthy controls. In contrast, the activity of monocytes from cerebral malaria sufferers was within normal limits. Also in acute P. falciparum infections, it has been reported recently that the clearance in vivo of IgG-coated RBC was accelerated in some but not all patients (Ho & Webster 1990 a). There was a significant positive correlation between the half-time for clearance of sensitised RBC from the circulation and the level of parasitaemia. The apparently normal rate of parasite clearance seen in patients with high parasitaemias suggests a failure to augment splenic Fc receptor function and consequent phagocytic activity in the face of a considerable antigenic challenge. Together, the in vivo and in vitro evidence indicates that immune clearance through phagocytosis is important in reducing parasitaemia to subpatency, thereby controlling the acute phase of infection. The failure of immune clearance in some instances may be related to the development of severe clinical illness, including cerebral manifestations.

(v) The role of cytokines

T cell-dependent manifestations of immunity in malaria include splenomegaly, lymphotoxin production, macrophage activation and circulatory monocyte production and activation (Jayawardena 1981). While evidence is indirect, it is thought that soluble macromolecules (cytokines) such as the interleukins, derived from lymphocytes and other cells, are instrumental both in the T cell-dependent regulation of the immune response and its effector phase. Most of these processes probably reflect the function of lymphokines secreted by activated T cells responding to malarial Ags.

Direct support for lymphokine production to malarial Ags has been provided by Wyler & Gallin (1977), who identified and partially characterised a mononuclear cell chemotactic factor in spleen cell extracts from malarious mice or monkeys but not from uninfected controls. Since spleen extracts of P. berghei infected nude mice lacked significant activity, it was concluded that the chemotactic activity was secreted by, or dependent upon, T cells and their precursors. Lelchuk et al (1984) showed that the ability of spleen cells from mice infected with P. yoelii or P. berghei to produce IL-2 when stimulated with Con A varied according to the time following infection. There was an increase in the capacity to release IL-2 early in both infections, a finding also shown for P. c. chabaudi infection (Langhorne et al 1989 a & b). Langhorne (1989) attributed IL-2 secretion to the T_H1 subset of Ly-4^+ cells which predominate during the clearance of the primary parasitaemia to subpatent levels. Splenic lymphocytes derived from P.

yoelii infected mice produced large volumes of IL-2 when stimulated with Con A at the time of remission of parasitaemia, but preceding the disappearance of parasites from the blood. Spleen cells harvested from malarious mice later in infection when they failed to secrete IL-2 had the capacity to respond to IL-2, suggesting that the lesion may be at the level of IL-2 synthesis. More recently, Lelchuk & Playfair (1985) showed raised levels of serum IL-2 inhibitor in euthymic but not in athymic mice infected with malaria. This factor, which was first described by Hardt et al (1981), acts both to inhibit IL-2 production and to block IL-2-dependent T cell proliferation and effector functions.

A family of cytokines increasingly being considered of importance in acquired immunity to the asexual erythrocytic stages of malaria are the interferons (Eugui & Allison 1982, Allison & Eugui 1983). Administration of exogenous IFN inducers or IFN-containing serum was found by Jahiel et al (1968 b, 1970) to delay the progress of P. berghei infection in mice. Furthermore, mice treated with sheep anti-mouse IFN globulins, thereby neutralising host IFN production, suffered accelerated P. berghei infections with increased parasitaemias (Sauvager & Fauconnier 1978). These authors concluded that IFN-α or IFN-β conferred resistance early in P. berghei infections, although death normally ensued, even in the presence of IFN at the start of acute infection, soon after challenge, when IFN was detectable in the sera of malarious mice (Sauvager et al 1979), and presumably this can be extrapolated to all malaria infections. Sera from P. falciparum infected children were found to have comparatively high titres of antiviral activity, which correlated directly with the degree of parasitaemia. The characteristics of the soluble antiviral factor indicated IFN-α to be predominating (Ojo-Amaize et al 1981). IFN-α could also be induced in vitro by free extracellular P. falciparum merozoites in a subpopulation of human peripheral blood lymphocytes, thought to be natural killer cells (Rönnblom et al 1983).

IFN-γ produced by Ag or mitogen activated T cells is an important regulatory lymphokine, and represents a useful indicator of cellular immunity. Lymphocytes from seropositive donors have been shown to produce IFN-γ in response to many viral Ags, e.g. vaccinia virus (Chang et al 1984) and Epstein-Barr virus (Andersson et al 1984). In malaria, the presence of IFN in the sera of infected humans and mice has been reported several times (Eugui & Allison 1982, Rhodes-Feuillette et al 1985). It was then shown that T cells from malarious patients and from immune individuals living in endemic areas were able to secrete IFN-γ and IL-2 upon stimulation with homologous Ag

(Troye-Blomberg et al 1985, 1987). This production of IFN-γ by primed T cells in vitro in response to P. falciparum asexual erythrocytic stages has been confirmed independently (Sinigaglia & Pink 1985, Riley et al 1988 a).

More recently, the highest levels of IFN-γ were found to be secreted by Ag-stimulated T cells from donors who were clinically immune to P. falciparum. IFN-γ production in vitro could be induced by both crude and defined Pf155-enriched Ag preparations (Troye-Blomberg et al 1987). There was no obvious correlation between T cell proliferation (as measured by [^{3}H]-thymidine incorporation) and IFN-γ production, indicating that these two phenomena, which both reflect T cell activation, may be partially independent processes taking place in different subpopulations of cells. Alternatively, it may reflect the natural course of differentiation of T cells, where mature memory T cells still secrete IFN-γ in response to specific malarial antigenic stimulation at a time when a proliferative response is no longer possible (Riley E.M., personal communication). Thus, it may be prudent to measure both DNA synthesis and IFN-γ titres when monitoring CMI of P. falciparum exposed individuals.

In contrast to its toxicity on the exo-erythrocytic stage of malaria parasites, IFN-γ has by itself no effect on the erythrocytic stages of plasmodia (Ferreira et al 1986). However, it has been hypothesised that IFN-γ is capable of activating macrophages with enhanced microbicidal activity. Moreover, it is thought that the production of IFN-γ is the primary role of $CD4^+$ ($Ly\text{-}4^+$) T cells in cell-mediated resistance to microorganisms (Murray 1988). Current opinion suggests that IFN-γ (and IL-2) secretion is a unique property amongst T cells of the $CD4^+$ T_H1 subset (Mosmann & Coffman 1987), and this generalisation has been confirmed for the specific case of malaria infection by Langhorne et al (1989 b), using the P. c. chabaudi-mouse model. Experimental evidence from in vitro and in vivo studies implicates IFN-γ in acquired immunity to blood stage malaria. Ockenhouse & Shear (1984) demonstrated that macrophages recovered from normal mice could be activated in vitro to destroy intraerythrocytic P. yoelii by oxygen-dependent mechanisms after incubation in IFN-containing S/N obtained from Ag-stimulated spleen cells from P. yoelii immune mice. In further studies, these investigators showed that the addition of anti-IFN-γ Ab to crude lymphokine S/N blocked macrophage-mediated parasite destruction, and demonstrated that recombinant IFN-γ activated human macrophages to induce the appearance of crisis forms of P. falciparum in cultures of human pRBC (Ockenhouse et al 1984).

Inflammatory mediators such as TNF can be induced in macrophages activated by IFN-γ

(Mosmann & Coffman 1987) in response to malarial parasite stimulation (Bate et al 1988). Both reactive oxygen intermediates and TNF may contribute to protective immune mechanisms, but the latter is also linked to the pathology of cerebral malaria. In vivo, it has been demonstrated that treatment of mice with exogenous IFN-γ has a protective effect during blood stage malaria. Clark et al (1987) treated mice infected with P. c. adami daily for 7 d with recombinant IFN-γ and observed a dose-dependent delay in the onset of parasitaemia, and, when treatment was extended for 17 d, a significantly lower peak parasitaemia. Similarly, Shear et al (1989) observed that daily treatment with an identical dose of recombinant IFN-γ resulted in lower parasitaemia and increased survival after infection with the lethal 17X strain of P. yoelii. Moreover, IFN-γ was thought to confer protection in rodent systems by acting in synergy with other lymphokines (Clark et al 1987). These studies to define the role of IFN-γ in anti-malarial immunity have been performed either in vitro or by using infected animals treated with exogenous IFN-γ. Recently, however, the in vivo importance of IFN-γ has been evaluated. In P. c. chabaudi AS-infected mice, the peak of endogenous IFN-γ production occurred just before peak parasitaemia, and correlated directly with a relatively high frequency of IFN-γ secreting T cells in the spleen (Slade & Langhorne 1989, Stevenson et al 1990). It is notable that the timing of peak IFN-γ levels is during the first patent parasitaemia when Ab-independent mechanisms are considered the major effector arm of acquired resistance to malaria infection. In vivo depletion of IFN-γ through injection of MAbs against this lymphokine exacerbated infection (Slade & Langhorne 1989, Stevenson et al 1990). Furthermore, in mice depleted of Ly-4$^+$ T cells, and thus unable to produce IFN-γ, treatment impaired but did not abbrogate completely host resistance to P.c. chabaudi AS infection (Meding et al in press).

This suggests that IFN-γ has a pivotal role in host immunity to malaria, but that factors in addition to this pluripotent lymphokine may be important in parasite clearance. Meding et al (in press) have also discovered that mouse strains both susceptible and resistant to P. c. chabaudi AS produce IFN-γ, suggesting that host susceptibility to malaria is not due to an intrinsic defect in IFN-γ synthesis. From other recent data, it seems that cerebral malaria in mice is associated with IFN-γ-induced TNF production (Grau et al 1987), suggesting that there may be a fine balance between the levels of serum IFN-γ and TNF, and protective immunity or pathological consequences. Thus, the levels of IFN-γ and TNF, which may vary from one strain of mouse to another, may

determine susceptibility or resistance to malarial infection (Langhorne. J., personal communication).

Riley et al (1989 a) have recently examined the role of $CD8^+$ T cells in determining the in vitro cellular immune response to P. falciparum Ags in malaria-immune adults. Removal of $CD8^+$ cells from the peripheral blood enhanced both the T cell proliferative and the IFN-γ response to malaria Ags in a group of normally low-responding immune individuals.

Although the in vivo significance of these findings is not clear, not only does this study confirm that it is the $CD4^+$ T cell compartment that is responsible for IFN-γ secretion, but also supports strongly the concept that Ag-specific $CD8^+$ T cells (putative T suppressor cells) are involved in the suppressive regulation of CMI to malaria (Troye-Blomberg et al 1983 a).

In addition to IFN-γ, another component present in the non-Ig fraction of certain malarious sera is TNF (Beutler & Cerami 1987), which is assuming increasing interest as a mediator of cellular immunity against asexual blood stage parasites in malaria. Before its chemical nature and origin had been revealed, it was suggested by Clark et al (1976) that a circulating parasiticidal factor was responsible for the intraerythrocytic death of parasites (so-called 'crisis forms'), and that this chemical was probably produced by activated macrophages and released during infection. It was later established that this crisis form factor was indeed able to retard intraerythrocytic parasite development and generate typical crisis form pRBC in vitro (Jensen et al 1982, 1983, 1984). This, it transpired, may not actually be TNF (Jensen et al 1987), although it has not been proven unequivocally. The direct parasiticidal effect of TNF is controversial, as the toxicity of recombinant TNF-α towards pRBC has yet to be demonstrated in vitro. However, TNF was recently shown to be present in very high amounts in human serum taken from malaria-infected individuals (Scuderi et al 1986). Furthermore, TNF-containing serum (TNS) and partially purified TNF are known to kill murine (Taverne et al 1981) and human (Haidaris et al 1983, Wozencraft et al 1984, Carlin et al 1985) blood stage parasites in vitro. There is good evidence that serum-extracted TNF inhibits the in vivo growth of the murine parasites P. vinckei (Clark et al 1981) and P. yoelii (Taverne et al 1982).

More recently, the administration of recombinant TNF-α in vivo has been shown to reduce the parasitaemia in mice infected with P. chabaudi (Clark et al 1987) and both lethal and non-lethal strains of P. yoelii (Taverne et al 1987). In the latter study, a

synergy between TNF and other factors in parasite killing was demonstrated; pRBC incubated with recombinant TNF showed no loss of viability, but repeated injections of TNF into mice challenged with a lethal strain of P. yoelii reduced parasitaemia and significantly prolonged survival. These observations suggest that TNS contains a parasiticidal factor(s) in addition to TNF, and that TNF is capable of activating immune effector mechanisms in vivo. That TNF is actually a macrophage-derived monokine was first demonstrated by Clark et al (1981). The mechanism by which TNF or TNS exert their deleterious effects on pRBC remains to be elucidated, but as TNF is known to be toxic to the host animal, whether or not it exerts a beneficial effect or is pathogenic may depend on the sensitivity of the individual to TNF and the level of the factor present in the serum.

(vi) The role of reactive oxygen intermediates

The consensus of opinion at present suggests that the major pathway of Ab-independent acquired immunity in plasmodial infection involves the release from Ag-activated $CD4^+$ T cells of lymphokines such as IFN-γ, which then stimulate cells of the mononuclear phagocytic cell system to exert anti-parasitic effects. Although this may be direct, by itself phagocytosis does not usually lead directly to parasite death. More often, macrophages mediate through the release of free oxygen radicals, which in turn may give rise to the formation of various stable parasiticidal components (Allison & Eugui1983, Clark et al 1987). That phagocytosis is not the principal mechanism by which CMI is effected was shown by the fact that recovery from non-lethal P. berghei and P. chabaudi infections does not correlate with increased phagocytosis (Lucia & Nussenzweig 1969, Cantrell et al 1970), but is coincidental with the presence of degenerate parasites within intact pRBC.

A major contribution to the understanding of how cell-mediated effector mechanisms cause pRBC destruction was derived from experiments in which agents known to generate free oxygen radicals, including alloxan (Clark & Hunt 1983) and t-butyl hydroperoxide (Wood & Clark 1982, Clark et al 1983) suppressed parasitaemia when injected into malaria infected mice. Examination of blood smears revealed degenerate forms of intracellular parasites and suggested that these chemical generators of reactive oxygen species, including hydrogen peroxide (H_2O_2), superoxide anions (O_2-) and hydroxyl radicals (OH·), may be mimicking one mechanism of CMI towards blood stage malaria. Indeed, reactive oxygen intermediates have been shown to be toxic to asexual stages of a variety of different Plasmodium species, both in vitro and in vivo (Dockrell & Playfair

1983). It is thought that during infection macrophages recruited to the spleen and liver, as well as myeloid cells in the blood, become highly activated through lymphokines (predominantly IFN-γ) produced by T cells specifically responding to malarial Ags. These activated macrophages act as non-specific effector cells by releasing reactive oxygen species during a respiratory burst (Ockenhouse et al 1984). Since oxygen radicals are extremely short-lived molecules, they may exert their activity locally in the liver and spleen, through lipid peroxidation leading to the generation of toxic aldehydes (Allison & Eugui 1983, Clark et al 1987). The chemicals may then circulate in the blood and effect parasite (and tissue) injury at distant sites. The oxidative burst capacity of splenic adherent cells, of which macrophages form a large proportion, has been shown to vary during the course of murine malaria (Dockrell et al 1986), and in the case of P. chabaudi infection, was maximal just as the animals began to recover; the amount and degree of respiratory activity appeared to correlate with the outcome of infection. During P. yoelii infection, enhanced oxidative capacity was observed throughout infection in both splenic and liver macrophages. In contrast, for mice challenged with P. berghei, the oxidative capacity decreased during infection, suggesting that the inability to generate reactive oxygen species may be associated with lethality of the parasite for the particular host. While the role of toxic oxygen metabolites is firmly established in vivo in rodent models of malaria, there are as yet no comparable human studies.

(vii) The role of toxic nitrogen oxides

Additional factors released by macrophages or other cells may play a part in immunity to asexual erythrocytic malarial infection, as evidenced by the parasiticidal component of TNS (Carlin et al 1985). In addition, it has been reported that in cases of chronic granulomatous disease, in which oxidative metabolism is impaired, IFN-γ-activated macrophages were able to inhibit partially the growth of (Ockenhouse et al 1984), and polymorphonuclear leukocytes kill (Kharazmi et al 1984), erythrocytic P. falciparum in vitro. These independent findings suggest the existence of an oxygen-independent parasiticidal mechanism. This has recently been elucidated, and involves the cytokine-induced synthesis of toxic nitrogen oxide (NO) from L-arginine by macrophages, hepatocytes and perhaps even endothelial cells. NO inhibits iron sulphur-dependent enzymes involved in cellular respiration and energy production.

For the obligate intracellular protozoan parasite of macrophages, Leishmania major, synthesis of L-arginine-derived nitrite (NO_2-), the oxidative end product of NO,

directly correlates with killing of leishmania amastigotes (Green et al 1990 a). In this instance, the rate of NO_2- production is a quantitative index for macrophage activation. It has been shown that competitive inhibition of NO synthesis by monomethylarginine inhibits both parasite killing and NO_2- production (Granger et al 1990). Now the same oxygen-independent mechanism of parasite killing has been found to be active against malaria schizonts and exo-erythrocytic stages of P. berghei (Green et al 1990 b).

In the case of leishmaniasis, it is known that the parasite itself participates in the regulation of this toxic effector mechanism by inducing TNF-α secretion by macrophages. This macrophage autocrine factor acts in combination with IFN-γ to induce nitrogen oxidation of L-arginine (Draper et al 1988, Ding et al 1988). Indeed, NO synthesis by IFN-γ-treated macrophages can be blocked by anti-TNF-α MAbs (Green et al 1990 b). At present, the activation signal involved in endogenous generation of NO is unknown. This mediation of TNF in intracellular destruction is of interest in malaria infections, since it has very recently been shown that pRBC induce the secretion of TNF, and furthermore, that the exoantigens that are liberated also induce TNF in vitro (Bate et al 1988, Taverne et al 1989, Taverne et al 1990), and in vivo (Taverne et al 1989, Bate et al 1989). As it is now considered, at least for Leishmania major infections, that H_2O_2 and other oxygen metabolites are not only not implicated in parasite clearance, but have been shown to exacerbate infection both in vitro and in vivo (Liew F.Y., personal communication), it may be that opinion will change as to the nature of the principal effector molecules secreted by lymphokine-activated cells involved in the elimination of blood stage malaria parasites.

(viii) The role of the spleen

It has long been recognised that the spleen plays a major role in resistance to malaria parasites. In general, splenectomy worsens infection; normally non-lethal challenges may become lethal and latent infections may relapse (Taliaferro 1929; reviewed by Wyler et al 1979 a). However, the findings are not totally consistent. In the rat, splenectomy prevented recovery from P. berghei infection (Quinn & Wyler 1980), even after vaccination (Stiffel et al 1972). Splenectomised or congenitally asplenic mice succumbed to P. yoelii or P. chabaudi challenge (Oster et al 1980), but in another study (Dockrell et al 1980), splenectomy did not prevent recovery from non-lethal P. yoelii infection, or the protective effect of a vaccine against the lethal strain of P. yoelii provided vaccination preceeded the operation. This paradoxical situation may be reconciled by the finding of Wyler et al (1979 a) that the spleen plays a beneficial role

for the host early in infection, but it may later have a deleterious effect by promoting chronicity of infection with some plasmodia. Also, the effect of splenectomy on the course of a subsequent malaria infection is dependent on the species of host (Zuckerman & Yoeli 1954, Langhorne et al 1979) and in the case of P. berghei, the age of the rat (Zuckerman & Yoeli 1954). Although splenectomy removes a large population of effector cells (Brown et al 1976 a), this quantitative loss is probably not as important as losing vascular access to the normal splenic architecture and filtering ability. In support of this, Oster et al (1988) showed that mice reconstituted with spleen cell suspensions after splenectomy experienced similar infections to the splenectomised controls, using several rodent malarias.

For P. berghei, splenectomy prior to infection has an ultimately adverse effect. Although in splenectomised adolescent rats, peak parasitaemia was lower than in similar intact controls, if the splenectomised animals were unable to reduce the first wave of parasitaemia to subpatency, there was a higher rate of mortality (Zuckerman & Yoeli 1954, Cantrell & Moss 1963, Cantrell & Elko 1966). Weiss & Hess (1978) demonstrated that in both rats and gerbils, vascular disconnection of the spleen from the liver reduced immunity to P. berghei almost as much as splenectomy, and, in another report, subtotal hepatectomy in rats had a similar effect to splenectomy (Cantrell & Moss 1963).

Rhesus monkeys vaccinated against P. knowlesi remained immune after splenectomy but only briefly (Butcher et al 1978), whilst immunity to P. knowlesi in marmosets was not affected by splenectomy (Langhorne et al 1979). With P. inui-infected rhesus monkeys, splenectomy before challenge increased mortality, but after infection facilitated self-cure within a year (intact monkeys had persistent infections for 1-13 years) (Wyler & Gallin 1977, Wyler et al 1977).

It was proposed by Barker & Powers (1971) that the generally deleterious effect of splenectomy on rodent malaria is attributable to a reduction of Ab response. However, the passive transfer of immune serum confers less protection to splenectomised rats than to intact rats, when infected with P. berghei (Brown & Phillips 1974), suggesting the spleen is important for the phagocytosis of malaria parasites. This latter finding correlates with the pioneering work of Taliaferro & Cannon (1936) who observed an increased number of differentiated macrophages phagocytosing parasites in spleen sections taken from P. brasilianum-infected Panamanian monkeys. It was also reported for the first time that during a primary infection, the spleen becomes massively

enlarged, splenomegaly, a hallmark of malaria. More recently, it has been shown that the total number of splenic macrophages increases dramatically during P. berghei (Wyler & Gallin 1977) and P. yoelii (Lelchuk et al 1979) infections of mice. Moreover, culture of the non-adherent spleen cell fraction from P. yoelii infected mice revealed a population of late adhering cells which resembled macrophages in morphology (Lelchuk et al 1979). These investigators concluded that malaria infection may stimulate the production of macrophage-monocyte precursors, whose development is regulated by the presence of mature macrophages.

Why the spleen is so often vital in malaria infection has only recently become clear with several claims for a physical role in trapping pRBC, enabling localised parasite elimination (Conrad & Dennis 1968, Schnitzer et al 1972, Wyler et al 1981). Observations suggest that the spleen efficiently filters out pRBC as soon as they can be recognised as foreign. Rat RBC parasitised by P. berghei are more rapidly removed from the circulation into the spleen than are nRBC (Quinn & Wyler 1979 b, Wyler et al 1981). A unique structural feature of the spleen not found in other lymphoid organs, the red pulp, is considered to be the site where filtration occurs (Weiss 1979). Within the red pulp, the intermediate circulation is such that arterioles open into cords that are connected with sinuses. The macrophage is the dominant leucocyte in the cords; monocytes entering the cords across arteriolar terminations appear to be selectively held in the interstices, or filtration beds, of the reticular meshwork (Weiss 1983 a & b) The migration of monocytes from the peripheral circulation to the spleen in vivo may be linked to the elaboration of a mononuclear chemotactic factor in vitro (Wyler & Gallin 1977). Thus, immigrant mononuclear cells with high capacity for a respiratory burst are concentrated in the post-arteriolar region, and pRBC entering through arterioles must pass through this site in close apposition to these effector cells, and can then be eliminated by direct phagocytosis by macrophages, or by the cytotoxic effects of monokines and other macrophage-derived factors. Phagocytosis of P. knowlesi-infected RBC by cordal macrophages has been observed in rhesus monkeys (Schnitzer et al 1972).

There also exists a second filter system of the red pulp; blood leaving the cord enters the lumen of the vascular sinus by passing between endothelial cells (Weiss 1979). This constitutes a slit-like space, and RBC passing through it must be pliant. If RBC deformability is reduced, as is the case for pRBC (Miller et al 1971 b), their passage is delayed and a pool of pRBC forms within an environment rich in effector cells. This

regional concentration of pRBC was first reported for P. brasilianum infection (Taliaferro & Cannon 1936).

During malaria infection, the changes in the spleen depend upon its singular capacities to trap circulating blood cells differentially in its filtration beds, where, for monocytes, they are stored for variable periods and proliferate (Weiss 1983 a & b) or, for pRBC, are modified or destroyed (Weiss 1978). Perhaps the most striking instance of splenic control of malaria is the phenomenon of crisis, the spontaneous and rapid disappearance of pRBC from the blood (Taliaferro & Cannon 1936, Taliaferro & Mulligan 1937, Taliaferro & Taliaferro 1944, Quinn & Wyler 1979 b, 1980, Wyler et al 1979 a, 1981, Wyler 1983 a). Crisis fails to occur in the absence of the spleen. The disappearance of circulating pRBC in crisis is due to their sequestration on the filtration beds of red pulp and their destruction by macrophages held there (Taliaferro & Cannon 1936, Taliaferro & Mulligan 1937).

As has been inferred from various challenges of splenectomised mice and rats already detailed, the capacity of the spleen to clear the blood varies considerably during the course of blood stage malaria. It has been shown that, after a brief initial phase of active normal clearance, splenic clearance capacity falls to subnormal levels until crisis, when active clearance is restored (Quinn & Wyler 1979 b, Wyler et al 1981). Furthermore, during malaria there is a change in blood flow through the spleen, with a switch from open arrangements (by which blood flows through the locules of the filtration beds) during the time of normal or heightened clearance, to closed arrangements (by which blood is shunted from the locules) during depressed clearance (Quinn & Wyler 1979 b, Wyler et al 1981).

In microscopic studies of lethal and non-lethal P. yoelii infections, a striking activation of reticular cells early in the disease was discovered, and this was at such a rate and magnitude as to provide an increasingly competent blood-spleen barrier (Weiss et al 1986, Weiss 1989, 1990). This barrier appears to exclude pRBC from filtration beds, thereby protecting the proliferating and differentiating populations of erythroblasts (and lymphocytes, plasma cells and monocyte-macrophages) held there against infection. The position of this barrier, separating the powerful filtering capacities of the spleen from parasites carried in the blood, permits the development of a rising parasitaemia and anaemia (McGhee 1960, Zuckerman 1960). Crisis is associated with the relaxation of this barrier and with two important, complementary consequences: allowing pRBC into the filtration beds of the spleen where they are destroyed; and

releasing the reticulocyte stores produced in the spleen by erythropoiesis into the circulation (Weiss et al 1986). Blood flow characteristics, filtration capacities of the spleen and control of malaria appear intrinsically related, and depend upon the formation of the reticular cell blood-spleen barrier. Into such close apposition does this barrier bring host effector cells and parasite, it has been speculated that the very structure of the spleen may have been driven evolutionarily by malaria (Weiss 1990).

(ix) The role of the liver

Sporozoite invasion and subsequent exo-erythrocytic schizogony within hepatocytes leads to the eventual destruction of the host cell. However, under normal circumstances, relatively few sporozoites are inoculated following a mosquito bite, and as the proportion of mosquitoes that are infected is often below 1%, sporozoite-induced injury of the liver is likely to be minimal (Hollingdale 1985). This is in contrast to the hepatomegaly commonly seen in malarial infections of mammals during the patent erythrocytic parasitaemia (Singer 1954, Russell et al 1963), when the liver becomes extremely friable and dark in colour. During P. berghei infection in rats, Kupffer cell hypertrophy occurs. There is also a progressive infiltration of the interlobular areas of the liver, initially with leucocytes and hypertrophying lymphocytes, but later increasingly with erythropoietic blast cells (Singer 1954, reviewed by Aikawa et al 1980).

Although of secondary importance to the spleen during malaria infection, the liver functions as a repository for the reticulo-endothelial system, with active phagocytosis of pigment and pRBC taking place within it. In splenectomised animals, the liver takes over most of the extra burden of phagocytosis (Taliaferro & Cannon 1936). In P. berghei infections in mice, the liver had a greater macrophage activity than the spleen (Singer 1954), whilst in rats challenged with the same parasite (Cantrell & Moss 1963), partial host hepatectomy resulted in a striking enhancement of parasitaemia early in infection.

(e) The immune response to gametocytes and gametes

Until relatively recently, research into acquired immunity to the sexual stages of malaria, the gametocytes and gametes, had been a neglected subject, presumably because these stages are not themselves pathogenic to the human host. However, since the gametocyte is the stage of the malaria life cycle which tranfers the parasite from the vertebrate host to the mosquito vector, stage-specific immunity against this form, or

against gametes, would tend to reduce the infectivity of the parasite to the mosquito. This would thus suppress transmission and therefore, indirectly, protect the secondary host, notably humans, against malaria. For this reason, such immunity has commonly been referred to as transmission blocking immunity (reviewed by Carter et al 1988, Targett 1988).

After uptake by the mosquito in a blood meal, under the influence of changes in temperature (Sinden & Smalley 1976) and pH (Carter & Nijhout 1977), including a potent mosquito factor(s) (Nijhout 1979) present in the mosquito midgut, both male and female gametocytes become extracellular. The gametes soon formed are then open to attack from any element of the vertebrate host's immune response also present in the blood meal (Sinden & Smalley 1976).

Both cellular and Ab-mediated mechanisms of transmission-blocking immunity have been proposed. In the case of Ab-mediated resistance, serum from animals recently immunised with malaria gametocytes (Gwadz 1976) or gametes (Gwadz & Green 1978) has been shown to suppress gametocyte infectivity when presented to mosquitoes in a membrane feeding apparatus, but the gametocytes themselves were not directly impaired by the effects of immunisation (as shown by their reinfectivity when fed to mosquitoes together with normal serum). Furthermore, when gametogenesis and fertilisation were induced in vitro before mixing the parasites with serum from gamete-immunised animals, the immune serum had little effect on the infectivity of parasites to mosquitoes (Grotendorst et al 1984). In these instances, the transmission-blocking immunity was mediated by Abs acting against the parasites only after they had entered the mosquito midgut but before fertilisation was completed, i.e. anti-gamete, fertilisation-blocking Abs, as determined by agglutination (Gwadz 1976, Carter et al 1979 b, Mendis & Targett 1982) or by immunofluorescence with live gametes (Munesinghe et al 1986).

In addition to their purely Ab-dependent action in preventing fertilisation (by an unknown mechanism), anti-gamete surface Abs can destroy gametes and also newly fertilised zygotes in complement-mediated reactions in the midgut of the mosquito (Kaushal et al 1983, Rener et al 1983, Mendis et al 1987, Graves et al 1988). Moreover, certain anti-gamete Ags require complement to be effective (Mendis et al 1987, Graves et al 1988).

Induction of Abs can be achieved by immunisation with ookinetes, a sexual stage exclusive to the mosquito. These Abs prevent the development of the fertilised zygote in the mosquito (Grotendorst et al 1984) in a complement-independent manner, the

mechanism of which is not known. In P. berghei, the Abs appear to impair the survival and morphological transformation of zygotes into ookinetes (Winger et al 1988). Ookinetes of P. falciparum, however, formed in normal numbers and morphology, but the number of resulting oocysts was very low, suggesting that the Abs prevented the ookinete from crossing the mosquito midgut (Vermeulen et al 1985).

A form of transmission-blocking immunity which reduced the dose and infectivity of gametocytes circulating in the blood stream was reported for the rodent malaria parasite P. yoelii nigeriensis (Harte et al 1985 a, b & c). This immunity was induced by vaccination of mice with microgametes and persisted for 12 months afterwards, although serum Ab to gametocytes was not detectable by immunofluorescence after six months. Adoptive transfer of immune T cells to naive recipients resulted in a near total (95%) reduction in transmission to mosquitoes following subsequent infection of grafted mice. This effect was best achieved with Ly-4^+ cells and was manifested as a marked decrease in gametocyte numbers without affecting the course of asexual stage parasitaemia. While serum taken from immune donors was minimally effective alone, transfer of both serum and T cells gave the most protection, suggesting a synergistic effect between the cell-mediated and humoral immune compartments in suppressing gametocyte infectivity to mosquitoes. A human T cell reponse specific to gametes of P. falciparum has also been demonstrated in vitro (Good et al 1987 a). With both T cells taken from naive donors and those previously infected with malaria, Ag-specific proliferation and IFN-γ secretion were readily stimulated by gametes, but not asexual blood stage parasites of P. falciparum.

The earliest experiments demonstrating transmission-blocking immunity were performed following immunisation with whole parasites of various sexual stages and in different degrees of purification from other stages, mostly asexual pRBC. Thus, fertilisation- blocking Abs have been induced by vaccination with whole parasitised blood, containing gametocytes (Huff et al 1958, Gwadz 1976), and with preparations of extracellular male (Gwadz & Green 1978, Carter et al 1979 b, Mendis & Targett 1982, Grotendorst et al 1984, Munesinghe et al 1986) and female (Carter & Chen 1976, Carter et al 1979 a, Mendis & Targett 1979, Kaushik et al 1982) gametes. These studies showed that the sexual stages are very immunogenic and demonstrated functional independence of sexual stage immunity from that against asexual erythrocytic parasites. Observations have recently been made on induction of immunity to sexual stages in human malaria infections. In Sri Lanka, transmission-blocking Abs are readily induced

by P. vivax infection and can readily be detected in about half of non-immune individuals recovering from a primary infection (Mendis et al 1987). In a study of malaria transmission in Papua New Guinea where individuals were exposed to multiple reinfection with both P. falciparum and P. vivax, about one third of sera collected contained detectable amounts of Ab to gamete surface Ags (Graves et al 1988). These findings indicate that Ab-mediated anti-gamete immunity is quite easily induced in man during malaria infection and that it appears to be potent in reducing infectivity of the parasites to mosquitoes in membrane feeding experiments. This degree of suppression could be expected to have a significant impact on malaria transmission amongst human populations. Frequent re-exposure to infection is required to maintain Ab levels (Mendis et al 1987), however, and long term anamnestic responses are poor (Ranawaka et al 1988). This suggests that there may be some defect in induction of memory T cell responses, although it is possible that antigenic polymorphism may prevent T or B cell responses being boosted by a different parasite strain.

Ab responses to gamete surface Ags are much more frequently detected in non-immune, convalescent malaria patients from non-endemic areas than in residents of malaria endemic areas (Mendis et al 1987, Ong et al 1990). Only a minority of malaria-immune adults possess significant levels of Abs to gametocyte surface Ags (Quakyi et al 1989, Riley et al 1990). This may result either from down regulation of Ab responses in individuals chronically exposed to malaria or from adaptive responses by the parasite such that gametocyte Ags are not recognised by persons carrying those HLA Ags which are common in endemic communities. The reasons for this non-responsiveness are not clear, and their elucidation will require longditudinal and age cross-sectional studies of malaria endemic populations.

1.12 Immunopathology

(a) Non-specific cell activation

As in other protozoan diseases, such as African (Kobayakawa et al 1979) and South American trypanosomiasis (Ortiz-Ortiz et al 1980) and visceral leishmaniasis (Galvao-Castro 1984), polyclonal B lymphocyte activation occurs in malaria leading to a hyperproduction of IgG, of which, in an immune adult, as little as 5% is specifically directed against P. falciparum Ags (Cohen & Butcher 1969). This increase in non-specific Ig synthesis was first reported by McGregor et al (1956), and it is now well established that early in the acute phase of the disease, asexual erythrocytic parasites

induce a polyclonal activation of B cells in humans (Greenwood & Vick 1975) and mice (Rosenberg 1978). This hypergammaglobulinaemia has been shown to be T cell-dependent in P. yoelii and P. berghei mouse models (Freeman & Parish 1978, Rosenberg 1978, Weinbaum et al 1978, Finley et al 1982). In contrast to infected naive mice, challenged athymic nude mice did not show hyperproduction of Abs against sheep RBC, different haptens and auto-Ags (Finley et al 1982, Rosenberg 1978).

Non-specific IgG has been shown to comprise a wide range of auto-Abs detected in sera from individuals with acute P. falciparum malaria, although autoimmunity is rare. Ags recognised include those of heart, thyroid and gastric parietal cells (Shaper et al 1968), lymphocytes (Wells et al 1980), and RBC (Rosenberg et al 1973, Ronai et al 1981, Zouali et al 1982, Wahlgren et al 1983). High titres of Abs to rheumatoid factors (Shaper et al 1968, Greenwood et al 1971 a), nuclear components (Greenwood et al 1970, Adu et al 1982), single strand DNA (Ribeiro et al 1984), mitochondria (Boonpucknavig & Ekapanyakul 1984), intermediate filaments (Mortazavi-Milani et al 1984) and smooth muscle (Phanuphak et al 1983) have also been found to be associated with malaria infections. In a longitudinal study, the incidence of auto-Abs peaked two to four weeks after patient discharge, even though in several cases the blood was aparasitaemic (Phanuphak et al 1983). The presence of such auto-Abs has been postulated to be related to the ability of plasmodia to induce polyclonal B cell activation. Indeed, normal human peripheral blood mononuclear cells were shown to synthesise Abs to nuclear components, intermediate filaments and rheumatoid factors when stimulated in vitro with P. falciparum culture S/N (Kataaha et al 1984). These results confirm the earlier findings of activation induced by pRBC lysate S/N in mice infected with P. yoelii and P. berghei (Freeman & Parish 1978).

Hypergammaglobulinaemia was initially thought to be due to a direct mitogenic effect of malarial Ags on B cells (Greenwood 1974) but has since been shown to be a T cell-mediated event (Weidanz 1982), probably through the production of B cell-activating lymphokines (Ballet et al 1987, Kabilan et al 1987). Moreover, plasmodial Ags can activate T cells from normal donors who have never been exposed to malaria (Wyler & Oppenheim 1974, Wyler et al 1979 b). These findings have recently been confirmed at the clonal level (Chizzolini & Perrin 1986). T cell activation is also indicated by high levels of soluble IL-2 receptors (Ho et al 1988) and IFN-γ (Rhodes-Feuillette et al 1985) in the sera of some patients with acute falciparum malaria.

(b) Immunosuppression

Although humans naturally infected in non-endemic regions and animals experimentally infected with malaria parasites may experience an immune hyporesponsiveness towards a wide range of Ags, quite often in residents of areas where falciparum malaria is endemic, an Ag-specific unresponsiveness is observed at the time of disease manifestation, regardless of severity (Ho et al 1986, Riley et al 1988 b). This is detectable both as a failure of lymphocyte proliferation in response to asexual blood stage malarial Ags in vitro (e.g. Ballet et al 1981, Brasseur et al 1983, Troye-Blomberg et al 1983 a), and as an apparent cessation of anti-malarial Ab production in vivo (Webster et al 1987,1988 b) and appears to be stage-specific (Webster et al 1988 a). This immunosuppression can last a long time after the clearance of parasites from the circulation and may partially explain the difficulty with which natural protective immunity to falciparum malaria develops in endemic areas.

Overall, immunosuppression is probably not that important in the near commensal state of chronic, asymtomatic, often subpatent parasitism reached by clinically immune adults. It may be important, however, in younger age groups and may also have important implications for the efficacy of potential malaria vaccines in populations having a high exposure to infection.

Malarious children have been shown to be deficient in their ability to mount primary immune responses to certain, but not all non-plasmodial Ags. Similarly, the immune response against several T-dependent and T-independent Ags has been reduced in mice infected with P. berghei, P. yoelii or P. chabaudi (reviewed by Wyler 1979, Weidanz 1982). Immunosuppression may account for the apparent increased frequency and greater severity of intercurrent microbial infections among children with malaria, as shown by a diminished Ab response to tetanus toxoid (McGregor & Barr 1962), the O Ag of Salmonella typhi (Greenwood 1984) and meningococcal vaccine (Williamson & Greenwood 1978). Data derived primarily from studies of mixed infections in mice have implicated malaria as an agent capable of enhancing the severity of other infectious diseases (Cox 1978).

Malaria-induced immunosuppression may also be instrumental in the proposed association between malaria, Epstein-Barr virus (EBV), and Burkitt's lymphoma, with which malaria has been implicated, perhaps because the geographical distribution of the two diseases coincide. Until recently, however, there have been few data to support the

contention that malaria enhances susceptibility to malignant disease (Burkitt 1969). In the P. yoelii-infected mouse model, Wedderburn (1970) showed an increased incidence of malignant lymphoma due to Moloney leukaemia virus, and that mice with both infections did not produce anti-virus neutralising IgG (Bomford & Wedderburn 1973). In Papua New Guinea, where Burkitt's lymphoma is endemic, individuals living in malaria-endemic regions were found to have an impaired EBV-specific T cell immunity without any major alteration of anti-EBV Ab titres, compared to residents in non-malarious zones, or with Caucasians (Moss et al 1983). Furthermore, a report from the Gambia showed that individuals with an acute attack of P. falciparum malaria exhibited a loss of T cell control of EBV-infected B cells, which proliferated abnormally and secreted large amounts of Ig (Whittle et al 1984). This finding, which was attributed to decreased numbers of $CD4^+$ T cells, may explain the relationship between Burkitt's lymphoma and malaria. Similarly, massive antigenic stimulation by malaria may serve to activate immunologically $CD4^+$ cells infected with HIV, resulting in the termination of virus latency and cell death (Zagury et al 1986).

Little is yet known about the mechanisms triggered by plasmodia in the development of immunosuppression, but several theories have been proposed, primarily on the basis of studies in rodents. These include clonal deletion of Ag-specific cells (Poels & Van Niekerk 1977), responses to polyclonal activation (Rosenberg 1978), antigenic competition (Terry 1978), and non-specific activation of suppressor cells of either T cell (Greenwood et al 1971 b, 1972, Theander et al 1986 a & b) or macrophage origin (Corrêa et al 1980, Loose et al 1984) (reviewed by Wyler 1983 b). In addition, splenic adherent cells, functionally defective as accessory cells in the response to Ags, have been implicated (Warren & Weidanz 1976, Weinbaum et al 1987, Wyler et al 1979 b).

Malaria induced immunosuppression has been shown to involve defects in IL-2 production both in P. falciparum infection of humans (Troye-Blomberg et al 1985) and in murine malaria (Lelchuk et al 1984). In the latter model, these defects were not due to deficient IL-1 secretion, which was either unaffected or enhanced, depending on the mouse strain (Lelchuk & Playfair 1985). Factors inhibiting IL-2 or depressing lymphocyte responses in vitro to both plasmodial and unrelated Ags have been found in sera of malarious patients as well as in mice (Lelchuk et al 1987, Theander et al 1987). However, the chemical identity of these factors and their involvement in suppression remains to be established.

(c) Cerebral malaria

Cerebral symptoms accompanying acute falciparum malaria represent severe complications with a frequently fatal outcome. This pathological manifestation of disease is not directly parasite-related but instead may be due to induction of a detrimental host immune response to the malaria parasite. The most common autopsy findings are vascular congestion and plugging of blood vessels with heavily parasitised RBC (Polder et al 1983). The neurones usually show no obvious lesions besides signs of anoxia associated with localised cerebral oedema, but brain oedema is a common finding (Oo et al 1987). In vivo damage of endothelial cells with subsequent alteration of capillary permeability has been reported in both human (Areekul et al 1984) and mouse (Depierreux et al 1987) malaria. However, the hypothesis of an increased blood-brain barrier permeability during human cerebral malaria has recently been questioned (Warrell et al 1986, Oo et al 1987), and the pathogenesis of these changes remains unknown. Various hypotheses have been proposed including endothelial lesions (Wash 1979) with attachment of monocytes to the endothelium (Rest 1982); sequestration of pRBC in capillaries (Yoeli & Hargreaves 1974, MacPherson et al 1985), possibly related to the particular adhesiveness of pRBC to endothelial cells (reviewed by Howard & Gilladoga 1989); T cell-mediated cellular immune reactions (Wright et al 1971, Finley et al 1982); and T cell-dependent humoral reactions involving circulating immune complexes (Contreras et al 1980, Adam et al 1981, Rest 1982).

In humans, indirect evidence of a T cell involvement in neurological complications is provided by the observation of a lower frequency of cerebral malaria in malnourished children (Edington 1967). Thymic atrophy (Watts 1969, MacFarlane 1971) and functional impairment of cell-mediated responses (MacMurray 1984) have also been described in brain cortex capillaries of fatal cases of cerebral malaria (Oo et al 1987).

For ethical reasons, experimentation on malarial complications in humans is not possible, and because of the cultural attitude towards autopsy in many Third World countries, post mortem examinations are also limited. For this reason, rodent malaria infections have been used to study cerebral malaria, and have highlighted the importance of the immune system in modulation of cerebral pathology (Mackey et al 1980, Rest 1983, Franz et al 1987, Cox et al 1987, Grau et al 1987, Curfs et al 1989). None of the rodent malaria species can be labelled as falciparum-like by parasitological, morphological or molecular criteria. However, some of these infections, e.g. P. berghei

ANKA strain in mice (Mackey et al 1980, Grau et al 1987) and P. berghei in hamsters (Rest 1983, Franz et al 1987) exhibit important properties of human cerebral dysfunction. T cell-mediated immunity has been implicated in the development of experimental cerebral malaria, since neurological complications are less severe in neonatally thymectomised hamsters (Wright et al 1971) and absent in athymic nude mice (Finley et al 1982).

In the P. berghei ANKA mouse model in CBA/Ca mice, a strain of mouse which is thought to be genetically susceptible to the development of neurological lesions, a cumulative mortality of about 90% was observed between 7-15 d p.i. (Grau et al 1986), when anaemia was moderate and parasitaemia relatively low. Several lines of evidence indicate that experimental cerebral malaria is strictly dependent on the presence of T cells with the Ly-4^+ phenotype (Grau et al 1986). The occurrence of the lethal neurological syndrome was abrogated completely in P. berghei-infected mice depleted of the Ly-4^+ T cell subset by MAb treatment in vivo, but not by similar depletion of the Ly-2^+ subset (Grau et al 1986). These results were confirmed in studies where adult-thymectomised, irradiated and bone marrow-reconstituted CBA/Ca mice appeared to be completely resistant to the onset of neurological signs upon infection with P. berghei. These findings extended those obtained by Finley et al (1982) using nude mice. Moreover, reconstitution of mice depleted of T cells by adult thymectomy with normal Ly-4^+ T cells rendered these mice fully susceptible to neurological complications. In contrast, mice reconstituted with Ly-2^+ T cells did not die acutely with immunopathological signs but later developed severe anaemia and overwhelming parasitaemia (Grau et al 1986). The implication of these experiments is that murine cerebral malaria is mediated by immune mechanisms induced by infecting asexual blood stage plasmodia. Indeed, the development of cerebral malaria is not related directly to the level of parasitaemia or degree of anaemia, but rather appears as an expression of the immunopathological reactions of the infected host.

It is believed that multiple factors determine why some acute P. falciparum infections exhibit cerebral symptoms whereas others with equal or even higher blood parasitaemia do not. Differences in P. falciparum parasites in genetically and phenotypically heterogeneous wild populations (Thaithong et al 1984), as well as variations in innate or acquired immune properties of the host, are all important. One event which does, however, appear to be consistent with all cases of acute cerebral malaria in man is the attachment of P. falciparum pRBC to endothelial cells lining brain capillaries, and

consequent reduction of blood flow (MacPherson et al 1985).

The most obvious cell-cell attachment involved in human cerebral malaria is that of pRBC with the endothelial cell lining of blood vessels (Trager et al 1966, Luse & Miller 1971, MacPherson et al 1985). Cellular adhesion between pRBC and other host cells, including nRBC (Handunnetti et al 1984, 1985) may also be of importance in sequestration and pathology of acute P. falciparum malaria.

In vitro models for sequestration have been developed using cultures of human umbilical cord endothelial cell line C32 (Udeinya et al 1981, 1983 a, 1985). These assays, although only representing an approximation of the in vivo situation, have revealed properties of P. falciparum pRBC that can affect cytoadherence. So far, three heterologous molecules have been discovered to be involved in the adhesion of pRBC to the endothelial cell surface. These are: the integral membrane glycoprotein CD36 (Oquendo et al 1989); the large platelet-derived secretory protein thrombospondin (Roberts et al 1985); and the intercellular adhesion molecule ICAM-1 (Berendt et al 1989). Emerging data suggest that some P. falciparum pRBC adhere to one or more of the three potential endothelial cell receptor proteins but not all (Howard & Gilladoga 1989). This could mean the expression of up to three structurally different receptors of pRBC, each recognising a different binding site on the endothelial cell proteins. Alternatively, all pRBC may express approximately the same single receptor site with subtle structural differences generating marked alterations in affinity for the three different cytoadhesion proteins.

ICAM-1 has a broad distribution on epithelial, endothelial, monocytic, fibroblast, and B and T cell lines, and was originally defined with MAbs that inhibited lymphocyte adhesion (Simmons et al 1988, Staunton et al 1988). It has recently been demonstrated that a wide variety of immune interactions involve ICAM-1-mediated cell adhesion (Dustin & Springer 1988, Dougherty et al 1988, Makgoba et al 1988), and induction of ICAM-1 expression by cytokines in inflammation is important in regulation of leucocyte localisation in inflammatory sites (Staunton et al 1988). Indeed, TNF-α, IL-1, IL-2 and IFN-γ can all upregulate ICAM-1 levels on diverse cell types, including endothelial cells, in vitro and in vivo (Dustin et al 1986, Rothlein et al 1988, Asarnow et al 1989). Berendt et al (1989) have suggested that severe malaria may occur when those individuals expressing elevated levels of ICAM-1 are also infected with a strain of malaria parasite that has a high affinity for ICAM-1.

Together, these findings implicate host cytokines in the induction of the

immunopathology observed in patients with acute falciparum malaria. Paradoxically, these cytokines are secreted by Ly-4$^+$ T cells from malaria patients in response to P. falciparum Ags (Troye-Blomberg et al 1985, Riley et al 1988 a), and can inhibit malarial development in vivo (Maheshwari et al 1986, Clark et al 1987). Therefore, the same mediators of asexual blood stage parasite destruction may prove disadvantageous to the host. This supposition about the possible mechanism of immunopathology in the cerebral malaria syndrome explains the essential role of Ly-4$^+$ cells in the neurovascular complications of experimental malaria.

Once again, examination of the role of cytokines in cerebral malaria has been restricted largely to the P. berghei ANKA/ CBA/Ca mouse model. It was demonstrated that excessive release of TNF plays a critical role in the pathogenesis of experimental cerebral malaria (Grau et al 1987). Markedly elevated serum TNF levels were seen only at the time of the neurological syndrome, whilst being undetectable in normal mice and only slightly increased in non-complicated cases of P. berghei infection of BALB/c mice, or in CBA/Ca mice infected with P. yoelii, a species which does not induce cerebral malaria. In addition, in vivo depletion of Ly-4$^+$ T cells, which blocked neurological manifestations, prevented the dramatic rise in serum titres of TNF usual in P. berghei-infected CBA/Ca mice (Grau et al 1986). Moreover, treatment by a single injection of anti-TNF Ab exerted a protective effect on the P. berghei-induced neurological syndrome (Grau et al 1987). This administration prevented all pathological abnormalities including haemorrages and the focal arrest of monocytes and other circulating leucocytes within brain capillaries and venules, but without affecting blood parasitaemia. Also, the conspicuous macrophage accumulation in the spleen red pulp and lymph nodes of mice developing cerebral malaria was suppressed by anti-TNF Ab treatment, suggesting that self-amplification of TNF synthesis and of macrophage recruitment had been interrupted. Most recently, it has been shown that administration of recombinant murine TNF to a P. berghei ANKA-infected strain of mouse resistant to cerebral malaria induced a lethal neurological complication with all the clinical and histopathological features of cerebral malaria (Grau et al 1989 a).

These observations may seem paradoxical with respect to the favourable role of T cells and macrophages in malaria infection. Indeed, these data show TNF to mediate host tissue lesion although it is known to participate in the cell-mediated killing of asexual erythrocytic stage plasmodia. Under these experimental conditions, at least, a beneficial role for TNF has not been demonstrated, since the level of parasitaemia is not changed by

the injection of anti-TNF Ab (Grau et al 1987). It is not difficult, however, to conceive that macrophages and their products may have both beneficial and deleterious effects, depending of the degree of activation, timing and location, and thus may confer protective immunity, or, alternatively cause immunopathology during malaria infection.

In man, elevated concentrations of TNF have been noted in the serum of malaria patients (Scuderi et al 1986, VanderMeer et al 1988, Grau et al 1989 b), but the relationship between serum TNF levels and human cerebral malaria has not yet been analysed. In human cases, accumulations of packed pRBC rather than macrophages observed in the murine model are described in cerebral vessels. However, both phenomena may result from a similar mechanism, such as increased endothelial adhesiveness, which may reflect TNF-mediated vascular alterations. Therefore, TNF may also be of pathogenic significance in human cerebral malaria.

Since the macrophage is the major source of TNF and because Ly-4^+ T cells are required for cerebral malaria to occur, the role of T cell-derived cytokines able to activate macrophages has been analysed. In experiments involving in vivo neutralisation using anti-cytokine Abs, Grau et al (1988, 1989 c) demonstrated that TNF hyperproduction is the result of a cytokine cascade. This implicates IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF), which act by enlarging the macrophage pool, as evident in lymphoid organs, and IFN-γ, which upregulates macrophage function, including the release of TNF. In turn, TNF may exert its deleterious effect on cerebral lesions by its action on endothelial cells either directly or through the stimulation of IL-1 production (Dinarello et al 1986, Nawroth et al 1986). Both cytokines have been shown to increase the adherence of polymorphonuclear leucocytes and monocytes to human umbilical vein endothelium in vitro through the induction of surface expression of adhesion-promoting molecules (Bevilacqua et al 1985, Gamble et al 1985). The cytoadherence of pRBC to endothelial cells may be similarly increased with resultant microcirculatory obstruction. With the establishment of the role of IFN-γ in TNF overproduction (Grau et al 1989 c), and the demonstration that both cytokines can potentiate cytoadherence, it suggests that $CD4^+$ or Ly-4^+ cells of the T_H1 subset may become pathogenic in a chronic or complicated malaria infection. This effect is not unlike the way in which T_H2 cells exacerbate the normal disease progression in cutaneous leishmaniasis (Liew 1989, Scott 1989).

1.13 Parasite evasion of immunity

Malaria infections are characteristically of long duration, the asexual erythrocytic stages of Plasmodium surviving in the blood in spite of the host's immune response making its environment as inhospitable as possible. In the case of P. vivax infections, latent liver stages intermittently complete their development and infect the blood. However, in other malarias, such as P. falciparum, there are no latent forms and the persistent fluctuating blood infection must reflect, therefore , either an incomplete immune response, for which there is some evidence, and/or evasion by the parasite of the full effects of the host's acquired resistance. Indeed, the survival of some plasmodia in the face of immunological elimination is implicit in the concept of premunition, and suggests that mechanisms of acquired immunity must be balanced by evasive strategies on part of the parasite.

(a) Sequestration

RBC containing mature parasites of some malaria species undergo deep vascular tissue schizogony during which schizonts tend to withdraw from the peripheral circulation (Garnham 1966). This peripheral withdrawal of pRBC is stage specific and is usually referred to as sequestration. Regardless of the state of immunity, only very immature sexual forms, rings, or mature gametocytes are usually found circulating in the blood of humans infected with P. falciparum. The more mature asexual stages localise to the post-capillary venular endothelium of the brain, placenta or gut. In the brain, sequestration causes cerebral malaria, a syndrome associated with high mortality (Warrell 1987). Gametocyte development takes place initially in the deep vascular bed (Smalley et al 1980); however, mature gametocytes are released into the peripheral blood, as they need to be accessible to mosquitoes.

In man, schizont withdrawal of P. falciparum is almost complete. This parasite also undergoes deep vascular schizogony in Aotus monkeys, in which the extent of schizont withdrawal is as marked, but the major sites, heart and adipose tissue, are different (Miller 1969). Parasites adapted to Saimiri monkeys are known to sequester, but not to the same extent as in the human host (David et al 1983). Thus, at least in the case of falciparum malaria, host factors clearly influence the distribution and extent of pRBC accumulation in deep vascular beds. Of the other human plasmodia, P. vivax shows some slight degree of withdrawal from the peripheral circulation from about 36 hr on in the 48hr asexual schizogony cycle (Garnham 1966). No evidence has ever been produced

that this occurs in P. malariae (Howard 1988), although with the typical low level infection this parasite normally produces, it may be difficult to detect.

Several primate and rodent species also show relative schizont disappearance from the blood stream during asexual maturation. This is most marked with P. coatneyi and P. fragile in both natural and unnatural hosts (Desowitz et al 1969, Fremount & Miller 1975). With both these parasites, tissue localisation was mainly in the cardiac muscle and to a lesser extent in adipose tissue or the small bowel mucosa. P. knowlesi shows weak schizont disappearance and similar tissue distribution in an unnatural host, the rhesus monkey; however, this only occurs at low parasitaemias (Miller et al 1971 a). At higher parasitaemias, trophozoites and schizonts are quite easily recognised in the peripheral blood.

In P. berghei, sequestration occurs predominantly in the liver and bone marrow in young rats, and in the spleen and bone marrow in mice (Alger 1963). In venous sinuses of the bone marrow of mice, Weiss (1983 b) observed reticulocytes parasitised with P. berghei adherent to endothelial cells and to non-parasitised reticulocytes. He suggested that merozoites may enter adherent reticulocytes in the local vicinity without significant extracellular exposure. The release of factors from pRBC or lymphocytes may explain the early reticulocytosis observed in this disease before the haematocrit falls greatly; thus, new reticulocytes would be released in a site favourable for parasitisation. By this anatomical arrangement, the spleen is largely avoided, and the pRBC are resistant to oxidant stress, so that cell-mediated immunity is less effective than in other murine malaria infections, which show far less predilection for bone marrow and may multiply in mature RBC.

The ANKA strain of P. berghei shows some accumulation of both pRBC and nRBC, and macrophages, at low parasitaemias in cerebral vessels of mice (Mackey et al 1980, Rest 1982). However, this may not represent preferential sequestration of parasites in the cerebral circulation (Warrell 1987), although this has been used as a model for human cerebral malaria (e.g. Grau et al 1987). P. c. chabaudi also exhibits peripheral withdrawal (McDonald & Phillips 1978) and schizonts have been noted to accumulate markedly in the liver (Cox et al 1987).

In the few systems where marked stage-specific peripheral withdrawal of pRBC is observed, deep vascular schizogony is clearly a parasite-induced process. It is often suggested that the reason sequestration occurs is to avoid splenic filtration, or because of the parasite preference for the relatively anoxic environment of the deep vasculature

(Howard 1988). Furthermore, it is often assumed that the less marked peripheral withdrawal shown by many species, e.g. P. knowlesi, also represents sequestration. No formal proof of this has been made in any model, and an alternative hypothesis is that this represents specific or non-specific host clearance mechanisms in the reticulo-endothelial system in the spleen or liver. The extent to which sequestration represents a characteristic feature of many malaria species, or a specific feature of only a few host-parasite combinations is thus unclear.

Mature trophozoite, schizont and segmented intracellular merozoite stage pRBC are all sequestered in various tissues by attachment to the endothelial cell lining of small blood vessels, particularly post-capillary venules. Several critical phases of parasite development, including nuclear division, growth in size, and segmentation of the multinucleate parasite cytoplasm into daughter merozoites, occur in this particular vascular microenvironment. P. falciparum asexual stage pRBC, particularly the mature forms, grow best in vitro under conditions of relatively low oxygen tension (Scheibel et al 1979), conditions similar to those of their site of sequestration in vivo. A number of hypotheses have been proposed to account for evolution of this sequestration characteristic of P. falciparum, unique amongst the human malarias; these include the requirement for a microaerophilic environment.

Sequestration of mature pRBC also prevents their passage through the spleen and could enhance parasite survival by this means as well. Mature P. falciparum infected pRBC contain a large parasite inclusion and have grossly impaired deformability compared to nRBC and immature pRBC (Cranston et al 1984). The splenic mechanisms for removal of RBC with reduced deformability (Sandza et al 1974, Driessen et al 1982, Card et al 1983), or inclusions (Schnitzer et al 1972) as the cells exit the spleen through the five fenestrations between the cells of the venous sinus wall are thereby avoided. The surface of mature infected RBC is also altered in expression of new Ags (Howard & Barnwell 1983) and alterations in surface carbohydrates (Sherman & Greenan 1986). The recognition of foreign cell surfaces by resident macrophages (Wyler et al 1979 a, Kreier & Green 1980) through Ab-dependent or independent mechanisms is also avoided by the capacity of P. falciparum pRBC to sequester to an immunologically privileged site. Although these concepts could account for the significantly greater virulence of P. falciparum as compared to other human malarias, in which all blood stages traverse the spleen and most mature pRBC may actually be destroyed, it does not account for the fact that other human malaria parasites survive at all as mature asexual stages in the

peripheral circulation if splenic selection is so potent. Splenic trafficking may be altered in infections of P. vivax, P. malariae or P. ovale so that a proportion of trophozoites and schizonts do survive in the bloodstream. Indeed, malaria-induced changes in splenic trafficking and in histology have been reported for different malarias, including acute P. falciparum malaria (Wyler et al 1981, Weiss et al 1986, Looareesuwan et al 1987). It is clear, however, that for P. falciparum, sequestration, and the mechanism of cytoadherence underlying it, are major determinants of parasite virulence. This is exemplified by the fact that parasite variants produced in the laboratory which do not cytoadhere in vitro or in vivo are of low virulence upon infection of Aotus monkeys (Lanners & Trager 1984, Green et al 1985, Langreth & Peterson 1985).

Cytoadherence of pRBC to endothelial cells during falciparum malaria represents the acquisition of a specific, functional cell surface property on the infected cell. Immature pRBC, rings and early trophozoites, and nRBC do not adhere to endothelial cells (Udeinya et al 1981) Thus, a specific receptor is expressed on the infected cell surface, capable of recognising a ligand(s) on the endothelial cells. The receptor is surface-exposed on pRBC for about 24 hr, half of the asexual schizogony cycle, and, since it must retain structural and functional homogeneity regardless of other phenotypic variations, it could represent an antigenically conserved target for an anti-malarial vaccine (Howard 1988).

A strong argument for the enhanced parasite survival conferred by the capacity of mature P. falciparum pRBC to sequester comes from studies with laboratory parasite variants lacking the cytoadherence property. Transmission electron microscopy of post mortem material of humans (Trager et al 1966, MacPherson et al 1985) and of Aotus (Luse & Miller 1971) have shown knob-like protusions of parasitised, sequestered RBC at the point of contact with the endothelium. Knobs are characteristic alterations of the pRBC membrane, only demonstrable by electron microscopy. They are conical protrusions of the RBC membrane, approximately 100 nm in diameter, overlying an electron dense core, which dynamically appear and change during parasite maturation (Langreth et al 1978, Gruenberg et al 1983). Knobs are absent from ring stage pRBC of P. falciparum or any blood stage of other human malarias (Howard 1988), none of which sequester in vivo. Early investigators correlated the presence of knobs with the property of cytoadherence, showing that adhesiveness is acquired at the same time in the asexual development cycle as the characteristic morphological alterations of the host

RBC membrane take place (Trager et al 1966, Luse & Miller 1971, Langreth et al 1978). The role of knobs in adherence to host cells was later confirmed when knobless variants of P. falciparum (denoted K^- to distinguish them from K^+ knobby wild-type parasites) were shown to not cytoadhere in vitro (Udeinya et al 1981), ex vivo (Raventos-Suarez et al 1985) or in vivo (Barnwell et al 1983 a, David et al 1983). Most important, K^+ parasites sequestered in Aotus and Saimiri monkeys, whereas K^- variants did not (Lanners & Trager 1984, Langreth & Peterson 1985). The K^- plasmodia have arisen by either continuous passage of K^+ parasites in human RBC in vitro (Langreth et al 1979, Trager et al 1981) or by passage of K^+ parasites in splenectomised monkeys (Barnwell et al 1983 a & b, David et al 1983).

It is inferred, on this basis, therefore, that K^- mutation is not selected for in the field. Studies with K^+ and K^- organisms infecting non-human primates have shown that although K^+ parasites produce fully virulent, sequestering infections, K^- variants lead to virulent infections with only very low parasitaemia (Lanners & Trager 1984, Langreth & Peterson 1985, Green et al 1985). Moreover, K^+ infections required drug cure of the animal host, whereas K^- infections recovered spontaneously. In addition, in splenectomised monkeys, K^+ and K^- infections were almost identical in the course of peripheral blood parasitaemia; indeed, K^- parasites often grew quicker in vivo in such hosts, consistent with a higher growth rate for K^- parasites in vitro (David et al 1983, Langreth & Peterson 1985). Therefore, in the absence of the selection pressure of the host spleen, K^- infections proved fully virulent, the presence of mature K^- pRBC in the peripheral blood having no disadvantage to the parasite.

It has also been shown that mature P. falciparum gametocyte-infected RBC derived from cytoadhering K^+ asexual parasites do not express knobs at the cell surface (Miller 1972) and do not sequester. Interestingly, immature gametocytes of the same species also fail to express knobs but are sequestered from the peripheral blood (Smalley et al 1980), obviously by a different mechanism to that for mature asexual stages.

The correlation between sequestration and knob expression apparent for P. falciparum infections of man or monkey models extends to falciparum-like malaria species in non-human primates. P. coatneyi trophozoites express cell surface knobs (Kilejian et al 1977) and sequester in vivo in rhesus monkeys (Desowitz et al 1969), whereas gametocytes are knobless and fail to sequester (Rudzinska & Trager 1968). Similarly, mature P. fragile pRBC express knobs and sequester in infected rhesus monkeys; gametocytes do not (Fremount & Miller 1975). Although knobs are required for

cytoadherence of P. falciparum, their presence as morphological entities is alone insufficient to confer this functional property. This was demonstrated by Udeinya et al (1983 a & b) who passaged in vitro some K^+ parasites which cytoadhere or bind (K^+B^+), whereupon they were converted to a K^+ B^- phenotype. Clearly, an additional surface membrane property other than knob expression is necessary for cytoadherence of P. falciparum, but this has not been identified. The quartan malaria parasites P. malariae and P. brasilianum both express knob protusions on the surface membrane of trophozoites, schizonts and gametocytes, yet none of these altered RBC sequester (Smith & Theakston 1979, Sterling et al 1972). These observations support further the concept that knobs are insufficient for cytoadherence, and raise the possibility that other important functions, yet undiscovered, are mediated by these parasite-induced structures.

Knob composition is complex and not fully elucidated (reviewed by Howard 1988). One of the Histidine Rich Proteins, HRP 1, has been identified as part of the knob protein (Kilejian 1979). Another component, called pf EMP2, has been localised to the knob structure (Howard 1987) and is identified to the mature parasite-infected erythrocyte surface antigen (MESA) (Coppel et al 1986). Pf EMP1 appears to be a surface-located protein associated with the knobs (Howard 1988). A further seven proteins have been identified in different laboratories as components of the RBC membrane of P. falciparum K^+-infected cells. Whether a full complement of knob proteins is essential for sequestration is unclear.

Much recent work has been dependent on the development of an in vitro cytoadherence assay. Human endothelial cells derived from umbilical cords (Udeinya et al 1981), melanoma cells, especially the amelanotic C32 line (Schmidt et al 1982) and monocytes (Ockenhouse et al 1984) are all cells to which P. falciparum schizont-infected RBC will specifically adhere. Using such a binding assay, it has been possible to show that the cytoadherence ligand is trypsin sensitive (David et al 1984).

Saimiri immune serum that can reverse schizont binding in vitro can also reverse sequestration in vivo (David et al 1983). Reversal or blocking of binding is strain-specific, suggesting that a functional part of the sequestering molecule is either itself strain-specific or sterically inhibited by Ab to a strain-specific antigenic determinant (Udeinya et al 1983 b).

The sequestering Ag is now thought to have been identified. This is a large protein of 300 kDa, which is localised to the cell surface by trypsin activity and iodination (Leech et al

1984). It is also retained after detergent extraction, suggesting an attachment to the pRBC cytoskeleton (Leech et al 1984). It was the only protein immunoprecipitated with strain-specific immune serum that correlated with the ability of sera to interfere with cytoadherence. This antigenic determinant has recently been called pf EMP1 (Howard 1988), although no definitive proof of its identity has been obtained. How exactly its presence is functionally related to sequestration is not yet established.

(b) Antigenic variation

One paradox in malaria is the ability of asexual stage parasites to remain patent in the peripheral blood for weeks or months of chronic low-grade parasitaemia, whilst the host stays clinically immune to any adverse effects. This is a near ideal state of parasitism, but is only reached after considerable morbidity is experienced during the acute infections that generate such immunity. The complex interaction of immunised host and asexual parasite appears to involve both the evasion of immunity by the malaria parasite and the ability of the host to limit plasmodial growth below life-threatening levels. The availability of well-characterised antigenic variants has proved important in the study of this immune evasion mechanism. This is because, despite the fact that recent data from field studies in endemic areas indicate that human responses to the analogous pRBC surface Ags are isolate-specific (Marsh & Howard 1986), and that these particular responses have a role in protective immunity, it is obviously impossible to delineate the role of antigenic variation under field conditions with uncloned parasites and constant exposure.

In humans, and in several other systems, it is relatively easy to establish solid immunity to one strain or clone of malarial blood stage parasite with repeated challenge, but this does not occur in natural transmission (Kitchen 1949), where there is a slow acquisition of acquired immunity. This is thought to be due to immune evasion on the part of the parasite, as, for example, splenectomy during established infection of the primate quartan malaria P. inui can greatly reduce the period of chronic blood stream infection (Wyler et al 1977). The inference from this and from other studies is that evasion mechanisms are selected for in the presence of immunological pressure by the host.

Although several explanations have been advanced for the chronicity of malaria and the capacity of asexual parasites to reinfect the host (Brown 1969), extensive serological and immunochemical data point to the ability of blood stage plasmodia to undergo

phenotypic antigenic variation as of paramount importance (Brown & Brown 1965, Brown et al 1968, Voller & Rossan 1969, Brown et al 1970 a & b, Brown 1971, Butcher & Cohen 1972). During the period of a single infection, asexual erythrocytic stage parasites repeatedly change functional Ags presented to the host, thereby requiring the host to make a specific immune response, usually Ab-mediated, to each new antigenic or variant type as it appears. The new antigenic variants are unaffected by immunity to previous variants, and by the time that the response to the new Ag reaches effective levels, a still newer variant is being produced. This mechanism keeps the parasite a step in front of the host's protective response and allows its survival regardless of the effect of the specific acquired immunity. Usually the immune response, a major component of which will be anti-malarial Ab, to each new variant as it appears is rapid and correspondingly the parasitaemia is maintained at a low level. Indeed, it is speculated that the presence of an anti-parasite Ab specific for the immunodominant variant type in the peripheral circulation may provide the signal to the parasite to change its variant type (Phillips 1983). Phenotypic antigenic variation by the multiplicative blood stage has been observed in several Plasmodium species, and involves Ags on the surface of the pRBC expressed late in the asexual cycle; these may (Newbold & Marsh 1990) or may not be parasite-derived (Sherman & Winograd 1990) in origin.

As a general strategy for maintenance of chronic infection in parasites, the phenomenon of antigenic variation has been studied in trypanosomes (Cross 1978) and borrelian spirochaetes (Stoenner et al 1982). Both these pathogens exist extracellularly in the bloodstream and are transmitted between host by blood-sucking arthropod vectors. Each organism has a surface-located immunodominant protein against which the host mounts a successful immune response. In such a way, one particular predominant antigenic variant is removed, thereby selecting for the expansion of another variant type which forms a patent wave or recrudescence of infection. Regular recrudescent peaks occur with the antigenically variant populations of both Trypanosoma and Borrelia in experimental infections with clones or strains.

In wild populations and multiple infections, such regular patencies may not be seen. A similar pattern is observed in many laboratory infections of malaria. However, unlike the extracellular blood stream pathogens, malaria is predominantly intracellular, and pRBC are not covered with immunogenic parasite material. Rather, surface-located Ags, which are expressed by trophozoites and schizonts, but which are not immunodominant,

have been described in several primate and rodent malarias.

The best described system for study of antigenic variation in malaria is P. knowlesi in rhesus monkeys (Brown1977), first reported by Brown & Brown (1969). One problem is that this is not a natural host-parasite combination, and P. knowlesi will kill the monkeys unless drug treatment is given. If subcurative chemotherapy is administered, such that the animal recovers but remains parasitaemic, successive waves of infection will occur, none of which need drug cover. Using this model, Eaton (1938) showed that schizont-infected RBC, but not the younger stages, can be agglutinated by immune serum. Later, the schizont-infected cell agglutination (SICA) test was developed to investigate the serology of patent recrudescent parasite populations; this was done initially with uncloned parasite lines (Brown et al 1968), but has been repeated with pRBC clones (Barnwell et al 1983 a). In all cases, each population stimulated specific agglutinating Ab in the infected monkey (Brown & Brown 1965). The new surface Ag responsible for Ab-mediated agglutination of pRBC was called the SICA or variant Ag (Brown et al 1968) and has been identified as a high MW protein of about 200 kDa (Howard et al 1983).

The ability of P. knowlesi to vary the SICA Ag is dependent on the presence of specific Ab of the appropriate variant specificity. Studies with both uncloned (Brown 1973) and cloned parasites (Barnwell et al 1983 a) imply strongly that this variation is Ab-induced rather than an immunoselective process as it is in both trypanosomes and borreliae. Rechallenge of SICA-primed monkeys with homologous variants induces change to another variant, whereas a heterologous variant is not induced to change phenotypes. In addition, the presence or absence of the spleen markedly affects expression of the variant SICA Ags. SICA-positive clones passaged in splenectomised monkeys lose both their susceptibility to agglutination and their detectable surface fluorescence. This occurs as an alteration of expression of the phenotype in all cells rather than appearance and selection of a SICA negative line. This phenotype is stable in splenectomised monkeys, and for variable periods in intact animals, during an acute infections, so is unlikely to be due to unusual properties of splenectomised host RBC (Barnwell et al 1983 a). Indeed, cloned SICA-negative lines can re-express the SICA positive phenotype after serial passage in intact monkeys. Howard & Barnwell (1985) showed by immunochemical analysis that the SICA-negative phenotype lacks expression of the variant SICA Ag, rather than expressing different non-functioning variants.

The induction of antigenic variation of SICA variants of P. knowlesi by Ab is also spleen

dependent (Howard 1984). Monkeys fully drug-cured and thereby vaccinated against that variant induce variation of the homologous phenotype upon challenge. Immunised monkeys splenectomised just prior to challenge do not, however, induce switching, despite the presence of variant specific agglutinating Ab.

The function of the SICA Ag has not been established, as little work has been carried out on the natural host-parasite combination, P. knowlesi in the Kra or cynomolgus monkey. Observations made in the atypical rhesus infection may not be valid, especially as protective immunity is not established naturally. Nevertheless, a role in virulence is suggested, as intact rhesus monkeys challenged with SICA-negative parasites experience two different outcomes (Barnwell et al 1983 a). With pRBC that do not revert to the SICA positive phenotype, the infections were controlled without drug intervention, whilst fulminating infections were suffered by monkeys in which parasites became agglutinatable. This did not relate to different multiplication rates, which were similar in both SICA-positive and negative clones in intact animals; similarly, both parasitic phenotypes form virulent infections in splenectomised monkeys. It has also been found that in this model, SICA negative phenotype is stable after mosquito passage (Barnwell et al 1983 b).

Other malaria parasites have been studied but in less detail. Ags localised on the surface of P. falciparum schizont infected RBC can be identified by binding of Ab-coated cells to protein A sepharose columns and by indirect immunofluorescence (Hommel et al 1982). IFAT-detectable Ags are trypsin-sensitive and strain-specific (Hommel et al 1983), but it is not yet known whether these are localised at knobs or distributed over the entire pRBC surface.

In the P. falciparum/Saimiri monkey model, whereas hyperimmune sera showed some cross-strain reactivity, with convalescent monkey sera, high strain specificity, albeit of low titre, was noted (Hommel et al 1983). This specificity was used to examine surface phenotypes of recrudescent parasites, or of those taken from secondary peaks of infection, each following homologous challenge or after passive transfer of imune sera. These different strategies were necessary because in this particular system recrudescences or secondary parasitaemia peaks are not common after homologous challenge with all strains. These results probably show that under immune pressure the parasite has the genetic capacity to switch from one serotype to another within the time span of challenge and appearance of a patent recrudescence of new serotype. However, this work was performed with uncloned lines of P. falciparum (Hommel et al 1983).

However, P. falciparum isolates cloned by limiting dilution have been shown to undergo modulation of antigenic phenotype at the surface of pRBC (Hommel et al 1983).

This switch in expression of Ags occurs during a change in host environment, between intact and splenectomised monkeys. This could represent a switch between two separate groups of Ags expressed in either intact or splenectomised hosts, or expression of variant forms of a homologous Ag (Howard 1984). Until this change can be shown to be due to the latter effect, it cannot be described as antigenic variation in the sense that is appropriate to P. knowlesi, T. brucei or Borrelia.

Another primate species, P. fragile, is the most recently described parasite in which variation in surface-located schizont specific Ags has been described (Handunnetti et al 1987). Cloned material was used, and is of special interest as all the work has been done using the toque monkey, Macaca sinica, a natural host for this parasite. Antigenic variation has been shown in other primate systems, but not located to a specific antigenic determinant.

There is no direct serological or biochemical evidence for antigenic variation with plasmodia other than P. knowlesi, P. falciparum, and P. fragile. Nevertheless, the findings of numerous biological studies are consistent with the existence of antigenic variation in several rodent malarias. This phenomenon was first described in P. berghei by Cox (1959, 1962), who showed that if acute infections were suppressed by drug treatment, a latent infection could be produced with periodic recrudescences. Challenge of naive mice with such recrudescences suggested that the parent and recrudescent populations were different in virulence and in immunity. Ab-induced variation in this parasite has also been demonstrated (Briggs et al 1968). Additional evidence for antigenic differences between P. berghei derived under the selective pressure of homologous immunity has come from experiments with the ANKA strain of this parasite. Mice were multiply immunised by repeated infection and drug cure. After infection with a cloned parasite, successive waves of parasitaemia with distinct subpatencies were observed (Wery et al 1979). Cross-challenge experiments between successive recrudescences suggested strongly that antigenic variation had occurred (Wery & Timperman 1979).

Using cloned lines of P. c. chabaudi AS in NIH mice, antigenic variation has been demonstrated in recrudescent populations by passive transfer of immune serum collected from mice following resolution of the acute infection before any recrudescence had occurred. Significant delay of the homologous parasite population to reach 2%

parasitaemia was noted with this serum in passive transfer compared to normal mouse serum. However, most cloned recrudescent populations were less sensitive to this immune serum (McLean et al 1982 b). A decline in the effector arm of the immune system takes place following resolution of the primary parasitaemia (McLean et al 1982 a). However, this was excluded in the passive transfer system, and cannot explain differences in sensitivity between the parental and recrudescent cloned parasite populations.

In similar experiments with CBA/Ca mice, malaria populations of P. c. chabaudi AS breaking through such passively transferred immunity seem to have changed. Breakthrough populations were found to be much less sensitive to the same serum than the original infecting clone in a similar passive transfer (Jarra et al 1986). In the NIH model, some heterogeneity was noted in the response of distinct parasite populations to passive transfer; this occurred to a certain extent for clones from the primary parasitaemia, itself a clone, and to a greater extent for clones derived from the recrudescence (McLean et al 1986 b).

As an alternative to the relatively cumbersome passive transfer system, a surface immunofluorescence assay has been adapted to live schizont-infected RBC of P. c. chabaudi and used to distinguish between antigenically different populations of this parasite species (McLean et al 1986 a, Gilks et al 1990). This has shown cloned recrudescent populations to be both different from the initial, infecting parental cloned population, and from each other, using both immune sera collected upon resolution of acute parasitaemias, and hyperimmune sera raised as described by Gilks et al (1990) (Brannan, L.R., personal communication). There does, therefore, appear to be strong evidence in favour of antigenic variation in the P. c. chabaudi model.

Overall, much work exists to suggest that antigenic variation occurs as a feature of recrudescing or relapsing chronic infection in malaria. Surface located parasite-derived neo-Ags, expressed at late trophozoite and schizont stages have been shown to be one group of Ags that is variant. However, no evidence shows unequivocally that phenotypic variation affects the duration of blood stream infection, and no function whereby such surface-located Ags may facilitate chronic infection has been defined. It is unlikely to be an immunoselective process, as, for instance, in trypanosome infection, because these surface Ags are not immunodominant; Ab also induces rather than selects variation. It is possible that the variant Ag has a specific function which needs a surface location. The obvious function is sequestration, and similarities between these two

processes of immune evasion have been noted (Hommel 1985, Howard 1988).

Many similarities are apparent between asexual erythrocytic stage malaria parasites showing the sequestering phenotype and undergoing antigenic variation. Both phenomena are parasite-induced changes on the surface of the pRBC that become apparent as schizogony commences, and both phenotypes are modulated by the spleen. Moreover, those parasites which sequester in vivo tend to have detectable variant surface Ag. Biochemical similarities exist between pf EMP1, the probable sequestering Ag of P. falciparum and the SICA Ag of P. knowlesi, the best characterised surface variant Ag. Knobless and SICA-negative pRBC cause attenuated infections which induce effective immunity.

It has been suggested that the phenotypes may be the same Ags (Hommel 1985, Howard 1988). Until recently, no system in which both phenotypes have been linked had been described; however, Gilks et al (1990) have reported the use of P. c. chabaudi AS in CBA/Ca mice as such a model. Preliminary findings showed that cloned parasite lines exhibit both clonal antigenic variation in late stage-specific surface Ags, and deep vascular schizogony in the liver. Furthermore, both these features were modulated by the spleen and surface Ag expression was crucially involved in the sequestering phenotype.

(c) Antigenic diversity

An important recent development in malaria research has been the increasing appreciation of the degree of antigenic diversity that exists in malaria parasites. This is the expression of antigenically different forms of an Ag by different malaria isolates. Plasmodia exhibiting antigenic diversity may be derived from different geographical locations, different individuals at the same location, or different bouts of malaria within the one individual. In addition, diversity may also be recognisable in pRBC taken at various times from a non-cloned parasite isolate cultivated in vitro. Under such conditions, it cannot be assumed that the phenotypically distinct parasites are derived one from another or from a single parental type. Ags that exhibit diversity may, but do not necessarily, confer a selective advantage to the parasite, and thus Ab-dependent selection of different phenotypes is not implied (Howard 1984).

Diversity within different species of malaria is a major reason why asexual blood stage plasmodia survive despite the ability of their hosts to mount immune responses which are effective in eliminating a particular infecting population. Significant antigenic

diversity in malaria parasites of humans was demonstrated when individuals deliberately infected with P. falciparum were found to be relatively more resistant to subsequent challenge with the homologous strain than with a heterologous strain (e.g. Jeffrey 1966). The slow development of immunity in people living in malaria endemic areas is consistent with the hypothesis that acquired immunity develops only after exposure to a large number of different parasite isolates.

For many years, geographical variation in drug resistance or transmissibility through mosquitoes has been recognised (Shute et al 1976). Immune serum from West Africa was less effective than East African serum on disease in East Africa (McGregor et al 1963), and morphological differences between isolates of the same species or subspecies have long been suggested and disputed (Garnham 1966). Major geographical variation has now been shown by such techniques as enzyme electrophoresis, Ag characterisation, two dimensional protein electrophoresis, drug sensitivity and variation in Ag sequence. Nevertheless, P. falciparum is still considered a single species worldwide in which gene exchange can freely occur (Walliker 1983).

A serological typing system has been generated using different MAbs raised against blood stage Ags, principally recognising schizonts, merozoites, or both (McBride et al1982). It has been shown that numerous malarial Ags exhibit antigenic diversity in different P. falciparum isolates; for example, the major glycoprotein on the surface of mature asexual stage pRBC (McBride et al 1982) and the S Ag released into plasma during rupture of schizont-infected RBC (Wilson 1980).

Ths S Ag system of heat stable proteins of unknown function is probably the best studied of the polymorphic Ags. Extensive work with serotyping (Wilson 1980) or with immunoblotting (Anders et al 1983) has been undertaken, and it is clear that even geographically quite localised populations have several different S Ag phenotypes at one time. For instance, in one area of Papua New Guinea, clear periodic small area variation of one (FC27) S Ag has been shown (Forsyth et al 1989).

In contrast to this enormous antigenic diversity, antigenic variation has been noted only in surface-expressed late stage-specific Ags and reflects the differential expression of a particular antigenic phenotype from a repertoire of genes capable of expressing alternative phenotypes in progeny organisms. However, antigenic diversity and variation are not necessarily mutually exclusive properties of a malarial Ag; in theory, an Ag that exhibits antigenic diversity could also undergo antigenic variation, though none has been found to date.

The great phenotypic diversity achieved by malaria parasites is thought to be of advantage to the parasite as many host protective immune responses are relatively specific, at least initially. With the limited information available, it has always been assumed that the antigenic diversity of various pRBC Ags reflects the expression of different allelic forms of a single gene. The diverse forms are generated either by accumulation of mutations or by genetic rearrangements during meiosis. That heterogeneity is maintained by genetic recombination in addition to mutation has been shown in P. c. chabaudi (Walliker et al 1975) and more recently with human malaria (Walliker et al 1987). In the latter case, two distinct gametocyte-containing cloned isolates of P. falciparum were mixed and mosquitoes fed on the blood meal. After transmission of resulting progeny sporozoites to a chimpanzee, the asexual erythrocytic stages were screened and recombination shown to have occurred.

1.14 Development of a malaria vaccine

P. falciparum affects millions of people, causes a potentially lethal disease and is becoming increasingly resistant to chemoprophylaxis and chemotherapy. Attempts to control the spread of the infection by combating the Anopheles mosquito vectors have failed. Some mosquitoes have become resistant to insecticides, and many species or subspecies are not strictly domicilary and are therefore very difficult to eliminate. It is clear that there is a pressing need for research towards vaccines, new anti-malarial drugs and novel methods to control mosquito vectors. The production of a safe and effective malaria vaccine must be the ultimate practical aim of research into the immunology of malaria, and during the last few years a great deal of fundamental work has been performed to this end.

Due to the complicated life cycle of malaria, each of the different stages presents possible target cells for immunisation, and experimental vaccines are being developed against all of them (Miller et al 1986). Thus, Ags for vaccine development are being identified from sporozoites, which are injected by mosquitoes into man; exoerythrocytic forms, which develop in hepatic parenchymal cells; asexual erythrocytic forms, which cause the disease; and sexual stages, which transmit the infection to mosquitoes. As acquired immunity appears to be stage-specific, this presents a problem in that a parasite which escapes immunity at one stage in the life cycle may be unaffected by this immunity during its development in the next stage. For example, if a parasite evades immunity induced by a sporozoite vaccine, it may proliferate to high parasitaemia in the

asexual erythrocytic cycle and cause death. Thus, unless a sporozoite or liver vaccine is completely effective, an asexual erythrocytic vaccine, if feasible, would be the most useful in Africa where the goal is to reduce mortality. Immunological neutralisation of gametocytes or their products would interrupt transmission of infection from man to the uninfected mosquito. The disadvantage of such a vaccine is that it would not directly protect the vaccinated individual, but would only prevent further transmission to the community.

The features required of a malaria vaccine may vary according to its role. In order to be successful in a public health capacity, a cheap, single inoculation preparation is required, which must be effective in conferring considerable and long-lasting protection in infants who may have been exposed to both infection and to maternal Ab. In contrast, a vaccine for the non-immune visitor to endemic areas may be required to preclude the development of parasitaemia completely, because of the considerable danger of early death in an adult experiencing a first parasitaemia of P. falciparum, and in order to minimise the chances of spread of disease upon return home.

Regarding the potential usefulness of vaccines for disease control, permanent residents in endemic areas constitute by far the largest populations that would benefit from successful vaccination and they are the major target group for eventual vaccine use. Most of those living in areas where P. falciparum is endemic develop effective immunity to malaria only after suffering multiple infections, usually over a period of several years. At such a time, blood infections may not be suppressed completely and adults who have been exposed to malaria throughout life frequently have asymptomatic parasitaemias. Experimental infection of humans has shown that a single infection with P. falciparum tends to modify the severity of subsequent infections, and among children living in an endemic area the clinical effects of successive infections becomes progressively less severe. It is possible that an asexual blood stage vaccine would have an effect similar to that produced by repeated natural exposure, dramatically reducing mortality and morbidity whilst not completely preventing parasitaemia. In such a situation, subsequent infections may boost vaccine-induced immunity. Obviously, vaccination would be of special importance for babies and pregnant women, but, if an anti-pRBC vaccine were to 'shortcut' an otherwise slow and hazardous acquisition of immunity, it would have to be administered soon after birth. Vaccine-induced asymptomatic infections of infants and children may be attainable, since it has been possible to immunise monkeys against P. knowlesi (Mitchell et al 1975), a malaria that

can infect monkeys repeatedly and produce a chronic infection. Successful vaccination of monkeys required the use of whole, mature parasites emulsified in Freunds complete adjuvant. If, however, safe, effective adjuvants for use in children are not available, then finding such an adjuvant may be critical to the development of a malaria vaccine.

Attenuated (Clyde et al 1973, Weinbaum et al 1976 b, Rieckmann et al 1979, Waki et al 1986) and killed (Desowitz & Miller 1980) malaria parasites have been shown to be efficacious as vaccines for humans or animals. The reason that such vaccine preparations are not currently used for disease prevention in humans is because of the difficulties of large scale in vitro cultivation of plasmodia (Trager & Jensen 1976) and the associated risks of preparing pRBC from cultures containing human serum, which is an essential requirement of in vitro cultivation. If such difficulties were to be overcome, it would be logical to develop a blood stage vaccine for human use from whole pRBC preparations. An attenuated live vaccine for P. falciparum could be a parasite isolate which, through gene deletion, has lost the capacity to adhere to endothelial cells (Pologe & Ravetch 1986). Such pRBC would not cause major organ pathology, notably cerebral malaria, and would circulate through the spleen, whereby they would be both cleared from the circulation and stimulate an immune response which would protect against a virulent parasite challenge.

At present, however, such a course is not possible. It is necessary, therefore, to stimulate protective immunity to the whole parasite from only one or a few parasite proteins, or derived peptides, prepared by recombinant or synthetic peptide technology, i.e. a subunit vaccine. The only possible active components of a vaccine are parasite-derived Ags, in particular pRBC surface Ags. It is asssumed that immune responses induced by the majority of these determinants are irrelevant to protection, or may even be undesirable, and that only a small number of the Ags induce protective responses. Subunit vaccines against falciparum malaria have already been tried with encouraging but limited success (Ballou et al 1987, Herrington et al 1987). These subunit vaccines, and others under consideration, have as their primary objective, the development of high titre anti-malaria Abs, which are thought to play a role in protection. However, the approach to malaria vaccines has been broadened significantly by the demonstration that a single $CD4^+$ T cell clone can adoptively transfer protection for the murine malaria parasite P. c. adami (Brake et al 1988). It has been inferred from this that a single T cell site on one parasite Ag can be a target for a protective immune response. Although there are manifest complications, not least of which is the

genetic restriction of the immune response to individual T cell epitopes, it appears that any candidate anti-blood stage vaccine will need to contain multiple T sites within the immunogen for natural boosting of the Ab response.

The strategy for the development of synthetic malaria vaccines is based on the identification and characterisation of these parasite Ags which specifically stimulate protective immune responses. Hybridoma technology for the production of MAbs has been applied in an effort to identify surface Ags from small amounts of starting material, and affinity column chromatography has permitted the isolation of protective Ags (e.g. Holder & Freeman 1981). The next phases involve the application of recombinant DNA technology to clone the genes encoding the antigenic epitopes, their expression in bacterial or yeast expression vectors, analysis of the nucleotide sequences, deduction of the corresponding amino acid sequences of the encoded peptides, and production of these molecules either by genetic engineering or by chemical synthesis. To date, a large number of Ags of the asexual erythrocytic stages of P. falciparum have been described and the genes encoding many of them have been coded (e.g Kemp et al 1983). None of these Ags appears dominant as a target of protective immune responses. Rather, several Ags appear to be capable of inducing immunity that limits the growth or development of P. falciparum, and it is possible that maximum efficiency will be achieved only with a vaccine that combines several such Ags. Other factors to consider in the individual tailoring of a subunit vaccine include immunogenicity for the population and appreciation of plasmodial mechanisms for immune evasion.

(a) Sporozoite vaccine

One malaria vaccine strategy involves immunisation with sporozoite-specific immunogens in order to elicit an immune response that blocks sporozoite entry into hepatocytes and/or leads to the destruction of infected liver cells (Nussenzweig & Nussenzweig 1989). Of all the different parasite stages, the development of a vaccine against the mosquito-borne infective form of P. falciparum is currently at the most advanced stage. Studies in rodents (Nussenzweig et al 1969 a), monkeys (Gwadz et al 1979) and humans (Clyde et al 1975) have demonstrated that repeated administration of irradiated sporozoites can result in at least short-lived resistance to rechallenge with homologous sporozoites, and that this protective immunity is Ab-mediated. Vanderberg et al (1969) showed that this Ab reacts with the surface of sporozoites giving rise to the circumsporozoite precipitin (CSP) reaction, in which surface epitopes are crosslinked

by Abs forming a characteristically long tail-like precipitate on the posterior end of the parasite. Since an immune response to sporozoites apparently occurs naturally only after many exposures to infected mosquitoes (Nardin et al 1979), this vaccine represents an example of the approach of artificially immunising the host with Ags that the immune system may not readily respond to under natural conditions.

With use of MAbs, a stage-specific 44 kD protein has been identified on the surface of sporozoites (Yoshida et al 1980). When transfused into mice, these MAbs or their Fab fragments gave protection from challenge with P. berghei sporozoites (Potocjak et al 1980), and it appears that the Ag inducing protective immunity and the CSP reaction were identical. Similar findings were obtained with P. knowlesi (Cochrane et al 1982), P. vivax and P. falciparum (Nardin et al 1982). It appears that the circumsporozoite (CS) protein which completely covers the sporozoite surface is involved in parasite invasion of hepatocytes, because, in the presence of Fab fragments, sporozoite attachment is prevented (Hollingdale et al 1982, 1984). Immature sporozoites found within oocysts in the midgut of anopheline mosquitoes bear little or no CS protein on their outer membrane (Aikawa et al 1981) and are not infective. There is a close temporal relationship between the development of sporozoite infectivity and the appearance of CS protein on the membrane of the parasite. More importantly, the expression of CS protein upon maturation of sporozoites also correlates well with their capacity to induce protection in otherwise susceptible hosts (Vanderberg 1974). The CS proteins of rodent, simian and human malaria parasites have similar structural, biosynthetic and immunological properties (Nussenzweig & Nussenzweig 1984).

The CS protein is the most advanced of all candidate vaccine proteins and its structure has been elucidated by cloning and sequencing various CS genes. Ellis et al (1983) cloned gene fragments encoding the P. knowlesi CS protein and Ozaki et al (1983) described the sequence of the entire gene. Only one copy of the CS gene is present per haploid genome, and its DNA sequence is uninterrupted. The central region of all CS proteins is formed by tandemly repeated sequences of amino acids, which vary in number and in composition according to the plasmodial species. The first gene which has been cloned, that of the CS protein of the H strain of P. knowlesi, contains 12 repeats of 12 amino acids each (Ozaki et al 1983). In P. falciparum, for which the entire CS gene has also been sequenced, very little variation has been noted in the tandem repeats which constitute the immunodominant epitopes of this molecule (Dame et al 1984, Enea et al 1984), each consisting of only four amino acids. This sequence, asparagine-alanine-

asparagine-proline (NANP) is reiterated between 36 and 41 times (Dame et al 1984). The immunodominant epitope of the CS protein of P. falciparum is represented by three consecutive repeats of NANP, denoted $(NANP)_3$. This was determined by comparing the reactivity of synthetic peptides, containing different numbers of repeats, with MAbs (Zavala et al 1985 a). The $(NANP)_3$ epitope occurs in sporozoites of many isolates of P. falciparum from widely different areas of the world, all of which react with the same set of MAbs (Zavala et al 1985 b).

The CS proteins of P. vivax have recently been cloned and sequenced. The central repeat region consists of nine amino acid sequences, repeated in tandem 19 times (Arnot et al 1985). The genes of P. vivax and P. knowlesi CS proteins are similar, suggesting they are closely related. However, within the P. cynomolgi species, the CS protein immunodominant repeats are all different, and seem to have diverged more rapidly than the remainder of the gene (Galinski et al 1987). Why this should have occurred in P. cynomolgi but not in P. falciparum is not clear.

Immunisation of experimental animals, rabbits and mice, with the synthetic peptide $(NANP)_3$ of P. falciparum coupled to a carrier protein, induced Abs which produce a CSP reaction, and furthermore, neutralise sporozoite infectivity to human hepatoma cells in vitro (Zavala et al 1985 b, Ballou et al 1985). Also, an Escherichia coli fusion protein containing a series of NANP repeats has been reported to be very immunogenic for mice and induced neutralising Abs (Young et al 1985). Similar results have been obtained for P. knowlesi and P. vivax CS repeats inserted into vaccinia virus and yeast expression vectors, respectively (Smith et al 1984, Barr et al 1987). Taken together, these findings indicated that the repeats of the CS molecule provide a promising candidate for vaccine development, which thus progessed to clinical testing.

Two sporozoite vaccines have been given to human volunteers, both aiming only at raising Abs to the repeat domain of the P. falciparum CS protein (Good et al 1987 b, Etlinger et al 1988). One of the tested vaccines was a synthetic construct consisting of cysteine-$(NANP)_3$ conjugated to tetanus toxoid (Herrington et al 1987), which was administered together with an aluminium hydroxide adjuvant. Seroconversions against $(NANP)_3$ took place in 71% of recipients of 100 µg, the highest dose of vaccine. Most positive sera reacted with sporozoites by immunofluorescence and showed significant rises in serum Abs to NANP repeats. Three vaccinees with the highest Ab titres and four unimmunised controls were challenged with P. falciparum sporozoites by the bite of infected mosquitoes. Blood stage parasites were detected in all controls after 7-10 d

(mean 8.5 d). In contrast, two vaccinated individuals did not show pRBC until 11 d, and the third was not infected at all. The significantly increased prepatent period in the two vaccinees is most likely due to the neutralisation of a large proportion of the invading sporozoites. Studies in sporozoite-induced malaria in both man and animal models (e.g. Schmidt et al 1982) have demonstrated an inverse relationship between the size of the sporozoite inoculum and the duration of the prepatent period. While the delay in prepatency was reported as statistically significant, its medical significance must be questioned since clinical manifestations of malaria were unmodified in these volunteers. One disappointing finding from this trial was that the serum titres of volunteers injected with $(NANP)_3$-tetanus toxoid were much lower than those of mice or rabbits immunised with the same Ag (Zavala et al 1985 a, Etlinger et al 1988). This may, in part, be due to the fact that a lower vaccine dose was used in the human volunteers; however, the dose of this vaccine cannot be increased because of the toxic effects of the tetanus toxoid carrier.

The other vaccine used in humans (Ballou et al 1987) was a recombinant protein, $R32tet_{32}$, produced in E. coli and consisted of a polypeptide containing 32 repeats and 32 irrelevant amino acids corresponding to part of a tetracycline resistance gene read out of frame (Young et al 1985). Of 15 individuals inoculated, 12 developed Abs to NANP repeats, but only in a single person was a high serum titre achieved. Six immunised volunteers and two controls were challenged. Parasitaemia did not develop in the volunteer with the highest titre of Abs and was delayed in two others. The rather poor Ab response could have been due to lack of T cell recognition or inadequate processing of the subunit vaccine, as suggested by studies showing that the immune response to NANP polymers in mice is severely restricted (Good et al 1986). Others have presented evidence suggesting that the 32 tetracycline-derived amino acids could have had a suppressive effect on the immune response to the NANP repeats (Russo et al 1988).

A comparison of anti-sporozoite Ab titres of volunteers following immunisation with either synthetic vaccine with those observed in adults from malaria endemic areas showed that even in the single volunteer who had the highest Ab response to $R32tet_{32}$, the level of Abs was below that observed in sera from Indonesia and Kenya (Ballou et al 1987). However, it is not clear from field studies that naturally acquired anti-sporozoite Abs are protective. Data from a malarious region of Kenya showed no difference between adults who did and did not develop parasitaemia in a longitudinal study (Hoffman et al 1987). A similar observation that levels of specific Abs were not

protective in preventing clinical malaria was reported from an area of more seasonal exposure in Thailand (Webster et al 1987). This study also showed that Ab synthesis ceased in the presence of pRBC, suggesting possible immunosuppression induced by blood stage infection.

There was no evidence for parasite-specific T cell sensitisation in any of the peptide-vaccinated individuals in either clinical trial, indicating that protection can be achieved with Abs alone, if the titres are sufficiently high. Similarly, complete protection against P. berghei sporozoite challenge has been achieved in a large proportion of mice vaccinated with a repeat peptide conjugated to tetanus toxoid. The serum titres of Ab to the P. berghei CS protein were high, but T cells did not respond in vitro to the synthetic repeat peptide (Zavala et al 1987). However, in another experiment, Egan et al (1987) showed that despite high titre Ab, CS peptide-immunised mice were only partially protected after sporozoite challenge. Furthermore, T cells, not Ab or B cells, from mice immunised with irradiated sporozoites transferred protection to naive mice. Recently, it has been observed that protective immunity in mice inoculated with irradiated sporozoites of P. yoelii is dependent on both $CD8^+$ T cells and IFN-γ (Schofield et al 1987 a, Weiss et al 1988). IFN-γ is a T cell product which has a potent inhibitory effect on the exo-erythrocytic stages of the malaria parasite, and if produced at the time of challenge, would prevent the development of sporozoites which escape the neutralising activity of Ab (Ferreira et al 1986, 1987, Schofield et al 1987 a). A disadvantage of both peptide-conjugate vaccines so far used in clinical trials is that they may not prime parasite-specific T cells, and a boosting of the Ab response and lymphokine production may not occur during subsequent exposure to sporozoites under natural infection (Miller et al 1986).

It may be possible to synthesise T cell epitopes from the CS protein together with the NANP repeats to form a T-B parasite-specific vaccine. In fact, T cell epitopes from the P. falciparum CS protein recognised by certain strains of inbred mice have been characterised. NANP repeats are recognised by T cells from mice bearing $I\text{-}A^b$ in MHC (Del Giudice et al 1986, Good et al 1986), while another sequence outside the repeat domain is recognised by mice bearing $I\text{-}A^k$ (Good et al 1987 b). Moreover, this latter sequence has been shown to be the immunodominant T cell domain (Good et al 1988).

These observations not only highlight the fact that T cell epitopes are very probably genetically restricted, and may not be recognised by all humans, but also that the CS protein may not provide the best basis of an anti-malaria vaccine as the immunogenic

regions are not those tandemly repeated. Additional epitopes recognised by T cells may be required and could be provided by other sporozoite or liver stage Ags. This suggests that although modifications in the carrier system, adjuvants, or vaccination schedules may provide some improvement, parasite-specific T cell activation is likely to remain the major challenge for protective efficacy of potential sporozoite vaccines.

(b) Asexual blood stage vaccine

As the asexual erythrocytic parasite is the cause of malaria disease, the primary objective of a blood stage vaccine is the reduction of morbidity and mortality through suppression of parasite proliferation. Vaccination that induces only partial immunity will still be useful, since it will suppress parasitaemia, the level of which is approximately proportional to disease severity. Moreover, during the course of infection, boosting of immunity should extend the life of a vaccine.

Asexual blood stage parasites of P. falciparum have been the main focus of research aimed at developing a malaria vaccine, which reflects the fact that pRBC are the main target of specific acquired immunity and the ready availability of these stages from in vitro cultures. One vaccine strategy is based on the absolute necessity of merozoite invasion of RBC for parasite growth and survival, and the fact that invasion can be blocked by specific anti-malaria Abs. Merozoite attachment and entry involves multiple specific receptor-ligand interactions of parasite membrane and RBC membrane surface components (Hadley et al 1986). Therefore, anti-receptor Abs elicited by vaccination should decrease the number of merozoites that successfully invade RBC and reduce both the blood parasitaemia and clinical manifestations. The intraerythrocytic asexual parasite may also be accessible to immune-mediated destruction. Infected RBC may be killed by Ab-dependent or Ab-independent mechanisms. During the normal course of infection, P. falciparum pRBC bind to venular endothelium via knob protusions on the infected cell surface and are thus protected from destruction in the spleen. By blocking sequestration, Ab forces the parasite from this privileged site to pass through the spleen (David et al 1983). Whether a common domain for cytoadherence exists on all pRBC remains to be determined. There are numerous other Ags of the asexual parasite which elicit Ab responses, but only a small number of these are presumed to be targets of protective immunity.

Vaccination with isolated merozoites has been successful in simian malaria. Targett & Fulton (1965) demonstrated the immunisation of rhesus monkeys against P. knowlesi,

which was later shown to be species-specific, stage-specific and long lasting (Mitchell et al 1975). Owl monkeys that were immunised with P. falciparum merozoites were similarly protected (Mitchell et al 1977). Unfortunately, optimal protection required the use of adjuvants that are unacceptable for human use, although the success of less toxic new adjuvants in this experimental model is encouraging (Siddiqui et al 1978).

Immunisation with purified Ag or passive transfer of Ab has provided evidence in several species of Plasmodium that a large MW Ag on the surface of the intracellular merozoite after schizont segmentation can induce protective responses. This Ag, first described in P. yoelii (Holder & Freeman 1981) appears to undergo specific processing from an early stage of schizont development. This 'precursor of the major merozoite surface Ags', PMMSA, is now known as the 'merozoite surface protein 1', or MSP1. It is a molecule between 185-250 kDa, depending on species and strain, but in P. falciparum, processed fragments of 83, 41 and 19 kDa are recognised by human immune serum as major surface Ags on the free merozoite (Holder & Freeman 1984). Immunisation of Saimiri monkeys with P. falciparum MSP1, purified from cultured pRBC, induced partial protection (Hall et al 1984, Perrin et al 1984). However, in addition to being polymorphic in size, this molecule has been shown to exhibit considerable antigenic diversity (McBride et al 1982, 1985), and naturally acquired immunity against it may be markedly strain-specific. The extent to which antigenic diversity of MSP1 frustrates attempts to use it as a component of a vaccine remains to be seen.

Extensive evidence implicates glycophorin A and perhaps other glycophorins in the process of merozoite invasion (Pasvol et al 1982). Several merozoite surface protein Ags of various sizes with affinity for glycophorins have been identified (Jungery et al 1983, Perkins 1984), but unequivocal evidence that any of these molecules is a glycophorin receptor is lacking. Perkins (1984) demonstrated that the major specificities of polyclonal antiserum raised against heat-stable Ags in culture are against two glycophorin-binding proteins of 155 and 130 kDa. This antiserum inhibits merozoite invasion in vitro and also reacts with the merozoite surface, although it has proven difficult to establish that either of these molecules plays a critical role in the invasion event in vivo (Van Schravendijk et al 1987), and may be dispersed products of the maturing parasite (Bianco et al 1987). The cloning of these Ags enabled their part in merozoite entry to be resolved (Ravetch et al 1985), and ultimately showed their unsuitability for inclusion in a candidate vaccine. It is now clear that receptor

functional phenotype varies amongst lines of P. falciparum (Mitchell et al 1986, Perkins & Holt 1988) and there is good evidence that at least two glycophorin receptor systems function in merozoites. The expectation that a receptor molecule would prove an excellent vaccine candidate rested, in part, on the assumption that there was a single and invariant merozoite receptor for RBC, and this is now known not to be the case.

Also of interest are parasite molecules that have a function in modifying the membrane of infected RBC. As the asexual blood stages of P. falciparum mature, the pRBC develops knobs on its surface (Trager et al 1966), which provide sites of interaction with the vascular endothelium and thereby enable mature pRBC to sequester out of the peripheral circulation (Luse & Miller 1971). The parasite Ag that mediates cytoadherence has not been identified unequivocally, but exhibits considerable antigenic diversity because naturally occurring Abs block cytoadherence in a strain-specific manner (Udeinya et al 1983 b). The knob-associated histidine-rich protein KAHRP or HRP1, one of several histidine-rich proteins (HRPs) identified in P. falciparum, has been shown to be a component of the knob (Leech et al 1984). It is not clear whether this is the same Ag as the strain-specific Ag that has been identified on the infected cell surface by immunofluorescence (Hommel et al 1983). Either way, Culvenor et al (1987) have found that KAHRP is located on the cytoplasmic surface of the pRBC membrane, and although anti-KAHRP Abs are generated in man by natural infection, this molecule is not now thought to be a target of protective immune responses.

The ring-infected erythrocyte surface Ag (RESA, also called pf155) has been located to the micronemes and dense bodies of merozoites and, after the merozoite invades to form the ring-stage parasite, RESA is transferred to, and associated with, the pRBC membrane and cytoskeleton (Perlmann et al 1984, Brown et al 1985). Presumably RESA is released via the merozoite apical pore at the time of entry.

This molecule ranks highly as a potential vaccine component, and it is apparent that most, if not all species of plasmodia, possess a RESA analogue, but its function remains unclear. Abs to RESA have been shown to be potent inhibitors of merozoite invasion in vitro (Wåhlin et al 1984), and fragments of the Ag produced in E. coli as fusion proteins partially protect Aotus monkeys against uncontrolled infection with P. falciparum (Collins et al 1986). Furthermore, a polypeptide homologue of RESA has been isolated by affinity chromatography from P. chabaudi and used to immunise mice protectively against subsequent challenge (Wanidworanum et al 1987). In contrast to MSP1, RESA has not been observed to exhibit antigenic heterogeneity among different isolates of P.

falciparum, adding further to its potential for vaccination studies. Sequencing of the RESA gene revealed that the polypeptide contains three sets of related sequence repeats, two of which are at the C-terminal end of the molecule and the other in the middle of the polypeptide (Coppel et al 1984, Cowman et al 1984). These repeats encode naturally immunogenic antigenic epitopes that are immunodominant. Ab responses to the major tetrameric repeat are most prominent in infected individuals (Anders et al 1986); however, experiments in Aotus monkeys indicate that Abs of this specificity are not important to protection in contrast to Abs with specificity for the two minor repeats (Collins et al 1986). The epitopes encoded by the RESA repetitive sequences are involved in a network of intra- and inter-molecular cross-reactions (Anders et al 1987), which may impair the development of high affinity Ab responses and thereby favour pRBC survival in the infected host (Anders 1986).

Studies on P. falciparum indicate that merozoites attached to RBC must reorientate so that the apical end is apposed to the RBC membrane before invasion can proceed. The membrane-bound rhoptry organelles are located in this region of the merozoite and discharge of their contents is implicated in invasion. Whatever the role of rhoptry proteins, it appears that they can induce protective immunity that is Ab-mediated. Passive transfer of Abs raised to a 235 kD rhoptry protein of P. yoelii protected against infection as did immunisation with the purified Ag (Holder & Freeman 1981). The analogous Ag in P. falciparum has not been identified, but a number of smaller MW Ags identified in the rhoptries of P. falciparum merozoites are being studied as potential vaccine Ags. At least five different Ags have been described that appear to exist in two soluble complexes. A low MW complex includes a polypeptide of 41 kD MW and an 83 kD Ag (Campbell et al 1984, Howard et al 1984, Schofield et al 1986). The lower MW component, after purification on a MAb adsorbent column, was tested in Saimiri monkey vaccination trials (Perrin et al 1985) and gave effective protection against P. falciparum challenge infections. The larger 83 kD component has more recently been used as part of a hybrid polypeptide polymer for human vaccination trials (Patarroyo et al 1988).

Three other Ags identified in the rhoptries of P. falciparum appear to be associated on a high MW complex (Campbell et al 1984, Holder et al 1985). One of these, of 155 kD MW, has been isolated from cultured parasites (Holder et al 1985), and another (105 kD) was cloned in E. coli (Coppel et al 1987). Apart from their location, the only evidence supporting their candidacy is the finding that a MAb precipitating the complex

weakly inhibited merozoite invasion in vitro (Cooper et al 1988), but the component with which it reacts has not been established. Two other Ags have been located to rhoptries that are not associated with these high and low MW complexes. One of these Ags is synthesised as a 240 kD polypeptide that is processsed into a 225 kD molecule (Roger et al 1988). The other, a 55 kD Ag, has been shown to have the solubility properties of an integral membrane protein (Smythe et al 1988) and has already been put forward as part of a subunit vaccine for further development.

The identification of several different asexual blood stage Ags that have potential as vaccine molecules is reason for optimism concerning the future development of a malaria vaccine. Immunisation with a vaccine containing several of these Ags may not only induce a more protective immune response, but also should make it less likely that antigenic variation will frustrate vaccination against P. falciparum. Unlike sporozoite-induced infections, monkeys of the Aotus and Saimiri species can be infected reproducibly with P. falciparum by blood challenge. Thus, pRBC Ags that are candidate vaccine molecules are being tested for efficacy in preclinical trials before the use of human volunteers.

A number of trials have been performed in which some protection of immunised monkeys against challenge with P. falciparum was achieved with well-defined Ags. These were either isolated from cultured parasites (Hall et al 1984, Perrin et al 1984, 1985, Siddiqui et al 1986, 1987), or were fragments of Ags expressed from genes cloned in E. coli (Collins et al 1986), or synthetic peptides corresponding to known epitopes or N-terminal sequences of blood stage Ags (Cheung et al 1986, Patarroyo et al 1987). In one of these trials, fusion proteins containing fragments of RESA were used to immunise Aotus monkeys (Collins et al 1986). Parasitaemias showed only a partial protection, but this correlated with Ab responses to repeat sequences in two different regions of the RESA polypeptide. A particularly impressive protective effect was achieved in another trial in which synthetic peptides conjugated to tetanus toxoid were used as the immunogen (Patarroyo et al 1987). Peptides corresponding to the N-terminal sequences of MSP1 and two other uncharacterised Ags were more effective when used together than alone. These trials in monkeys have provided important information about Ags that may subsequently be tested in clinical trials. Monkey models are, however, less than ideal in that only a relatively limited number of strains of P. falciparum will give reproducible infections in available primate species, and the number of monkeys that can be used for vaccine trials is limited (Collins & Pappaionou 1985). Evidence from

these non-human primate experiments suggests that the use of a single candidate Ag for use as a blood stage vaccine may be restricted. One reason for this is that as $CD4^+$ T cells play a key role as effectors of anti-pRBC malaria immunity (Brake et al 1988), sufficient T cell epitopes to give a good response in a majority of the population would have to be included in a vaccine. This is likely to require more than one component. Nevertheless, both MSP1- and RESA-derived Ags remain clear candidates for inclusion in an eventual vaccine. This may in future be developed by combining several asexual erythrocytic stage Ags with the CS protein or Ags of other malaria life cycle stages.

No human studies have yet been reported using P. falciparum MSP1 or RESA as the sole immunogen, although sequences based on fragments of these molecules were major components of the hybrid high MW protein polymers used by Patarroyo et al (1988). The first of these, SPf(66)30, was composed of three synthetic peptides corresponding to fragments of MW 83, 55, and 35 kD P. falciparum merozoite-specific proteins. The second, SPf(105)20, contained the synthetic 83 kD equivalent, plus a CS NANP repeat, two other CS sequences and the 5' region repeat of RESA. Ag was absorbed onto aluminium hydroxide and multiple doses given, without local or systemic side effects. In blood-challenged volunteers, there was evidence that individuals suffered modified infection. Since parasitaemia was not allowed to rise untreated, the necessary cure of some immunised volunteers complicated interpretation of the findings. Nevertheless, three recipients of SPf(66)30 controlled their infections, parasitaemia resolving spontaneously without reaching the 0.5% criterion for treatment. These subjects showed a significant rise in Ab titre to SPf(66)30 by ELISA, and an increase in schizont-specific Ab by IFAT. However, two of these persons were positive by ELISA or IFAT to at least two of the three malaria Ags present in the hybrid prior to immunisation, according to the criteria specified by the investigators. This suggests the possibility of previous exposure to malaria, in which case the vaccine may have acted by boosting an already acquired partial immunity in three volunteers. There is little evidence that the other hybrid vaccine administered, SPf(105)20, provided protection when compared to controls (Patarroyo et al 1988). In subjects receiving this immunogen, no Abs were detected against the CS repeat molecule or against the RESA determinant, and clinical symptoms were present in all volunteers. Moreover, lymphocyte proliferation assays did not suggest a correlation with protection. Overall, it is of interest that the three volunteers inoculated with SPf(66)30 who cleared pRBC, exhibited a response intermediate between unprotected individuals and controls.

How the synthetic proteins will work in larger studies remains to be seen. If this pragmatic approach is successful, the processes whereby protection is achieved can be established retrospectively. Further trials are currently being conducted and the results of these are awaited with great interest by the malaria research community.

(c) Sexual stage vaccine

Vaccination which aims to block transmission of a malaria infection to the mosquito vector introduces a novel concept quite separate from the more readily appreciated aims of immunisation with sporozoite Ags and asexual blood stage Ags, of preventing infection or alleviating clinical malaria, respectively. Transmission-blocking vaccines may have no prophylactic use in that they would have no effect on the clinical course of disease in an individual, but would aim to eradicate malaria from endemic areas by reducing the number of infected mosquitoes to the point where transmission of infection could no longer be sustained. The site of attack of these vaccines is the interruption of the malaria life cycle in the mosquito midgut. Abs taken with the blood meal into the mosquito midgut block fertilisation in the absence of complement, lyse gametes and zygotes in the presence of complement, and block zygote development (reviewed by Carter et al 1988). Since this approach, if used alone, offers no protection against disease to the individual vaccinated, it has been termed an altruistic vaccine. This may be used in malaria eradication programmes or in combination with vaccines to other stages to slow the appearance of parasite mutants. Of all vaccine candidates, those inducing interruption of transmission are the easiest to test. Abs from animals or humans are mixed with cultured gametocytes, and fed to mosquitoes, and their efficacy assayed by evaluating the reduction of oocyst burden in mosquitoes after a blood meal.

Transmission blocking was demonstrated first by vaccination with attenuated whole gametes of avian (Carter & Chen 1976), rodent (Mendis & Targett 1979) and simian (Gwadz & Green 1978) malarias. The immunised animals were challenged and then exposed to the bites of vector mosquitoes. A highly effective immunity was achieved in avian and rodent studies without the need for adjuvants, but Freund's complete adjuvant was necessary in vaccination of rhesus monkeys with gametes of P. knowlesi (Gwadz & Green 1978). In a latter study, immunity to P. yoelii nigeriensis was still fully effective when an interval of a year intervened between vaccination and the challenge infection from which transmission was attempted (Harte et al 1985 a). These and other studies also showed that parasite challenge served to boost vaccination-induced

immunity (Mendis & Targett 1979), and this enhancement had the characteristics of an anamnestic response. Sera from animals vaccinated with intact microgametes prevented their release during exflagellation and/or agglutinated liberated gametes, indicating that one important effector mechanism is an Ab-mediated blockade of fertilisation in the mosquito gut (Gwadz 1976, Carter et al 1979 b).

MAbs raised to both micro- and macro-gametes of various plasmodia, including P. falciparum, have confirmed the effectiveness of Abs in blocking transmission by conferring passive immunity when inoculated into infected host animals (Harte et al 1985 b), or introduced into cultures used for membrane feeding of mosquitoes (Kaushal et al 1983, Rener et al 1983, Vermeulen et al 1985). Single MAb treatment will block transmission totally if given in adequate dosage (Harte et al 1985 b), generally, though not always (Carter et al 1984), without a requirement for complement. Such Abs suppressed both ookinete production and oocyst development. Other MAbs raised against zygotes and ookinetes (Sinden et al 1985, Vermeulen et al 1985), which also interrupted transmission, were found to have no effect on the fertilisation process, indicating that the target Ags were those involved in the transformation of zygotes and ookinetes into oocysts rather that in pre-fertilisation development.

Among the surface proteins of gametes, zygotes and ookinetes, several have been identified as the targets of transmission-blocking MAb. On gametes and newly fertilised zygotes these are the 230 kD proteins in P. gallinaceum (Kaushal et al 1983) and in P. falciparum (Carter et al 1988), and the 48 and 45 kD molecules in P. falciparum (Vermeulen et al 1985) and their P. vivax and P. y. nigeriensis analogues (Harte et al 1985 c). Soon after gamete fusion, these Ags are lost and replaced on the zygote by lower MW glycoproteins. For P. falciparum, this is a 25 kD protein (Carter & Kaushal 1984, Vermeulen et al 1985).

A notable feature of the target epitopes of transmission-blocking MAbs against the 48, 45, and 25 kD Ags of P. falciparum is that their reactivity with MAb is abrogated by reduction (Vermeulen et al 1985). This implies that these determinants have a tertiary protein structure maintained by disulphide bridges between adjacent cysteine residues. Until very recently, the conformation structure of these target epitopes had frustrated efforts to clone genes coding for these proteins from recombinant DNA expression libraries in E. coli due to the inability of such libraries to reform disulphide bonds as found in the original malaria Ags. However, this problem has now been overcome, making it possible to clone the gene encoding the 25 kD zygote Ag of P. falciparum, now

called Pfs 25 (Kaslow et al 1988). This molecule is conserved in several isolates of P. falciparum, and the immune response to it does not appear to be genetically restricted, unlike the response to other surface epitopes of P. falciparum sexual stages (Carter et al 1988). The gene encoding a 24 kD Ag shared by P. vivax gametes and asexual stages, GAM 1, has also been cloned. This polypeptide is conserved in several P. vivax isolates, and, because it is also present in asexual blood stages in humans, a boosting of the vaccine-induced immune response by subsequent natural infection can be expected (Carter R., personal communication).

An important recent advance has been the establishment of the extent of natural transmission-blocking immunity in individuals living in malaria endemic areas. Mendis et al (1987) observed that immunity to the sexual stages of P. vivax in Sri Lanka is Ab-mediated and is directed agsinst surface Ags of gametes and zygotes, preventing their development in the mosquito vector. Patients developed the transmission-blocking Abs during acute primary infections, and titres increased further in subsequent attacks. The development of Abs to gamete surface epitopes, a stage found only in mosquitoes, is explained by the presence of these Ags, or precursors of them, in gametocytes, the sexual stages of the parasite in humans. This phenomenon has also been observed in P. falciparum malaria (Vermeulen et al 1985). A second attack of P. vivax within four months of initial recovery was correlated with even greater transmission-blocking effects in experiments evaluating infectivity of patients to mosquitoes (Ranawaka et al 1988). However, the boosting effect declined with longer intervals between successive infections and there was a suggestion of infectivity enhancement in one of the subjects studied. While it is assumed that young non-immune individuals are the greatest reservoir of gametocytes, it has been argued that the large adult population with low level infections may contribute significantly to transmission (Carter & Gwadz 1980). However, if the adult community displays effective natural immune responses to sexual stages of human malaria parasites, as now appears to be the case, this would possibly alter the target population to be vaccinated in future attempts to reduce or eliminate disease transmission in malarious regions.

To date, no clinical trials using transmission-blocking vaccines have been conducted. However, with the recent success of cloning genes encoding the epitopes of sexual stage Ags, studies can begin on the immunogenicity of synthetic constructs representing defined sexual-specific Ags.

With the global resurgence of malaria, the rising costs of control, and the failure of

available techniques in areas such as Africa and South East Asia, the need for a malaria vaccine is apparent. The choice of vaccine; sporozoite, asexual or transmission-blocking, may vary depending on the requirement. Vaccines directed against asexual blood forms would probably reduce mortality in endemic areas. From sporozoite and transmission-blocking vaccines, a genuine reduction of malaria prevalence may be expected. The prevention of epidemics or the short period protection of non-immune persons entering an endemic area, e.g. tourists, would very likely require the deployment of multiple component vaccines, together with the ongoing use of other control measures. Due to the many unknowns about the immunogenicity and variability of Ags of various stages and the duration of protection engendered, it cannot be predicted what the ultimate form of the vaccine will be or the combination of Ags that will be most effective in each situation.

1.15 History of Plasmodium chabaudi chabaudi

Plasmodium chabaudi chabaudi was isolated from the blood of thicket rats, Thamnomys rutilans, caught in the Central African Republic by Landau in 1965. The parasite infects mainly mature RBC (Landau 1965), although it can invade reticulocytes later in infection (Jarra & Brown 1989; Phillips, R.S., personal communication). Multiple infection of RBC with P. c. chabaudi does occur (Carter & Walliker 1975).

P. c. chabaudi AS strain was selected for this project because it has important similarities to P. falciparum in the human host. The parasite forms a chronic blood stream infection with recrudescences, but without liver stage relapses. The infection is synchronous, although the asexual erythrocytic cycle is completed in only 24 hr. Schizont peripheral withdrawal occurs to a small but significant extent as schizont maturation takes place (McDonald 1977, McDonald & Phillips 1978). Cloned, well characterised lines of the AS strain have been established by Carter & Walliker (1975) in laboratory mice from wild-caught isolates without any need for adaptation. These clones have been cyclically passaged in Anopheles stephensi and are free from contamination with other rodent malaria species or pathogens such as Eperythorozoon coccoides and Haemobartonella muris (Cox 1978). Isoenzyme patterns have been established for these AS strain clones, which have, unlike many laboratory strains of rodent malaria, remained close to the original isolate (Beale et al 1978; Walliker, D., personal communication).

Most inbred strains of mouse, including the NIH strain used in this study, show a

genetically determined resistance to P. c. chabaudi AS (Stevenson et al 1982). The asexual erythrocytic infection shows an acute primary parasitaemia lasting 10-14 d (after inoculation of 10^5 pRBC), the parasitaemia increasing logarithmically to reach a peak of between 30-50 %. A period of subpatency is followed by one or two usually mild recrudescences. Infection in normal, immunocompetent mice can last up to two months and always features at least two patent waves of parasitaemia. Figure 1.2 shows a graph of a typical course of infection of P. c. chabaudi AS strain in naive NIH mice. Some groups of mice infected with the AS strain parasite show a second peak of parasitaemia shortly after the first (Fig. 1.3). In these animals, the parasitaemia becomes subpatent shortly later, but a recrudescence still occurs.

No correlation between the pattern of infection and the age or sex of the host, or with the size of the infective inoculum has been observed in this model system (McLean 1985; Phillips, R.S., personal communication).

The cloned AS strain of P. c. chabaudi was selected for use since it is considered the best murine model for P. falciparum (Mons & Sinden 1990). NIH mice were chosen because of the previous experience Professor Stephen Phillips' laboratory had with this host-parasite combination.

1.16 Experimental rationale

It is now clear that the major protective immune mechanisms to the erythrocytic stages of malaria parasites require the presence of T lymphocytes. This is substantiated by many rodent malaria models of malaria in which protection can be conferred by the adoptive transfer of specific T cells from immune mice (McDonald & Phillips 1978, Brinkmann et al 1985, Cavacini et al 1986). In addition, in T cell-deficient animals, parasitaemia cannot be controlled (Brown et al 1968, Weinbaum et al 1976, Jayawardena et al 1977, McDonald & Phillips 1978).

Whilst the role of the T cell in acquired immunity to blood stage plasmodia is generally considered to be that of a helper cell for the production of specific Abs, it is becoming increasingly apparent that Ab-independent mechanisms of immunity also make a significant contribution to host defences against the erythrocytic stages of the malaria parasite (reviewed by Weidanz & Long 1988). Strong support for this comes from the observation by Grun & Weidanz (1981) that mice rendered B cell-deficient by treatment with anti-μ serum were able to resolve primary P. c. adami infections with the same kinetics as those of normal control animals. This experiment showed that

whilst immunosuppressed mice could never totally sterilise their infections, humoral immunity was not required to control acute infection with this subspecies of Plasmodium. It was subsequently shown in both P. c. adami and P. c. chabaudi systems that Ly-4$^+$ T cells are necessary to reduce the parasite burden (Cavacini et al 1986, Brake et al 1986, 1988, Süss et al 1988, Langhorne et al 1989 b). In the experiments described in this thesis, the P. c. chabaudi AS/NIH mouse model has been used to define further the role of cellular immunity in response to malaria infection.

The starting point for this project was based on an original idea by Professor Stephen Phillips. He and coworkers had studied previously the lymphocyte migration patterns during the acute primary infection of C57/BL mice with P. c. chabaudi AS (Kumararatne et al 1987). During this investigation, it was discovered that there was a considerable increase in total lymphocyte numbers in the peripheral blood just after the time of peak parasitaemia. It was supposed that this transient lymphocytosis afforded a population of freshly immunologically primed cells which may confer protection upon adoptive transfer to challenged naive mice. This was of interest, for all previous reconstitution studies had employed lymphoid populations taken after parasite clearance from recovered animals.

Initial findings showed that protection could be transferred with lymphocytes taken remarkably early in primary infection, before detectable levels of serum Abs arise. However, as greater levels of acquired resistance were achieved using cells taken from the spleen rather than the peripheral blood of semi-immune donors, this organ was used in most subsequent applications. Thereafter, the degree of protection, and its mechanisms, were dissected using a variety of immunologically suppressed murine recipients.

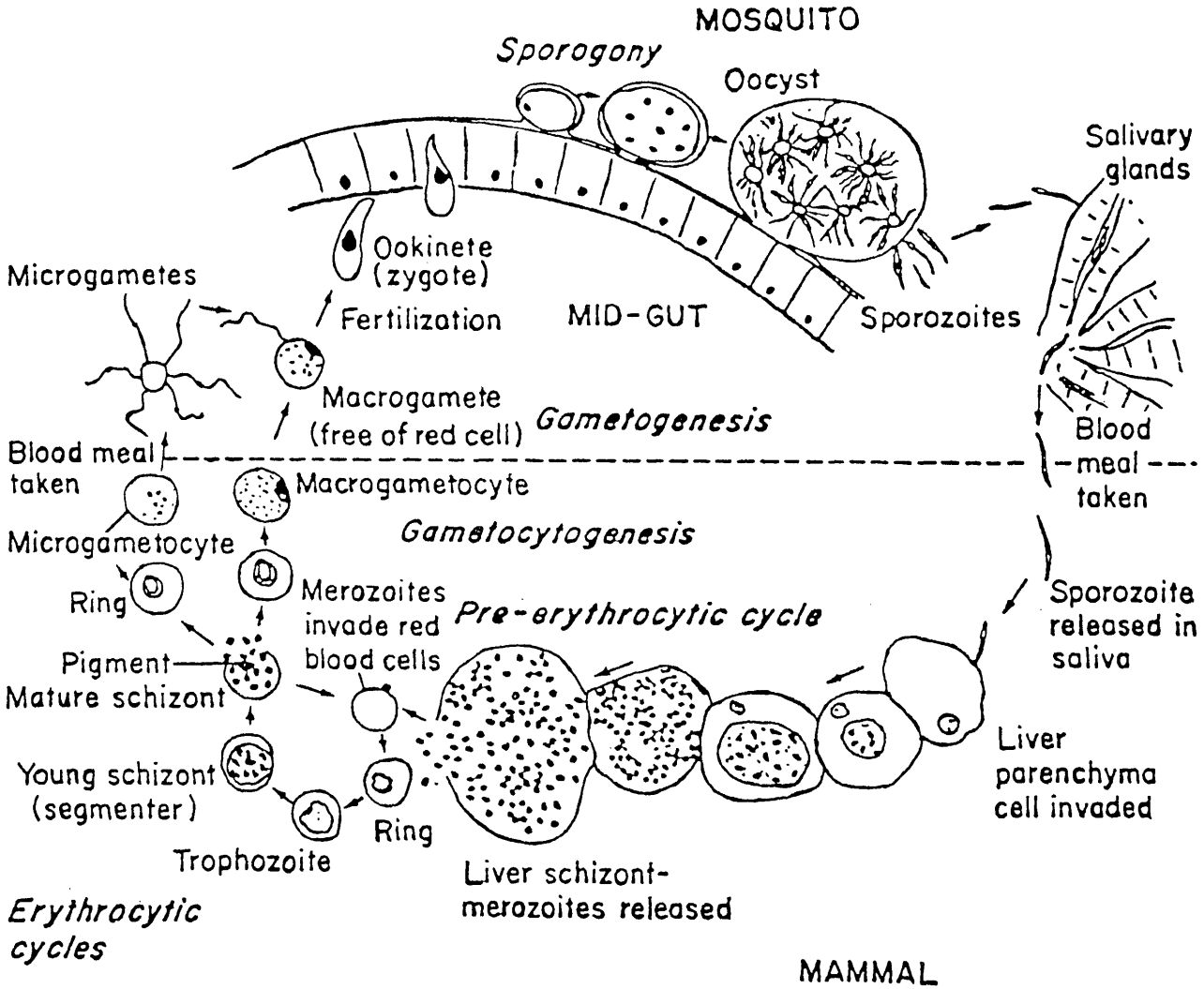
To analyse the mechanism of what appeared to be a T cell-dependent immunity, spleen cells taken during a primary P. c. chabaudi AS infection, as well as after further infections, were established in vitro as Ly-4$^+$ T cell lines, and then after cloning by limiting dilution, as cloned lines. Each of these homogeneous populations was examined with respect to surface phenotype, ability to moderate and alter a challenge infection after adoptive transfer to syngeneic naive mice, capacity to release cytokines in vitro in response to stimulation with P. c. chabaudi AS Ags, and helper activity in terms of anti-parasite Ab production.

To examine further the role of the Ly-4$^+$ T cell subset in the mediation of protective immunity to P. c. chabaudi AS infection, a different approach to that of adoptive transfer

was subsequently taken. Mice were depleted selectively of specific T cell subsets by MAb treatment prior to challenge, and the effects of this serotherapy on the course of infection elucidated.

The dynamics of different T lymphocyte subsets in the evolution of an effective immune response during a malaria infection, and the implications of the successful transfer of immunity with cloned Ly-4^+ cells, are discussed with regard to the development of asexual blood stage malaria vaccines.

Figure 1.1
The life cycle of a mammalian Plasmodium
[after Phillips, R.S. (1983) Malaria, Edward Arnold, London, p.8; with permission of the author]



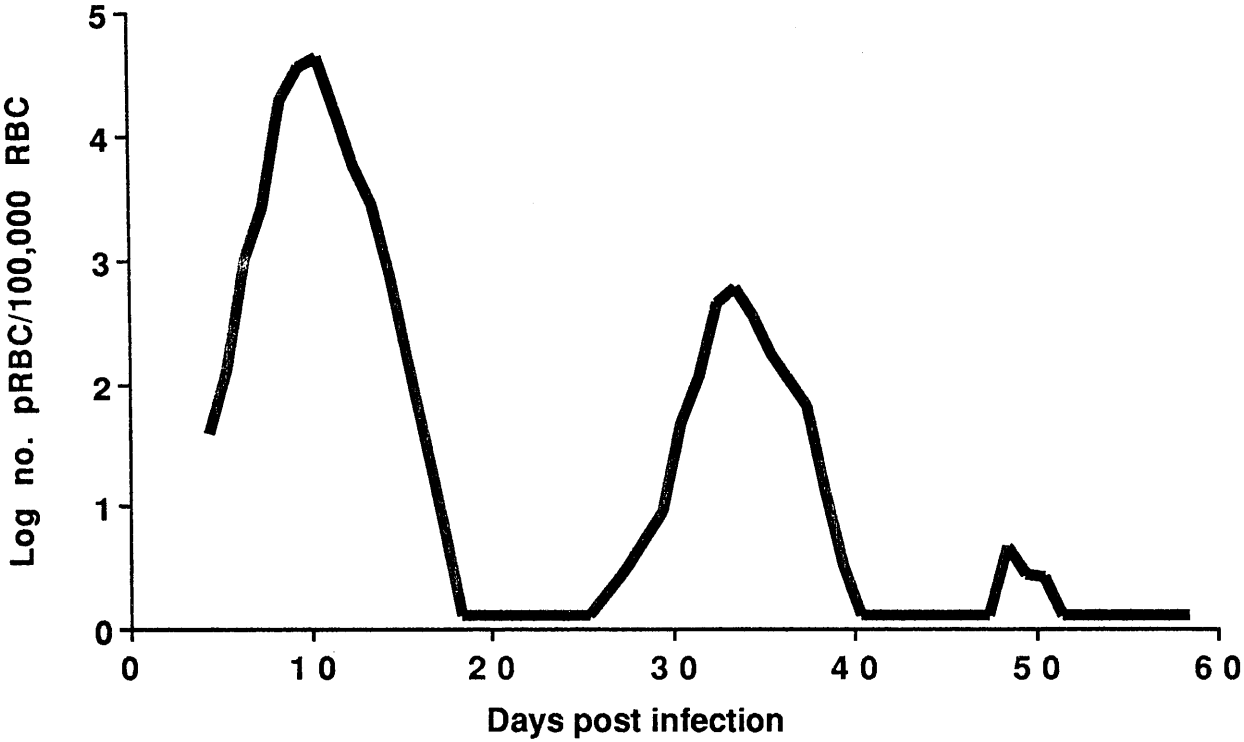


Fig. 1.2 The course of Plasmodium chabaudi chabaudi AS strain infection usually observed in naive NIH mice.

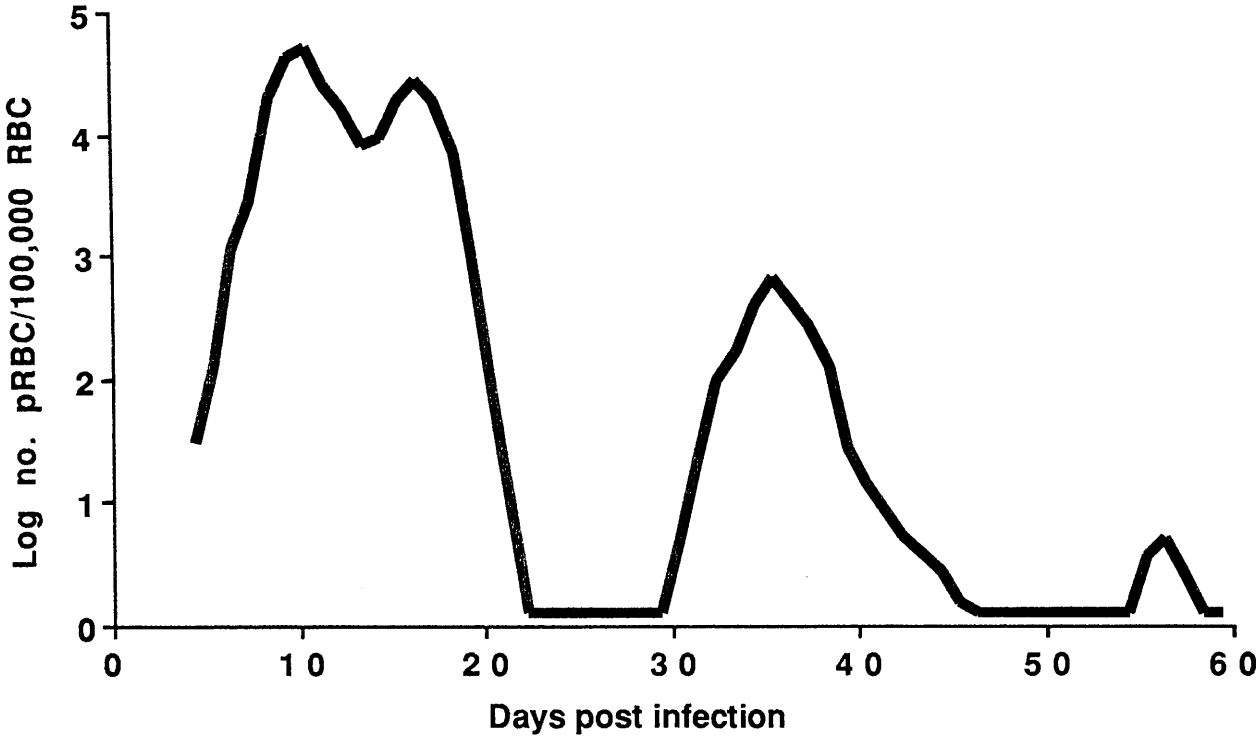


Fig. 1.3 The course of Plasmodium chabaudi chabaudi AS strain infection occasionally observed in naive NIH mice.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Mice

Inbred NIH mice were used for most experimental procedures. These were bred in the WLEP animal house breeding facility. The original mating pair on which the colony was founded came from Hacking & Churchill Ltd. in 1980, and all the studies described used animals inbred to between 18-25 generations. Congenic B10.S and B10.HTT strains were also supplied inhouse from colonies four and seven generations old, respectively. In each case, the original breeding pairs were purchased from Harlan Olac Ltd.. All mice born in the breeding facility were weaned at three weeks of age. C57BL/10 and all other B10 congenic mice were supplied by Harlan Olac Ltd. at six weeks of age. All mice were kept in the WLEP animal house at 22 °C ± 2 °C and 50-60% relative humidity, and maintained in 12 hr artificial light from 0800 to 2000 hr.

For all experimental procedures, female animals were used between 8-12 weeks old, when they weighed approximately 25 g. They were maintained on pelleted CRM breeder diet (Labsure Ltd.) and given both food and water ad libitum.

2.2 Parasites

The AS strain of Plasmodium chabaudi chabaudi was originally isolated from adult thicket rats (Thamnomys rutilans) at La Maboke, Central African Republic in March 1969 for Professor David Walliker (Institute of Cell, Animal & Population Biology, University of Edinburgh), established in laboratory mice and then cloned by limiting dilution (Walliker et al 1971). The parasites were supplied to the Department of Zoology, University of Glasgow by Professor Walliker in 1973, since when the parent AS clone has been maintained by cryopreservation and subpassage through mice (2.3 & 2.4). Refer to Fig. 2.1 for a history of P. c. chabaudi AS since isolation.

The AS recrudescent parasite clone 10 was originally derived from the recrudescent patent infection of a mouse challenged with the AS parent parasite GUP 1349 and cloned by limiting dilution once. This was performed by Dr. Sheila McLean at WLEP. The parasites were maintained as an antigenically distinct clone by frequent subpassage and cryopreservation. Fig. 2.2 details the history of P. c. chabaudi AS recrudescent clone 10.

The CB strain of P. c. chabaudi was a kind gift of Professor Walliker (on behalf of the World Health Organisation Registry of Standard Strains of Malaria Parasites). This isolate was obtained from a thicket rat caught in the C.A.R. in 1970 and transported to Edinburgh (Beale et al 1978). The parasite line was cloned by limiting dilution in

1979 and the parasites frozen (Walliker, D., personal communication). It was one of these stabilates that was acquired from Edinburgh in 1989. Refer to Fig. 2.3 for a history of P. c. chabaudi CB since isolation.

2.3 Maintenance of parasites

Suspensions of pRBC taken at a specific time from a known source were designated stabilates as proposed by Lumsden & Hardy (1965). For longterm preservation, parasite stabilates were kept in liquid N_2 (-196 °C)(BOC). When required for experimental use, infected blood was recovered from stabilate following the method of Mutetwa & James (1984 a & b). Each stabilate was defrosted by immersion of the frozen cryopreservation vial (Nunc, Gibco) in a 37 °C waterbath, then diluted with an equal volume of 15% w/v glucose in PBS (pH 7.2)(Appendix A) before immediate i.v. injection into one, or more usually two, recipient naive mice.

Parasites were maintained by blood passage in mice every 3-4 d. Mice were bled by cardiac puncture, under ether anaesthesia, into a syringe containing the anticoagulant sodium heparin (1000 i.u./ml, Evans Medical Ltd.) in PBS at 10 i.u. heparin per ml of blood. The infected blood was sub-inoculated immediately into recipient mice i.v. into the lateral tail vein.

2.4 Cryopreservation of infected blood

Parasites were stored as stabilates after the method of Phillips & Wilson (1978) using blood obtained from cardiac bleeding. Since ring-stage parasites survive the cryopreservation and subsequent thawing the best, mice were sacrificed before 1000 hr when early rings predominate in the infected blood. Blood was collected into heparinised PBS (10 i.u./ml) and diluted 1:1 with a solution of sorbitol-glycerol (Appendix C), added slowly dropwise with frequent mixing (Gray & Phillips 1981). Aliquots of 0.2-0.3 ml were dispensed into 1.2 ml cryopreservation vials and each labelled carefully with the WEP code (Wellcome Experimental Parasitology) and a number. These were immediately snap frozen by immersing in liquid N_2 cannisters (Union Carbide or Taylor-Wharton).

Careful records of establishment and use of each stabilate were kept. Parasites from one expanded stabilate were never used for more than two subpassages. Parasite stabilates were made from blood with a parasitaemia of 5-10%, and experimental mice infected with blood one passage from stabilate.

2.5 Challenge infections

Infected blood for challenge infections was obtained by cardiac puncture and collected into 10 i.u./ml heparinised PBS. The blood parasitaemia of the donor mouse was determined by examination of a Giemsa's stained thin blood smear collected from the tail (2.7). The blood was diluted to the required concentration of pRBC/ml in complete RPMI 1640 medium (Gibco)(Moore et al 1967)(Appendix B) supplemented with 10% FCS (Gibco). For all infections, naive mice were challenged with 1 $x10^5$ pRBC administered i.v. as a 0.2 ml inoculum, using a 1ml syringe fitted with a 26 G needle (both Becton Dickinson). For experimental challenges, as little time as possible was allowed between the bleeding of donor animals and the challenge, during which time parasites were kept on ice.

2.6 Inoculation

I.v. infection was performed via one of the tail veins, while each animal was held in a Perspex restrainer box. To realise a near 100% success rate of inoculation, prior to injection mice were warmed gently under a heat lamp, so causing vasodilation of the tail veins.

2.7 Determination of parasitaemia

Malaria infections were evaluated by determining parasitaemias from thin blood smears made directly from infected mouse tail blood. For daily parasitaemia counts monitoring the course of infection, smears were taken between 0900-1100 hr before any peripheral withdrawal had occurred.

Smears were made on microscope slides with ground glass edges (Chance Propper Ltd. or BDH Ltd.) from drops of tail blood drawn by piercing the mouse tail with a lancet (Monoject Scientific). The lancet was routinely sterilised in alcohol before bleeding each mouse or group of mice in order to minimise the possibility of transfer of pRBC between animals. Blood smears were allowed to dry in air at RT, fixed in 100% methanol (Analar, BDH Ltd.) for 1-2 min and then stained in Giemsa's stain (Gurr, BDH Ltd.) diluted 1:10 in phosphate buffer (pH 7.2) (Appendix A) for 30 min. Fixing and staining was carried out in Coplin jars. The blood smears were rinsed in tap water, air dried and examined under oil immersion using x100 objective and x10 eyepiece lenses on a Leitz S.M. Lux binocular microscope.

When monitoring the course of infection, parasitaemias were obtained by calculating the number of pRBC from a total number of RBC. If the parasitaemia was 2-3% or greater (determined by observing more than 3-4 parasites per field), direct counts of the number of parasites per 500 RBC were made. Lower parasitaemias were enumerated by counting the number of parasites observed in a total of 30 fields. At this magnification, each field represented about 200 RBC when the cells were confluent but not overlapping. Infections were described as subpatent when no parasites were seen in 50 fields of view (approximately 10,000 RBC).

For experimental procedures, the day of infection was termed d 0 and smears taken from d 4-60 p.i. (when all mice had cleared infection). Smears were not taken prior to d 4 after inoculation since this was the time most mice began to carry a patent infection. This follows from the inverse linear relationship between time to patency and the $\log_{10}$ dose of parasites inoculated (Warhurst & Folwell 1968).

2.8 Presentation of parasitaemic data

The course of infection of a group of mice was represented graphically by plotting the geometric mean of the parasitaemia (mean $\log_{10}$ of the number of pRBC/10^5 RBC) against time (expressed in days). Vertical bars showing one standard deviation are included where necessary. However, to preserve the clarity of graphs where much data is presented, standard deviations are put in only if differences in parasitaemia courses are not self-evident. From the data collected and their graphical presentation, four main criteria were used to evaluate the effects of protective immune responses on challenge parasitaemias. These were (i) the time taken for mice to show a 2% parasitaemia (the pre-2% latent period) (Warhurst 1966) (ii) the day and level of peak parasitaemia (iii) any extension of the time taken for the primary parasitaemia to be resolved relative to the controls (assessed by recording the total time for the parasitaemia to rise from the 2% level to peak and then to fall to subpatency) (a modification of the data evaluation of Jarra et al (1986)) (iv) any extension of the pre-recrudescent subpatent period relative to the controls.

For all in vivo studies in which the course of infection was followed, the minimum number of mice in each experimental group was 5-6. For such a group size, the reproducibility of individual parasitaemia determinations, which includes the variable of smear preparation as well as the parasite counting, is close to the predicted theoretical minimal level calculated using the binomial distribution (Gilks 1988).

Groups of 5-6 mice were used so that the random scatter of parasitaemias would be expressed to cancel each other out. Thus, parasitaemia curves are relatively smooth. Despite these precautions, some variability wereencountered in the counts, especially after crisis and resolution of the primary parasitaemia. To minimise the impact of individual readings distorting the mean, the data was transformed by either of two computer programmes: a Fortran programme developed by Dr. Chris Newbold (University of Oxford) on the Dec 20 computer system at N.I.M.R., Mill Hill, London (this programme was a kind gift of Dr. Bill Jarra of the same Institute); and Cricketgraph version 1.2.1 (Cricket Software) on an Apple Macintosh SE microcomputer (Scotsys Computer Systems). When differences in courses of infection between groups of mice were not obvious, statistical analysis using the non-parametric sign test (Colquhoun 1971) was performed using the same programmes (Jarra & Brown 1985).

2.9 Haematology

Small volumes of blood were taken from mice by lancing the tail and collecting blood into microcentrifuge tubes (Eppendorf Gerätebau or Scotlab) containing 10 µl 10 i.u./ml heparinised PBS. RBC and WBC counts were made with a haemocytometer (Improved Neubauer, Weber) after dilution of RBC 1:200 in PBS (pH 7.2) and WBC 1:10 or 1:20 in white cell diluting fluid (Appendix C). Differential WBC counts were made by examining Giemsa's stained thin blood smears. These were scanned their full length to eliminate problems arising from the uneven distribution of cells. Leucocytes were differentiated into lymphocytes, monocytes and granulocytes. Results are shown as both total numbers of peripheral blood leucocytes/ml or of each cell type/ml, and as differential numbers (% values).

For the adoptive transfer of peripheral blood leucocytes, a cytosmear of the WBC preparation was made using a cytospin centrifuge (300 g for 10 min at RT) (Shandon-Elliot Ltd.) (Doré & Balfour 1965). The Giemsa's stained slide was then viewed to enable a differential count of the relative proportions and absolute numbers of leucocytes inoculated at adoptive transfer.

2.10 Collection of serum

Large volumes of sera were collected by exsanguination by cardiac puncture under ether

anaesthesia, yielding about 1-1.2 ml blood from a 25 g mouse. Either a 1ml or a 2ml syringe was used, fitted with a 25 G needle. Blood was pooled, where appropriate, and allowed to clot. The clot was loosened from the edges of the container using a wooden stick, and left to contract for 1hr at 37 °C (or overnight at RT). The overlying serum was collected by pipetting, any contaminating RBC being removed by centrifugation (300 g for 5 min)(MSE Mistral 3000i, Fisons). The serum was then aliquoted, labelled and stored frozen at -20 °C until required. Subsequent freeze-thawing was kept to a minimum. Large volumes of hyperimmune and normal sera were collected using this methodology. A stock of hyperimmune serum for use as positive controls in the anti-P. c. chabaudi slide IFAT was raised from donors which were challenged on three occasions successively with 1 $x10^5$, 1 $x10^7$ and 1 $x10^8$ pRBC at two monthly intervals. The mice were bled one week after the last challenge when the peripheral blood parasitaemia had been cleared. Normal serum was obtained from normal non-infected mice.

Smaller volumes of serum (up to 100 µl) were obtained by bleeding mice from the tail into hard glass capillary tubes (BDH Ltd.) (Gray 1979). Mice were prewarmed under a heat lamp, then the distal 2-3 mm of the tail snipped off using a pair of clean, sharp scissors. Capillary tubes were 1/4 filled with blood, labelled and allowed to clot for 30 min at 37 °C and then placed in a 4 °C refrigerator for a further 30 min to contract the clot. The serum was removed from the sedimented RBC and transferred to a microcentrifuge tube and spun (300 g for 5 min)(MSE Microcentaur, Fisons) to pellet any remaining RBC. The serum samples were then frozen at -20 °C until needed.

For the collection of immune serum from infected mice to show the levels of specific anti-malarial antibodies during the course of infection, sera were collected from different experimental groups at 2-3 d intervals from d 0-60. Within each experimental group, every mouse was bled on an equal number of occasions to ensure that anaemia did not result from the repeated bleeding of an individual animal, and also that the circulating leukocyte count was maintained in a steady state (Sluiter et al 1985).

2.11 Irradiation of mice

Mice were irradiated with whole body gamma irradiation from a ^{60}Co source chamber (Nuclear Engineering) in the Department of Veterinary Physiology, University of Glasgow. During the course of this study, the source emitted at a rate varying between

3.78-4.58 Gy (1 Gy = 100 rad), and mice were exposed for suitable lengths of time to receive doses of 4, 6 or 7.5 Gy depending on the experiment (details in text). Mice were lowered in pairs into the source chamber and irradiated in an upright position. To lessen the risk of gut damage upon irradiation, mice were starved of food for 24 hr immediately preceeding treatment. This precaution increased the radiation resistance of the mice (Hudson & Hay 1989). Irradiation of recipient mice prior to adoptive transfer and challenge infection took place no earlier than 24 hr previously, but more often on the day of infection. Following immunosuppression, mice routinely received terramycin (3 g/l) (Pfizer) in their drinking water for one week to prevent superinfection-associated deaths (McDonald & Phillips 1978).

2.12 Preparation of peripheral blood lymphocytes for adoptive transfer

High purity lymphocyte preparations suitable for adoptive transfer were obtained by separating WBC from whole blood by differential centrifugation on density gradient media. To enable the adoptive transfer of a large number of peripheral blood lymphocytes collected from a relatively few donor animals, mice were bled at the time of peripheral blood lymphocytosis; this occurred just after peak primary parasitaemia, usually d 12-13 p.i. (Kumararatne et al 1987) (see Chapter 3). These semi-immune donor mice were bled aseptically by cardiac puncture into heparinised PBS (10 i.u./ml) and the blood pooled. After preliminary investigations using several different separation media, it was found that a preformed discontinuous gradient of 30%, 40% and 50% Percoll (Pharmacia Fine Chemicals) (Pertoft et al 1978) (Appendix D) gave the cleanest separation of lymphocytes from pRBC and nRBC (Ulmer & Flad 1979, Kurnick et al 1979 b). 6 ml whole blood was layered on a total of 9 ml Percoll (3 ml each layer, 30% fraction uppermost) in a plastic universal tube (Sterilin) and spun at 400 g for 15 min at 20 ^{o}C on an MSE Mistral 3000i centrifuge using an angle-headed rotor to band the different cell types at their isopycnic densities. The centrifuge run was slowed without breaking to prevent disruption of the separated layers. The lymphocytes formed an opalescent band above the 30% Percoll layer, the pRBC banded at the 40-50% interface, whilst the nRBC pelleted at the bottom of each tube. The uppermost lymphocyte layer was drawn off, removing as little of the gradient as possible. The cells were then washed twice in excess PBS (300 g for 5 min), resuspended in a minimal volume of 10% FCS RPMI 1640 medium, and kept on ice until used. For the purification of control populations of normal lymphocytes taken from non-infected donor mice, a

satisfactory separation was achieved using a one-step gradient of Ficoll Hypaque (Nycomed UK Ltd.) (Bøyum 1968) (Appendix D). Whole blood was layered onto Ficoll Hypaque (21 parts 9% Ficoll: 10 parts 33.9% Hypaque) and centrifuged without breaking at 420 g for 20 min at 4 ^{o}C. The turbid lymphocyte fraction was removed carefully using a flammed Pasteur pipette (Bilbate Ltd.), and the cells recovered as for the infected donor lymphocytes (Chi & Harris 1978).

2.13 Preparation of splenic lymphocyte suspensions

Mice were killed by overdosing with ether (May & Baker Ltd.) and the spleens dissected out using aseptic techniques. If several mice were used, each excised spleen was placed in a 9 cm Petri dish (Sterilin), containing incomplete RPMI 1640 medium until used. The spleens were dissociated by mashing through a stainless steel sieve (mesh size 0.025 mm^2) using the inside plunger of a syringe (Becton Dickinson) to push the cells through, and collected into the incomplete medium. The cells were disaggregated by aspiration with a sterile Pasteur pipette and, after removing connective tissue debris and clumps of cells by sedimentation, the supernatant was collected. The spleen cells were washed twice in fresh medium (250 g for 5 min), then resuspended in a relatively small volume of 10% FCS RPMI 1640 medium to give a single cell suspension.

Contaminating RBC were removed by haemolysis. 1 ml of spleen cells (approximately $1x10^9$ cells) was incubated in 9 ml 0.83% Tris-ammonium chloride (pH 7.4) (Appendix C) for 5 min at RT (Boyle 1968). Most macrophages and other adherent cells were removed from suspension by filtration through glass wool, as described by Julius et al (1973) and Trizio & Cudkowicz (1974). A glass wool column, made by packing a 10 ml plastic syringe barrel to the 8 ml mark with glass wool (Travenol Laboratories), was clamped in a vertical position and 30 ml incomplete RPMI 1640 medium, followed by 10ml 10% FCS RPMI 1640 medium, washed through the column. The spleen cell suspension, now lacking RBC, was loaded onto the soaked glass wool column under gravity and washed out with 10% FCS complete medium. This procedure, carried out at RT, as well as removing phagocytic cells, cleansed the suspension of dead cells.

2.14 Enrichment of spleen cell populations by nylon wool separation

The T and B subpopulations of prepared splenic lymphoid suspensions were fractionated by filtration through nylon wool. The enriched T cell population was collected by passage through nylon wool columns, based on the modification by Handwerger & Schwartz

(1974) of the technique of Julius et al (1973). Recovery of the enriched B cell fraction was achieved by following the method of Trizio & Cudkowicz (1974).

The barrels of 10 ml plastic syringes were packed tightly to the 7 ml mark with scrubbed nylon wool (Leuko-pak, Fenwal Laboratories), autoclaved and stored wrapped in aluminium foil. The sterile nylon wool columns were rinsed with 30 ml 10% FCS RPMI 1640 medium, followed by 20 ml 20% FCS RPMI 1640 medium (used in all subsequent procedures) and any air bubbles dislodged by gentle tapping. All washing and eluting procedures were carried out in upright columns within a sterile lamina flow bench (Intermed microflow pathfinder). The wet columns, sealed at the top with parafilm (American Can Co.) and at the bottom with a 25 G needle stuck in a rubber bung, were kept in a humidified incubator (Hearson Ltd.) at 37 °C for 90 min prior to use. They were then washed through with 10 ml fresh medium at 37 °C before 2-3 $x10^8$ cells in 2 ml volumes were added dropwise onto each column. The cells were washed into the columns with 1 ml prewarmed medium, the columns resealed and again incubated at 37 °C for 45-60 min. After incubation, the non-adherent cells were run out dropwise from the syringe. These cells were washed out with 20 ml medium warmed to 37 °C and were considered thereafter as the enriched T cell subpopulation. Nylon wool-adherent cells were eluted from the columns by physically disrupting the nylon wool with flamed forceps and by forcing fresh warm medium through the column using a 10 ml syringe plunger. The released adherent cells were collected in 10 ml and thence referred to as the enriched B cell subpopulation. Both cell populations were washed, resuspended in 10% FCS RPMI 1640 medium and kept on ice until either injected into recipient animals or cultured in vitro. Spleen cell numbers were counted in a haemocytometer using white cell diluting fluid (2.9). The counts were adjusted after cell viability was determined by the trypan blue exclusion method (2.17).

2.15 Adult thymectomy of mice

Four to five week old female NIH mice were surgically thymectomised by the method of Millar (1960), as modified by Monaco et al (1966). Before the operation, animals were injected i.p. with 0.1 ml of a 1:10 dilution in sterile distilled water of Hypnorm (Janssen Animal Health) (Appendix G) and subsequently anaethetised by an i.p. injection of 0.2 ml of a 1:5 dilution of Valium (Roche) (Appendix G) in sterile distilled water (McKeand, J.B. , personal communication). Each animal was harnessed to a cork operating board in the supine position with the four limbs stretched out and secured.

After sterilising the chest wall with a few drops of 70% alcohol (May & Baker Ltd.), a midline longitudinal incision was made through the skin just above the anterior sternum. The skin was then opened, the salivary glands deflected, and an anterior-posterior cut made through the sternum. The location of the thymus was revealed by parting the muscle underlying the sternum. Thymectomy was achieved by suction using a bent glass cannula connected via a glass T-piece to a vacuum line. The amount of suction at the cannula tip could be varied by changing the pressure of the thumb on the end of the T-piece. With fairly light suction, each thymic lobe was located and held firmly at the cannula tip, the mouse's diaphragm being pressed so that the contents of the thorax forced the thymus into a more convenient position for manipulation. When the lobe was loosened from its bed by gentle teasing, the suction was increased so that the gland was sucked out through the cannula. Both the left and right lobes of the thymus were removed in this way. The wound was closed by pinching together the skin and held with two Michel suture clips (Thackray). After the operation, mice were revived under a warm lamp. The Michel clips were removed one week later using clip inserting forceps. After practice, post-operative mortality was < 15%.

For all experimental groups of adult-thymectomised mice, at the time of sacrifice, selected mice were checked for thymic remnants and none were seen. The same operative procedure was carried out on littermates on the same day to prepare sham adult-thymectomised mice controls, except that the thymic lobes were not removed. These animals experienced the same surgical trauma as did the thymus-depleted mice.

2.16 Irradiation and bone marrow reconstitution

Four weeks after thymectomy , thymus-ablated mice were given a 7.5 Gy (750 rad) dose of whole body irradiation delivered by a gamma-emitting ^{60}Co source (2.11). Bone marrow reconstitution was achieved within 2 hr by i.v. injection of 10^7 syngeneic, adult, nucleated bone marrow cells. These were obtained by flushing the marrow cavities from dissected tibiae and femora with incomplete RPMI 1640 medium (Benner et al 1981; Phillips, R.S., personal communication). Donor mice were killed by ether overdose and the skin wetted with 70% alcohol. To free the tibia or femur respectively, the elbow or knee tendons were cut as was the joint capsule at the top of each long bone. Each bone was then pulled away and residual muscle trimmed away. The marrow was isolated after cutting the head of the femur or tibia and small pieces of the greater trochanter on the upper side, and the condyle on the lower side. By means of a 2 ml

syringe equipped with a 26 G needle, a hole was pricked in both spongious ends of the long bone, then the marrow collected by flushing the marrow cavity with 2 ml incomplete RPMI 1640 medium. Whether or not the marrow had been completely extracted could be judged from the colour of the shaft. The pooled cell plugs were disaggregated by forcing through a 21 G needle and the cells further dispersed by Pasteur pipette aspiration. The resulting single cell suspension was washed twice by centrifugation (300 g for 5 min) and resuspended to a small volume for counting/viability testing prior to reconstitution of the recently irradiated mice by the i.v. route.

The recipient mice were protected from infection by administration of a single 0.2 ml dose of 5000 i.u. Penbritin (ampicillin sodium b.p., Beecham Animal Health) i.p., and for two weeks after irradiation 3 g/l terramycin in their drinking water.

Mice were used experimentally, by adoptive transfer and challenge infection, approximately four weeks after reconstitution. The described protocol of thymus ablation, gamma irradiation and bone marrow reconstitution effectively depleted the treated mice of all but some mature peripheral T cells; thus, each individual has historically been termed a 'B-mouse' (Hudson & Hay 1989).

2.17 Determination of cell viability

Viabilities of preparations of cells (peripheral blood, spleen, bone marrow) were measured by the trypan blue exclusion test (Naysmith & James 1968; recommended by Jerne et al 1974). After making an appropriate dilution (usually 1:10 or 1:100) of the cell suspension in PBS (pH 7.2) if necessary, the cells were further diluted 1:1 in a solution of 0.2% w/v trypan blue (Gurr, BDH Ltd.) in PBS (pH 7.2) and mixed thoroughly. The suspension was incubated for 2-3 min at RT and then examined by phase contrast under oil immersion (x100 objective, x10 eyepiece) on a light microscope. Dead cells were unable to exclude the vital dye and stained blue, whereas viable cells remained clear. Leukocytes could be distinguished from erythrocytes by size, morphology and colour. The proportion of live to dead cells was expressed as a percentage viability, and adjustments to total cell numbers (determined by haemocytometry) made accordingly.

Viability of spleen cells passed successively through glass and nylon wools was usually > 95% and always > 90%. Viabilities of peripheral blood lymphocytes and bone marrow cells was > 92% and > 97% respectively.

2.18 Determination of anti-malarial antibody titres

Total anti-parasite antibody determinations were carried out on serum taken from infected mice at 2-3 d intervals throughout the course of infection using the slide IFAT procedure of Van Meirvenne et al (1975) modified by McLean et al (1982 a), based on the indirect fluorescent antibody method for malaria parasites as described by Voller (1964) and O'Neil & Johnson (1970).

(a) Preparation of malarial antigen slides

Late-stage (trophozoite/schizont) pRBC were collected from infected mice and used as the source of antigen. Infected blood was prepared by cardiac bleeding donor mice with an ascending parasitaemia of between 5-15% into 10 i.u./ml heparinised PBS. This was performed late in the afternoon at a time when the normal lit mice contained a high proportion of late trophozoite and schizont stage parasites. pRBC were washed three times in 20 ml PBS (pH 7.2) by centrifugation (250 g for 5 min) and resuspension. After the last wash, the pellet was resuspended to less than the original blood volume ready for making thin blood smears.

Teflon-coated 12 well multitest slides (Flow) were washed in detergent, rinsed successively in tapwater, distilled water and ethanol (May & Baker Ltd.) and finally hand dried with Crestex tissue (British Tissue Co.). Using a P100 'pipetman' pipette (Gilson), 100 µl aliquots of pRBC suspension were pipetted up and down onto every reaction zone on each slide. The liquid was then aspirated from the slides, leaving a layer of cells in the circles. Slides were either air-dried or with a hair dryer at RT, and then wrapped in batches of five in tissue and dehydrated overnight in a dessicator packed with silica gel. The antigen slides were stored in sealed polythene bags with silica gel at -20 °C until required (Manawadu & Voller 1978).

(b) Assay

To avoid condensation when recovering slides from frozen, they were brought up to RT in a dessicator for 1-2 hr before use in the IFAT. Slides were fixed in absolute acetone (May & Baker Ltd.) and air dried. The smears were then rehydrated by placing in three successive baths (Coplin jars) of PBS (pH 7.2) and left to stand in the final PBS wash for 15 min. It was important that from this point onwards, the slides did not dry out; if the slides remained hydrated, non-specific fluorescence was prevented (McLean, S.A.,

personal communication). Slides were drained briefly and the area between wells dried to leave 12 separate reaction zones. Serial 1:2 dilutions of normal serum (negative control), hyperimmune serum (positive control) and the immune sera to be tested were prepared in a 96 well microtitre plate (Nunc), starting at an initial dilution of 1:10 in PBS (pH 7.2). 20 µl of test and control sera at the appropriate dilutions were added to the reaction zones using a Gilson P20 'pipetman' pipette. For every serum sample tested, each taking one or two slides, the first reaction zone in each slide contained PBS in place of diluted serum, to act as a control for non-specific fluorescence. The slides were incubated in a humid chamber for 15 min at RT, then washed and rehydrated for 15 min as before. Each slide was recovered from PBS and the edges dried (there was no need to separate each reaction zone as all were to receive the same conjugate mixture. A 1 ml solution of FITC-conjugated rabbit anti-mouse IgG (Sigma) diluted 1:200 in PBS containing Evans blue (1:10000 w/v) (Merck) was applied to each of the slides which were then incubated for a further 15 min. The FITC-conjugate mixture was prepared by mixing 5 µl neat conjugate solution with 100 µl of a stock solution of 1:1000 dilution of Evans blue in PBS, and making up to 1 ml with 895 µl PBS (pH 7.2). The Evans blue was required as a counterstain for the RBC in the antigen slide preparations (El Nahel & Bray 1963). The slides were washed again to remove unbound conjugate, and rehydrated in fresh PBS before mounting under a long coverslip in a 1:1 solution of non-fluorescent PBS/glycerol (Merck).

(c) Examination of slides

Fluorescence was observed using a Leitz ortholux microscope linked to an Epson PX4 computer through a Leitz MPV Compact 2 microscope photometer. All apparatus was located in the WLEP darkroom. The overhead ultraviolet source was a Wotan HBO-50 mercury lamp with 2 x KP490 exciting filters and a TK 510 dichroic beam-splitting mirror and a K515 suppression filter. The microscope was switched on 15-20 min prior to use to allow the mercury lamp to warm up slowly.

The slides were examined using a x50 water immersion objective and a x12 binocular eyepiece. For each specimen, 100-200 cells were counted, both in ordinary incident light and in u.v. light. The titre (endpoint) of the serum was considered to be the last serial dilution of serum at which specific parasite fluorescence was observed. For each slide, the control zones of hyperimmune or normal serum, as well as that of PBS alone, were examined for comparison.

2.19 Preparation of parasitised erythrocyte lysate

Soluble P. c. chabaudi AS strain antigens were prepared from whole blood cells enriched for mature trophozoite/schizont-infected RBC using a modification of the method described by McDonald & Sherman (1980). Since peripheral withdrawal of schizonts of some strains of P. c. chabaudi has been shown to occur in vivo (Shungu & Arnold 1972), short-term in vitro culture was used to obtain considerable numbers of schizont stage pRBC.

Mice to be used as a source of Ag were kept under reversed light conditions (12 hr light between 2000-0800 hr) for a minimum of 10 d acclimatisation before infection. Using this system, schizogony is usually synchronous and occurs between 1100-1300 hr (compared to maintenance on a normal daylight cycle, when peak schizogony occurs at 0100 hr, an unreasonable time to perform an experiment) (Jarra & Brown 1985).

Cardiac blood was collected under sterile conditions from several donor mice undergoing primary peak parasitaemia (average parasitaemia 40%) by bleeding from the heart into heparinised PBS (10 i.u./ml). Bleeding took place between 0800-0900 hr, when most parasites were at the late ring stage (i.e. prior to deep vascular tissue sequestration). After washing twice in 5% FCS RPMI 1640 medium, RBC were resuspended to a 10% haematocrit in the same medium and cultured using the candle jar method of Trager & Jensen (1976). 15 ml of the 10% w/v suspension of the infected blood in medium were dispensed in 9 cm Petri dishes (Cel-Cult, Sterilin), which were placed, together with a candle, in a humidified glass dessicator. The candle was lit and the lid put on with the stopcock open. When the candle flame extinguished, the stopcock was closed. This procedure provided a gas phase of 3% CO_2 and 15 -17% 0_2, i.e. an atmosphere low in O_2 and high in CO_2 (Trager 1987). The candle jar was placed in a 37 °C incubator until the parasites had reached the schizont stage; parasite maturation was monitored every 30-45 min by examination of Giemsa's stained thin blood smears. After harvesting, the cultured parasites were washed in 5% FCS RPMI 1640 medium (200 g for 10 min) and resuspended to their original volume in sterile PBS. The blood solution was filtered through sterile Whatman CF11 powdered cellulose paper columns to remove leucocytes (Beutler et al 1976) and the filtrate subsequently washed. The pellet containing the malaria parasites was restored to its previous volume in PBS (pH 7.2) and then freeze-thawed five times. Each cycle of freeze-thawing entailed snap freezing the preparation by plunging into liquid N_2, then immediately defrosting the solution by placing in a 37

°C water bath (Gallenkamp). The rapid temperature transition brought about by this process acted to fracture the RBC, so releasing parasitised material from those lysed infected RBC present. The disrupted pRBC suspension was centrifuged at 1500 g for 10 min and the S/N fluid collected. This was termed the pRBC lysate and was stored in 20-50 µl aliquots at -20 °C until required.

Since all procedures used were sterile, the lysate samples were suitable for direct in vitro use without prior filtration. The total protein concentration of the lysate was determined by the standard BCA assay (see 2.20). Using this method, the amount of contaminating RBC Ag in the crude Ag preparation was minimal as determined by PAGE (Sayles & Wassom 1988). Erythrocytes from normal uninfected mice (nRBC) treated in the same way were used as a control for testing Ag specificity (Dodge et al 1963).

2.20 Determination of total protein concentration

A quantitative estimation of total IgG and IgM in protein samples (pRBC and nRBC lysates and rat MAbs) was determined by spectrophotometric measurement at 595 nm. The procedure used was the BCA standard assay, as described by Smith et al (1985) using the Coomassie blue G-250 Pierce protein assay reagent (Pierce Chemical Co.) and BSA as a protein standard. This method was a modification of the original technique of Bradford (1976), which measures an absorbance shift from 465-595 nm that occurs when Coomassie blue binds to proteins in an acidic solution. The assay can be used to determine protein concentrations in the range of 150-1500 µg/ml, a range over which the intense purple response is relatively linear and thus permits accurate total protein quantitations of unknown samples as measured against a standard curve.

A known protein concentration series was prepared by diluting a 2 mg/ml stock BSA standard (Pierce) in deionised water. Convenient standard concentrations were used to cover the range 150-1500 µg/ml (Appendix F). 5.0 ml protein assay reagent was added to 100 µl of each of the diluted standards and also to 100 µl of the unknown protein sample(s), in clear, clean test tubes and mixed well. 100 µl deionsed water sample diluent was used as a blank. All tubes were then incubated for 30 min at 37 °C. Absorbance was read at 595 nm on a uv spectrophotometer (Pye Unicam PU 8600) against the deionised water blank. This latter value was then subtracted from each standard or unknown protein sample absorbance to give the net absorbance at 595 nm of each sample tested. As the protein concentration of all samples tested during the course of this study exceeded the 1500 µg maximum, each solution had to be diluted

appropriately (usually 1:10 or 1:100) to bring the diluted sample within the assay range suitable for spectrophotometric measurement.

2.21 Production of IL-2 for maintenance of T cells in vitro

For the routine maintenance of T cell lines and clones in vitro , S/N from rat spleen cell cultures stimulated with the T lymphocyte mitogen concanavalin A (Con A S/N) were used as a source of IL-2 (Douglas et al 1969).

Spleen cell suspensions were prepared from inbred female Wistar rats supplied by the WLEP animal house breeding facility (methodology as described previously) and these cultured at 2 $x10^6$ cells/ml in 10% FCS RPMI 1640 medium containing 2.5 µg/ml Con A (Sigma Chemical Co. Ltd.) at 37 °C in a humidified atmosphere containing 5% CO_2. The cultures were maintained horizontally as 40 ml volumes in 75 ml tissue culture flasks (Cel-Cult, Sterilin). After 48 hr, the cell suspensions were transferred to 50 ml centrifuge tubes (Sterilin) and spun at 400 g for 10 min, the S/N aliquoted into 20 ml Universal containers (Sterilin) containing 0.4 g of α-methyl mannoside (Sigma) and stored at -20 °C. α-methyl mannoside was added to sequester free Con A which may otherwise induce non-specific T cell activation when the Con A S/N was used subsequently. Every aliquot was filter-sterilised through a 0.22 µm microfilter (Millipore) prior to use. Each batch of Con A S/N was screened for IL-2 activity before use by determining the IL-2 titre in a microassay (Hamblin & O'Garra 1987) using polyclonally-activated T lymphocytes as test cells and recombinant murine IL-2 (Genzyme)(Appendix E) as standard.

2.22 Production of IL-2 for use in T cell cloning

Helper T cell lines produce high levels of autocrine IL-2 after 48 hr exposure to Ag (Taylor et al 1987). This source of IL-2 was reserved for those stages of culture involving cloning of the autologous Th lines. A helper T cell line specific for ovalbumin was raised and maintained in vitro by a repeat 'feed-starve-feed' cycle (Kimoto & Fathman 1980)(2.26). 2 $x10^5$ cells/ml 10 ml cultures were fed with 100 µg/ml chicken egg ovalbumin (Sigma) and 2 $x10^6$ APC/ml and incubated in an upright 25 ml flask for 2 d. The medium (auto IL-2) was aspirated, 0.22 µm filter-sterilised and kept at -20 °C until required. Auto IL-2 was used in cloning procedures at 20% (v/v), as dictated by the IL-2 assay (2.23).

2.23 Screening of stock IL-2 sources

Two sources of IL-2 were used for cloning and maintaining T cells in vitro : S/N from Con A-stimulated rat spleen cell cultures (Con A S/N) and auto IL-2, produced by Th cell lines after 2 d culture with Ag and APC. Prior to use in culture work, each batch of S/N was tested for IL-2 activity, so enabling subsequently an appropriate dilution to the optimal concentration shown to stimulate T cell proliferation.

Serial dilutions of S/N in quadruplicate were tested for their ability to support the growth of murine peripheral blood T cell blasts (Hamblin & O'Garra 1987, modified from Boylston et al 1981).

(a) Preparation of blast cells

Murine mitogen-activated T cell blasts were prepared as described by Malek et al (1983). Naive, uninfected NIH mice were bled aseptically by cardiac puncture into heparinised PBS (10 i.u./ml) and the blood pooled. Mononuclear cells were collected by separation of the blood on a Ficoll Hypaque (Nycomed U.K. Ltd.) gradient (Bøyum 1968) (Appendix D)(2.12). Cells were resuspended at 1 $x10^6$/ml in 10% autologous serum in 10% FCS RPMI 1640 medium. The cell suspension was placed in 75 ml tissue culture flasks with 20 µg/ml PHA-R (Wellcome) and cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C for 3 d. After incubation, the cells were recovered by washing twice with incomplete RPMI 1640 medium (300 g for 5 min) and counted.

A layered gradient of Percoll solutions (Pharmacia) (100%, 70%, 60%, 50%, 40% and 30%)(Appendix D) was prepared in plastic Universal tubes, allowing 2 ml of each dilution in every gradient. The blast cells were resuspended in the 30% Percoll fraction with no more than 5 $x10^7$ cells/gradient, prior to layering this dilution uppermost in each tube. The suspensions were spun at 450 g for 17 min at 20 °C using an angle-headed rotor centrifuge (MSE Mistral 3000i) and slowed without breaking, after which the cells were banded at the various density interfaces. The fraction most enriched for blast cells was the 40-50% Percoll interface; this layer was removed with a flamed Pasteur pipette into a sterile Universal tube and diluted with 20 ml 10% FCS RPMI 1640 medium. Each tube was washed three times (200 g for 10 min) in complete medium. The blast cells were reincubated at 1 $x10^6$/ml in 10% FCS RPMI 1640 medium in 75 ml flasks at 37 °C in a humidified atmosphere at 5% CO_2 in air for 4 d.

(b) Blast cell assay

PHA-R-stimulated peripheral blood blast cell cultures were washed twice (200 g for 10 min) and resuspended in 10% FCS RPMI 1640 medium at 2.5 $x10^5$ viable blasts/ml. Serial two-fold dilutions of both unknown and standard recombinant IL-2 (Genzyme) (Appendix E) were made in complete RPMI 1640 medium. 100 µl of blast cell suspension was placed in each well of a 96 well flat-bottomed tissue culture plate (Nunc) and to this added 100 µl of a suitable dilution of IL-2. A Gilson P100 'pipetman' pipette was used for all micromanipulations. For each batch of IL-2 to be screened, four replicate cultures were set up for each dilution, together with appropriate negative controls without IL-2. Each assay plate was cultured in a humidified CO_2 incubator at 37 °C for 72 hr, whereupon 1.0 µCi [^{3}H-methyl] thymidine (Amersham International) as a 20 µl volume was added to each well. The radioactivity incorporated over an 18 hr pulse time was determined as described in 2.34. Samples containing IL-2 gave dose-related stimulation of tritium incorporation into the mitogen-activated lymphocyte blasts. From the data, the optimum dilution of each batch of Con A S/N or auto IL-2 to add to T cell cultures could be determined.

2.24 Antigen presenting cells (APC)

Irradiated syngeneic spleen cells (Schwartz et al 1978) are the most convenient source of large numbers of APC for routine maintenance of T cell lines or clones. APC were obtained from the spleens of naive syngeneic NIH mice (H-2^q haplotype). Spleen cell suspensions were prepared in 10% FCS RPMI 1640 medium and placed on ice. The cells were then irradiated to 30 Gy (3000 rad) by exposure to a ^{60}Co (thereby inhibiting accessory lymphocyte proliferation in vitro). Preliminary investigation established that a dose of ≥ 25 Gy irradiation was required to inhibit completely feeder cell growth in vitro using this particular gamma emitter. The cells were then washed twice (300 g for 5 min) and resuspended in complete medium. After taking a total cell count, the suspension of APC was added to the cultured T cells at an appropriate final concentration.

2.25 In vivo priming of Th cells to Plasmodium c. chabaudi

Prior to generating a helper T cell line by culturing spleen cell suspensions with pRBC lysate as a source of plasmodial antigens and irradiated splenic APC, the donor mice were primed in vivo to the antigenic stimulation, namely parent AS strain parasites of P. c.

chabaudi.

Two strategies were employed in an attempt to prime spleen cells in vivo to varying degrees to the full range of plasmodial asexual erythrocytic stages. The standard way of priming mice was to allow for a complete course of infection to be cleared (> 60 d) and then to boost the immune response by inoculating one or more reinfective doses of pRBC; these were then cleared very rapidly by the pre-sensitised host immune system (similar methodology as for raising hyperimmune serum; 2.10). Alternatively, mice were primed for shorter periods of time by not waiting till parasite clearance before splenectomy but sacrificing donor animals at various points throughout the course of primary infection (discussed in text).

2.26 Initiation and propagation of Ag-specific helper T cell lines

The original protocol followed for generation of Th cell lines in vitro was the cyclic stimulation and rest method first described by Kimoto & Fathman (1980; Fathman & Kimoto 1981), as modified by Taylor et al (1987). This technique was subsequently adapted progressively in consultation with Dr. Richard Grencis (Dept. of Cell & Structural Biology, University of Manchester) in order to optimise conditions for raising clones by limiting dilution from established T cell lines. Although the majority of experiments described in this thesis were performed after the latter technique was standardised, some initial results were attained using cells generated by the former method, so each is described here.

(a) Original protocol

Spleen cell suspensions were prepared and cultures established at 4 $x10^6$ cells/ml in 10% FCS RPMI 1640 medium containing 200 µg/ml pRBC lysate (previously determined optimal concentration of lysate shown to induce T cell proliferation). 40 ml aliquots were dispensed into 75 ml tissue culture flasks and these incubated in a humidified 5% CO_2 atmosphere fo 4 d. Cells were harvested, dead cells removed on Metrizamide (2.7) and viable cells resuspended at 2 $x10^5$/ml. Freshly prepared APC were added to the responder cells at a final concentration that ranged between 5 $x10^5$ -2 $x10^6$/ml, and Con A S/N added at a suitable dilution to maintain cell viability. The cultures were incubated for a further 4 d.

The Th cells were recultured (2 $x10^5$/ml) with APC (2 $x10^6$/ml) for 6-8 d without pRBC Ag but with Con A S/N if necessary - this was the 'starve' period. Thereafter, the

Th lines thus generated were maintained by a 'feed' (3-4 d, Ag + APC), 'starve' (6-8 d, APC alone), 'feed' cycle.

Cell stocks were frozen down 3 d after the second or subsequent antigenic stimulations. Proliferation of established Th cell lines was assayed 8 d after the start of a starve period, i.e. when the cultures were spent of residual Ag and therefore optimally responsive to fresh stimulation.

(b) Modified protocol

The methodology outlined above was modified in order to increase the viability of routinely cultured responder cells, so promoting the probability of attaining clones by limiting dilution. To replace the 'feed-starve-feed' cycle used previously, the starve step (in which T cells were cultured without Ag but with Con A S/N) was omitted and cultures simply incubated without alteration between successive pRBC lysate feeds. The validification for including a starve period was to promote the growth of Ag-specific populations of cells (Fathman & Kimoto 1981) which could be achieved as efficiently by not disturbing the cultures for 12-15 d between successive subcultures with fresh APC and Ag (Grencis, R.K. & Wood, P.J., personal communication). An exogenous source of IL-2 was not considered essential as Th cells secrete autogenous IL-2 upon antigenic stimulation which would be sufficient to support the cells at times of Ag deprivation.

Th cells were established and maintained as stable lines in vitro using the protocol outlined in Table 2.1. After initiating the cultures at 4 $x10^6$/ml spleen cells, the number of responder cells was reduced stepwise at every subculture to 2.5 $x10^4$ cells/ml, whence the concentration of proliferating cells was maintained at this level. From an initially low starting concentration, successfully primed responder cells, once established, were capable of proliferating rapidly to outgrow the fixed APC in culture. As it was found that the presence of residual dead cell debris had no detrimental effects on the rate of cell proliferation, non-viable cells were not removed between feeds. This was a benificial finding as Metrizamide treatment utilised in the original protocol removed all dead cells but resulted in a sometimes unsatisfactory recovery of viable cells.

2.27 Removal of dead cells from in vitro cultures

Following the original protocol for in vitro propagation of T cells, dead responder cells and APC were removed from suspension at every subculture. The methodology used was

first described by Kurnick et al (1979 a & b) using the centrifugation gradient medium Metrizamide (Nycomed (U.K.) Ltd.).

A stock solution of 35.3% w/v analytical grade Metrizamide was prepared in distilled water, filter-sterilised and kept at 4 °C for up to three months. For use, the colloid was diluted to 18% (v/v) by making up 1.02 ml of stock, 0.94 ml PBS and 0.04 ml FCS.

1 ml of this dilution was dispensed to a 12 x75 mm plastic centrifugation tube (Nunc), carefully overlayed with 1 $x10^7$ cells (washed and resuspended to 1 ml with 10% FCS RPMI 1640 medium) and the gradient centrifuged at 450 g for 15 min. Viable cells were recoverable from the interface and washed twice (300 g for 5 min) with 1 ml complete medium in similar centrifuge tubes. Recovery of intact, living cells was always > 83%.

2.28 Anti-CD3 monoclonal antibody assay

A cell suspension S/N taken from an exhausted culture of the 145-2C11 hybridoma cell line was a gift of Dr. Richard Grencis (Dept. of Cell & Structural Biology, University of Manchester) and was used routinely as a source of the anti-CD3 MAb for cloning Th cell lines by limiting dilution. Prior to using this S/N, its efficacy in stimulating lymphocytes had to be tested. To do this, a standard proliferation assay was carried out, using the MAb as the mitogen and naive splenic lymphocytes as the target cells.

A suspension of spleen cells was prepared at 4 $x10^6$/ml and 100 µl of this dispensed to wells of a 96 well flat-bottomed microtitre plate. The anti-CD3 S/N was titred out over a range of dilutions (1-100% original S/N concentration), 100 µl/well of each dilution was added to quadruplicate wells and the plate cultured in a humidified CO_2 incubator for 72 hr. Thereafter, the method followed for quantification of cellular proliferation was identical to that described in 2.34.

From this assay, it was possible to determine the optimal dilution (v/v) of 145-2C11 culture S/N to use to promote Th cell clonal proliferation.

2.29 Cloning of helper T cell lines by limiting dilution

For all attempts at cloning, cells were taken 3 d after the third or more round of antigenic stimulation; if attempts were to be made to obtain clones earlier, the efficiency of cloning would be extremely low, although a wider sample of the antigenically primed clonal repertoire may be obtained (Mills et al 1986). Initial attempts at cloning used

auto IL-2 to stimulate clonal growth (Sredni et al 1980); this was superceded by a hamster anti-murine CD3 MAb (Leo et al 1987) with which clones were successfully expanded. The latter was a S/N taken from a spent culture of the 145-2C11 hybridoma. A suspension of irradiated virgin spleen cells (30 Gy, 4 $x10^6$/ml) was prepared in 10% FCS RPMI 1640 medium supplemented with either 20% IL-2 or 20% anti-CD3 (Wasik & Morimoto 1990) and pRBC lysate Ag (200 µg/ml). This APC preparation was then plated out at 100 µl/well in flat-bottomed 96 well microtitre plates (Nunc) using an eight channel 50-200 µl pipette (Titertek, Flow). The Th lines to be cloned were washed (300 g for 5 min) and counted. Cells were diluted in complete medium containing 20% dilutions of either auto IL-2 or anti-CD3 to cover the concentration range 500-1 cells/ml (50-0.1 cells/well). These dilutions were plated out at 100 µl/well into the previously prepared APC-containing microtitre plates, allowing two plates each for the lowest cell numbers. The cultures were incubated at 37 °C in a humidified CO_2 incubator (Flow) for 7 d, when 25 µl of either auto IL-2 or anti-CD3 (both at 100% S/N) was added to all wells. On d 10 and on subsequent days, plates were scored for wells containing growing cells. This was done by examination of the wells through a 25 x lens of an inverted phase contrast microscope (Leitz) with constant adjustment of focus. Cells forming a clone could be distinguished from clumps of background feeder cells by their larger, usually irregular shape and bright appearance under phase.

Although only the lowest dilution plates were used for expansion of clones, all plates were examined for the presence of clones to ensure that the greatest numbers were obtained at the highest cell concentrations, i.e. a Poisson distribution of growing clones for the number of cells seeded.

2.30 Expansion of helper T cell clones

A manageable number of clones (< 20) was chosen from the lowest dilution plates, i.e. those containing the lowest number of positive wells (< 10/96). Cell lines established from dilutions which gave rise to cell growth in < 30% of wells had > 83% probability of being clones (Henry et al 1980) and are herein referred to as such. As this step of the limiting dilution procedure was reached only using the modified protocol, anti-CD3 MAb was used throughout to stimulate clonal expansion.

APC (30 Gy; 2 $x10^6$/ml) were prepared in 10% FCS RPMI 1640 medium containing 20% anti-CD3 MAb (145-2C11 cell line S/N) and 200 µg/ml soluble lysate Ag. This

suspension was plated out at 1.5 ml/well in flat-bottomed 24 well plates (Linbro, Flow); growing clones considered to be of sufficient size were then transferred to prepared wells. It was found that if clones were transferred prematurely from 96 well to 24 well plates they failed to expand further. After 6 d incubation, 0.5 ml 20% anti-CD3 MAb was added to each well. A further 10-12 d later, each of the clones had grown sufficiently for the wells to be confluent. They were restimulated with APC/Ag (details as before) and transferred to 10 ml cultures (2 $x10^6$/ml) in upright 25 ml culture flasks. After sufficient proliferation, the flasks were tilted at an angle to the vertical to allow a larger surface area for cell growth. Finally, flasks were laid flat to enable the clones to establish completely in flasks.

The expansion and feeding of the Th clones was determined by their individual growth rates. When each clone was established as a flask culture, it was propagated following the modified maintenance protocol of the parent cell line. All new clones were tested as soon as possible for proliferation upon parasite stimulation (to verify Ag-specific responsiveness) and cell stocks established in liquid N_2.

2.31 Cryopreservation of Th cell lines and clones

A library of helper T cell lines and clones was maintained for storage of cells for an indefinite period in a frozen state under liquid N_2.

A cryoprotective solution was prepared by mixing 20% v/v DMSO (Sigma), 50% v/v FCS (Gibco) and 30% v/v incomplete RPMI 1640 medium (Hudson & Hay 1989), usually as a 10 or 20 ml final volume. This solution was kept on ice till use. Viable, actively growing cells were harvested from healthy cultures, pelleted by centrifugation at 350 g for 10 min and resupended in freezing medium. Cells were dispensed in 1 ml aliquots to 1.2 ml cryopreservation tubes (Nunc, Gibco)(labelled with the WEP code and a number) and frozen by controlled cooling (Theander et al 1986 a). A cooling rate of 1 ^oC/min was obtained by placing the ampoules in a freezing tray (Taylor-Wharton), this fitted on top of a liquid N_2 cannister, and incubating in the vapour phase above liquid N_2, either for 6-8 hr or overnight. Once frozen, the tubes were transferred to -196 ^oC for permanent storage.

Cells were recovered from liquid N_2 storage by thawing rapidly by incubating at 37 ^oC; a warming rate of 12000 ^oC/min could be achieved by removing the cryopreservation tubes from frozen storage and dropping them directly into a water bath at 37 ^oC (Mutetwa & James 1984 b). The contents of an ampoule was transferred to a plastic

Universal tube and the cell suspension diluted by adding 2 ml warm 10% FCS RPMI 1640 medium dropwise with shaking, and another 7 ml of warm complete medium slowly. Cells were washed twice (250 g for 5 min at RT) using the minimum of centrifugation and pipetting, resuspended in a minimal volume and counted as described previously (2.9; WBC counts). The suspension was adjusted to a suitable cell concentration immediately prior to culturing in vitro.

Cell lines and clones recovered from stabilate and intended for adoptive transfer were not inoculated directly from frozen but always cultured in vitro beforehand. This had the advantages of ensuring that cells were in their logarithmic growth phase at the time of grafting and enabling the collection of sufficient Ag-primed cells for adoptive transfer. It also eliminated the possibility of injecting trace quantities of the toxic DMSO into recipient animals.

2.32 Surface phenotyping of T cell lines and clones

In vitro-propagated T cell lines were periodically evaluated for the presence of cell membrane Ags by indirect immunofluorescence. Clones derived by limiting dilution from such lines were tested for homogeneity in expression of surface markers. Immunofluorescent staining was necessary to characterise at the cellular level the T cell subsets responsible for the cell-mediated immune responses functioning both in vivo and in vitro that are described in this thesis.

Cultures of T cell lines or clones were washed twice (300 g for 5 min) in chilled 10% FCS RPMI 1640 medium, cleansed of dead cells on Metrizamide and washed again. Cells were resuspended in complete medium to 1 ml, and their concentration adjusted to 2 $x10^7$/ml after total and viable cell counts were made. Naive spleen cells were used as a control for surface phenotyping; these were prepared as single cell suspensions as described previously.

As the IFAT can be adapted for use with live cells or fixed material, at this stage the cells requiring phenotyping were either kept on ice for immediate testing (modified from Brake et al 1986) or coated onto glass slides for slide IFAT (modified from McLean et al 1982 a). For the latter, thin smears of each lymphocyte suspension were aliquoted onto individual zones of teflon-coated multitest slides (Flow) and air-dried (2.18). These Ag slides could then be stored dessicated at -20 °C for testing at a later date.

(a) Live cells

Serial 1: 2 dilutions of normal rat serum (negative control) (prepared as for normal mouse serum, 2.10) and the primary MAbs to be tested were prepared in a 96 well tissue culture plate, starting at an initial dilution of 1:10 in PBS (pH 7.2). 50 µl of each MAb at the appropriate dilutions were mixed with 50 µl test suspension (2 $x10^7$ cells/ml) in a microcentrifuge tube and incubated for 1 hr in a 37 °C waterbath. In all cases, a 1: 200 dilution of FITC-conjugated goat anti-rat IgG (Sigma) was used as the secondary MAb. After washing twice in PBS (300 g for 3 min in an MSE microcentaur microcentrifuge), the cells were resuspended in a 100 µl volume of this solution and incubated for 30 min at 37 °C. Cells were washed twice as before to remove any unbound conjugate, resuspended to 0.5 ml in cold PBS and kept on ice until ready to view.

The % of positive cells was determined by fluorescent microscopy with the use of a Leitz incident light u.v. microscope equipped with a mercury lamp with an appropriate excitation filter for fluorescein. As the FITC-conjugated antiserum is unable to cross the intact cell membrane of viable lymphocytes, staining is confined to the external surface determinants. Positively stained cells, therefore, were visible as green rings and could be distinguished from dead cells showing bright homogeneous intracytoplasmic fluorescence. For each microscope field, the number of surface-fluorescent lymphocytes was counted and then the total number of viable lymphocytes enumerated under phase contrast viewing. For each specimen, ~ 200 cells were counted under visible light and the % of fluorescing lymphocytes calculated.

(b) Fixed cells

The methodology used for the slide IFAT was essentially similar to that detailed for anti-malarial Ab titre determinations, save using FITC-labelled goat anti-rat IgG as the second step reagent (without the addition of Evans blue counterstain for RBC).

Using either assay method, each cell sample was tested against a panel of primary MAbs, all of rat origin, specific for different surface Ags. Anti-mouse IgG_{2b} MAbs specific for Ly-4 (L3T4; helper T cells) (Dialynas et al 1983) and for Ly-2 (cytotoxic T cells) (Ledbetter & Herzenberg 1979) were either purchased from Sera-Lab in the first instance or purified from ascitic fluid (rat hybridomas a kind gift of Dr. Steve Cobbold, Dept. of Pathology, University of Cambridge), as outlined later. IgG_{2b} MAbs to lymphocyte markers Thy-1 (pan T cell) (Chayen & Parkhouse 1982), Ly-17 (anti-Fc

IgG_{2b} receptor) and Ly-40 (Mac-1; macrophages) were also used throughout (all from Sera-Lab). Normal rat serum was used in every assay as a negative control in place of each primary reagent to confirm the lack of non-specific fluorescence in the absence of specific MAbs. All reagents were diluted 1:10 in PBS upon receipt and stored in working aliquots (20-50 µl) at -20 °C ready for use.

2.33 Complement-mediated cytotoxicity assay

As a confirmatory test to phenotypically characterise the T cell lines and clones raised to P. c. chabaudi , the cells were assessed for expression of surface markers using the technique of complement-mediated cytolysis described by Rose et al (1976). MAbs directed against cell surface Ags were used to kill cells carrying these Ags with the aid of complement; cell death was assayed by exclusion of the dye trypan blue (2.17).

Rat anti-mouse MAbs specific for Ly-4 and Ly-2 determinants were purified from ascites secreted by rat hybridoma cell lines. These hybridomas were acquired from Dr. Steve Cobbold (Dept. of Pathology, University of Cambridge) and the MAbs attained from them were evaluated for protein content by measuring absorption at 595 nm. Normal rat serum was used as a negative control for cytotoxicity.

In vitro-propagated T cells were prepared for assay by washing twice (300 g for 5 min) in 10% FCS RPMI 1640 medium and dead cells separated out by Metrizamide gradient centrifugation. The viable cell fraction was washed once more and resuspended to 1 $x10^7$ cells/ml. Naive spleen cells, presumably containing approximately equal proportions of both major T cell subsets, were used as a control of specific MAb-induced cytolysis in the presence of complement. A spleen cell suspension was prepared, a T-enriched fraction collected over nylon wool and its concentration adjusted to 1 $x10^7$ cells/ml as for each sample on test.

Six serial dilutions of each Ab or antiserum sample were made in 1 ml volumes in PBS (pH 7.2) to cover the titration range 1:10-1: 5000 initial concentration. A 200 µl aliquot of every MAb dilution was added to 0.5 ml volumes of each T cell sample in a microcentrifuge tube and incubated at RT for 30 min. After incubation, each tube was spun (300 g for 5 min) in a microcentrifuge and the cell pellet resuspended in a minimal volume. 0.5 ml of a 1: 40 dilution in PBS of unabsorbed guinea-pig complement (Wellcome) was overlayed, then mixed and the suspension incubated at 37 °C for 45 min. Complement was used at the dilution recommended to cause efficient lysis when treating 10^7 spleen cells (Waldmann, H., personal communication). After

incubation, the microcentrifuge tubes were placed on ice to prevent further complement fixation and cell lysis. Once more, the cells were washed and resuspended to their original volume (0.5 ml) in PBS (pH 7.2) prior to cell counting. 0.1 ml of each cell suspension was mixed with 0.1 ml of 0.2% w/v trypan blue in PBS and incubated for 2-3 min at RT.

The number of viable lymphocytes (phase bright, unstained) was counted using a haemocytometer and a phase contrast microscope, as described in 2.17. The number of viable cells/ml could be calculated and from this the % lysis for each tube, according to the following equation (adapted from Hudson & Hay 1989):

$\% \text{ lysis} = C_N - C_A \times 100/C_O$,

where C_N = number of live cells in normal rat serum

C_A = number of live cells in MAb dilution

C_O = original number of live cells.

For each cell line or clone and each MAb used, a graph of % lysis against MAb dilution was plotted.

2.34 Helper T cell proliferation assay

This assay measured the ability of cultured Th cells to respond to specific antigenic stimulation and was a necessary inclusion in all in vitro studies to demonstrate that the cell lines and clones employed experimentally were primed to the asexual erythrocytic stages of P. c. chabaudi to which they were raised. In principal, the assay measured, at the microtitre level, the same cellular proliferation after restimulation with Ag and APC that accounted for cell growth after freshly feeding flask cultures. Apart from confirming the Ag-specific proliferation of Th lines and clones, this assay was a fundamental prerequisite to longterm bulk culturing of T cells in vitro since it was necessary to determine at the outset the optimum concentration of pRBC lysate required to induce maximal cellular proliferation in vitro. Once this variable was evaluated, the stock of lysate Ag could be diluted routinely to that final concentration when feeding cell suspensions.

When testing for cellular proliferation by incorporation of tritiated thymidine, it was necessary to deprive of Ag the lines or clones for 6-8 d before assay. This precaution avoided high background counts which would occur in the presence of residual stimulatory pRBC lysate.

For the assay, APC were prepared (2.24) at 4 $x10^6$/ml in 10% FCS RPMI 1640

medium. The Th cells to be tested were washed twice in complete medium (300 g for 5 min) to remove residual Ag or IL-2, resuspended in the APC suspension at 2 $x10^5$/ml, and the complete preparation then plated out at 100 µl/well in 96 well flat-bottomed microtitre plates (Nunc).

Using standard stocks of pRBC and nRBC lysates for which the total protein concentration had been determined previously, a range of Ag dilutions was prepared to cover the range of concentrations 5-400 µg/ml RBC lysate. This was performed in a microtitre plate using 10% FCS RPMI 1640 medium as the diluent. For each concentration, 100 µl of diluted Ag was added to four successive test wells containing T cells and APC (i.e. the assay was performed in quadruplicate for each sample of Th cells at every dilution of lysate).

The 96 well plates were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 72 hr, at which time 1.0 µCi (37 kBq) of [^{3}H-methyl] thymidine (20 µCi/ml, specific activity 5 Ci/mmol; Amersham International) was added to each well. Addition of tritiated thymidine provided a freely available alternative nucleotide which could be incorporated into the DNA synthesised. To add the radiolabelled nucleotide, 20 µCi/ml [^{3}H] thymidine stock was diluted 1: 20 in complete medium and 20 µl aliquoted to each well using a P20 'pipetman' pipette (Gilson). 18 hr later, the wells were harvested with a semi-automatic cell harvester (Titertek, Flow) onto glass fibre filter paper (FG/A, Whatman), washed twice with distilled water and dried. Each filter disc was transferred to a plastic beta vial (LKB) and 2 ml non-aqueous scintillation fluid (Optiscint 'safe', LKB) added using an automated dispenser (Jencons (Scientific) Ltd.). All the insert tubes were lidded, labelled and placed inside outer plastic scintillation vials ready for counting. The beta activity in each sample was detected using a liquid scintillation counter (LKB Wallac 1219 Rackbeta) (1 or 5 min count time) and quantified on a programmed computer (Olivetti DM282 100).

For individual wells, total counts and c.p.m. were measured, from which the arithmetic mean c.p.m. for quadruplicate wells could be calculated. From the data collected from all dilutions for a given assay, a dose-response curve of tritium incorporation against Ag concentration could be plotted; this showed the proliferative response of the cells tested to specific antigenic stimulus titred out at different concentrations, from which the optimum dilution of pRBC lysate stock could be determined.

In all cases, control wells containing responder cells alone, T cells and APC, plus T cells and Ag were set up to enable enumeration of background responses. Also, in certain

instances, control cultures with APC, Ag or medium alone were added to give even lower background responses. The inclusion of negative control wells was necessary for statistical analysis of the proliferation measured; cells were considered to have given a positive cellular response if the counts for their wells were ≥ 2 S.D. more than the c.p.m. values for appropriate negative controls.

2.35 MHC restriction

To examine whether or not presentation of plasmodial Ags to Th lines and clones in vitro in the P. c. chabaudi/NIH system is an MHC-restricted process, similar proliferation assays were performed as that detailed in 2.34 but using not only APC of the same haplotype (H-2^q) as the responder cells (NIH), but allogeneic APC covering a range of varying haplotypes. If Ag presentation is a class II MHC-restricted phenomenon, it would be expected that only APC from syngeneic mouse strains (NIH, B10. G, B10. T(6R)) would be able to process and present pRBC lysate to the parasite-primed Th cells in such a way as to induce their proliferation in vitro. However, if all APC, irrespective of the mouse strains used, potentiated responder cell stimulation, lymphocyte proliferation would be considered outwith genetic control.

The methodology followed was identical to that described for the standard proliferation assay, i.e. measurement of [^{3}H] thymidine uptake (c.p.m.) over an 18 hr pulse period.

2.36 Collection of T cell culture S/N for assaying lymphokine secretion

Cells were harvested from maintenance culture between 6-8 d after the last feed with malarial Ag and stimulated for lymphokine production. The culture preparation was essentially similar to that for Th proliferation assays.

Cells were washed twice (300 g for 5 min) in 10% FCS RPMI 1640 medium, non-viable cells removed on Metrizamide and the remainder resuspended in a previously prepared suspension of APC (4 x10^6/ml) in complete medium at 4 x10^5/ml. The suspension was dispensed into individual wells of 96 well microtitre plates at 100 µl/well. Thereafter, quadruplicate cultures were overlayed with different dilutions (5-400 µg/ml) of pRBC lysate Ag (100 µl/well) and the plates incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 hr. After the incubation period, the culture-conditioned S/N were harvested by aspiration using a Gilson P200 'pipetman' pipette (with a fresh sterile tip for each well) and clarified by centrifugation at 350 g for 5 min. If the S/N were not to be tested immediately, they were stored at -20 °C

until used; under these conditions, the lymphokine activity of cell culture S/N remains stable for extended periods of time (Araneo et al 1989).

The same S/N samples were either used in all three lymphokine bioassays employed or samples from identical cultures compared, so reducing batch variation of lymphokine activities. In all instances, spleen cell culture controls were set up and S/N taken from these for assay; naive and post-infection spleen cells, the latter taken by splenectomising a mouse recently recovered from a primary infection. S/N derived from culturing APC alone and medium containing pRBC lysate were used as background controls for all lymphokine assays.

2.37 Bioassays for IL-2 and IL-4

IL-2 and IL-4 have convergent effects in the immune system, both maintaining the proliferation of activated T cells, although the molecules are structurally different and presumably act through separate receptors. This enables the assaying of both cytokines by measuring the increase in proliferation of the same dependent cell line (Gillis et al 1978). The line used was the C57BL/6 mouse T cell lymphoblast CTLL (Gillis & Smith 1977). Prior to the discovery of IL-4, the CTLL-2 cell line was reported to be solely an IL-2-dependent line; however, it is now known to respond to both lymphokines, although to a much lesser extent to IL-4 (this particular clone gives a maximal response to saturating concentrations of IL-4 approximately 1/10 -1/20 of that to saturating IL-2) (Cushley, W., personal communication; Kelso & Gough 1988). Monospecificity for each lymphokine was achieved by incubation of the target cells with or without an anti-IL-4 MAb (Ohara & Paul 1985).

(a) Maintenance of the CTLL-2 cell line

The CTLL-2 cell line was obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire (originally deposited by Prof. D. Kilburn, Dept. of Microbiology, University of Vancouver).

Upon receipt, the frozen cell suspension (2 $x10^6$ cells) was thawed rapidly at 37 ^oC, washed in 10% FCS RPMI 1640 medium (250 g for 5 min) and the cells resuspended in fresh complete medium (pregassed and prewarmed) containing 10 i.u./ml murine recombinant IL-2 (Genzyme) (Appendix E) to a 10 ml volume (cell density $\sim$ 2 $x10^5$/ml). The cells were incubated in an upright 25 ml tissue culture flask overnight

at 37 °C in a humidified CO_2 incubator. After overnight culture at a relatively high starting concentration (to allow rapid conditioning of the medium), the cells had reached saturation density, when they were subcultured at 2 $x10^4$ cells/ml in 10% FCS RPMI 1640 medium supplemented with Con A S/N as a laboratory-prepared source of IL-2. Thereafter, the cell line was subcultured every 2-3 d by splitting the cultures to 5 $x10^3$ cells/ml in fresh medium. As new cultures were seeded, stocks of CTLL-2 cells were frozen down (protocol as for 2.31).

(b) Maintenance of the 11B11 cell line

The anti-IL-4 MAb-secreting 11B11 lymphocyte line was a kind gift of Dr. William Paul (National Institutes of Health, Bethesda, Maryland). This was supplied as a growing 10 ml culture in a 25 ml tissue culture flask; upon arrival, this was washed (250 g for 5 min) and the cells resuspended to 3 $x10^5$/ml in 10% FCS RPMI 1640 medium prior to incubation at 37 °C in a humidified atmosphere containing 5% CO_2. Suspensions were subcultured every 4-5 d when the line had reached saturation density, at which time conditioned 11B11 culture S/N was drawn off and this used as a source of the anti-IL-4 MAb (11B11 S/N). The exhausted culture S/N was 0.22 µm filter-sterilised and stored as 1 ml aliquots at -20 °C. Cultures from which the S/N had been decanted were either subcultured at 3 $x10^5$/ml in fresh medium or frozen as stabilate in liquid N_2, using DMSO as a cryoprotectant (2.31).

(c) Assays for IL-2 and IL-4

CTLL-2 cells were harvested 3 d after feeding with IL-2. The cells were washed twice by centrifugation (250 g for 5 min) in 10% FCS RPMI 1640 medium prior to the assay to remove any remaining IL-2.

Nine serial two-fold dilutions (1:1-1: 256 v/v) of each assay sample were prepared in complete medium. In addition, similar dilutions of a laboratory standard preparation of murine recombinant IL-2 (Genzyme) were titrated out. This acted as a positive control for IL-2-dependent cellular proliferation, whilst the negative control used was culture medium alone. 100 µl aliquots were distributed in quadruplicate wells for each titration into individual wells of a flat-bottomed 96 well microtitre plate (Nunclon, Nunc).

100 µl washed CTLL-2 cells were added to each well at 2 $x10^4$ viable cells/ml in 10% FCS RPMI 1640 medium (containing 11B11 S/N if necessary; see below), and the

plates incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 hr. After this time, 20 µl of [^{3}H-methyl] thymidine (Amersham International) containing 1.0 µCi in complete medium was added to each well and the plates reincubated for a further 20 hr at 37 °C in a CO_2 incubator. The contents of each well was harvested onto glass fibre filters using a semi-automated cell harvester (Titertek, Flow) and the radioactivity incorporated into DNA determined by liquid scintillation spectroscopy (details as for 2.34).

Using the recombinant IL-2 control, a standard curve of proliferation could be generated (radioactive incorporation plotted against IL-2 dilution). Lymphocyte culture S/N containing IL-2 or IL-4 gave similar patterns, though different levels, of dose-related stimulation of thymidine incorporation.

To achieve monospecificity for the two different lymphokines assayed, paired assays were run simultaneously, either incubated in the absence or in the presence of the anti-IL-4 MAb. For the latter, 11B11 culture S/N was added to microtitre cultures at a final concentration of 1: 20 v/v (optimal concentration to block IL-4 activity; Cushley, W., personal communication). To do this, a 1:10 dilution in 10% FCS RPMI 1640 medium was made and this used to resuspend washed CTLL-2 cells; when the cell suspension was added to the distributed titrations of sample S/N to be measured, a final concentration of 1: 20 v/v 11B11 S/N was realised. The addition of the anti-IL-4 MAb abbrogated completely all IL-4 activity in vitro. Thus, any target cell proliferation when incubated with this MAb could be attributed to the presence of IL-2 in solution; this gave effectively an IL-2-specific bioassay. As the comparable anti-IL-2 MAb (Mosmann et al 1986) was not available, a correspondingly monospecific IL-4 assay could not be attained. However, the presence of this lymphokine in culture S/N could be determined by direct comparison of dose-response curves for cells cultured in the presence or absence of the anti-IL-4 MAb. Any proliferation in the absence of the MAb which was lost when the Mab was added, was an IL-4-dependent response by the CTLL-2 cell line. Although this method was not as unequivocal as using an anti-IL-2 MAb, it proved a valid assay, partly because all Th cell lines and clones gave unambiguous lymphokine profiles (see text).

2.38 Quantification of gamma interferon by anti-viral assay

To determine an accurate titre for IFN-γ in Th cell lines and clones, its long-established anti-viral properties (DeSomer et al 1962, Lockart et al 1962) were used as the basis

of an assay to measure the effect of multiple serial dilutions of S/N samples on viral replication. Specifically, IFN-γ was assayed by inhibition of Semliki Forest virus-directed RNA synthesis in susceptible murine adipose tissue L-929 fibroblasts (Earle et al 1943), using a modification (Warren & Sanderson 1985) of the technique of Allen & Giron (1970). Semliki Forest virus lends itself to ready quantification of intracellular viral RNA production, achieved by measuring incorporation of [^{3}H] uridine into viral RNA (distinguished from any host RNA synthesis by its resistance to inhibition by actinomycin D). IFN-γ titrations were performed in the Dept. of Biochemistry, University of Glasgow, under the supervision of Dr. Bill Cushley, or by Dr. Peter Wood, Dept. of Immunology, University of Manchester.

(a) Growth of the L-929 fibroblast cell line

The target L-929 cell line was obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire as a 10 ml culture in a 25 ml tissue culture flask. Upon receipt, it was incubated overnight at 37 °C in a CO_2 incubator. The confluent cells were then subcultured by splitting the suspension between three flasks (cell density ~ 3 x10^5/ml) using 10% FCS RPMI 1640 as the growth medium. Further subculturing took place every 3-4 d and frozen stocks of L-929 fibroblasts established. In preparation for biochemically assaying the challenge virus, a stock of L-929 cells were grown up in complete medium as described.

(b) Preparation of Semliki Forest virus

Semliki Forest virus belongs to the rhabdovirus class and is a lipid-enveloped, negative strand RNA virus. It infects a wide range of animal cells and is easily grown; humans are, however, not generally susceptible to infection.

Virus stocks were prepared in L-929 fibroblast cells using MEM with Earle's salts (Gibco) (Appendix B) supplemented with penicillin (100 i.u./ml), streptomycin (50 μg/ml) and 10% FCS.

Fibroblast cells were seeded in 10 cm Petri dishes (Sterilin) and grown to confluency. A dilution of Semliki Forest virus in sterile PBS (pH 7.2) at 3 x10^6 p.f.u./ml (m.o.i. = 0.1 p.f.u./cell) was prepared. After removing culture medium from the cell monolayers, they were rinsed once with prewarmed PBS and then 1.0 ml of the diluted virus added per dish (it was important to use a low input multiplicity to avoid the

generation of defective, interfering virus particles). The virus was adsorbed at 37 °C by incubating for 1 hr in a humidified atmosphere containing 5% CO_2, after which the inoculum was removed by aspiration and the cultures rinsed gently once with warm PBS. Each fibroblast monolayer was overlayed with 5 ml 2% FCS RPMI 1640 medium and the cultures incubated for 24 hr at 37 °C in a humidified CO_2 incubator, at which time the target cells were observed to show a strong cytopathic effect.

The culture dishes were successively frozen and thawed by placing in a -70 °C freezer and then in a 37 °C dry incubator, after which the freeze-thaw cycle was repeated. The culture fluid was collected aseptically in a sterile 50 ml centrifuge tube and spun at 2000 g for 10 min at 4 °C. The virus-containing S/N was collected, aliquoted in 1 ml volumes in 1.2 ml cryopreservation tubes and these stored at -70 °C. Each preparation was titred routinely by plaque assay in order to determine the concentration of infectious virus particles in the stock solution.

(c) Plaque assay of challenge virus yield

For each stock of Semliki Forest virus to be titred, twelve confluent cultures of L-929 indicator cells were prepared in 6 cm Petri dishes (Sterilin). The sample to be tested was recovered from -70 °C by thawing rapidly in a 37 °C waterbath and then kept on ice until used. A series of five sterile plastic bijoux (Sterilin) were labelled -2, -4, -6, -7 and -8. 1.0 ml of sterile PBS (pH 7.2) was added to the first three containers and 0.9 ml to the others. The virus sample was mixed thoroughly (but avoiding vigorous vortexing) and 10 µl transferred to the -2 tube (giving a 100-fold dilution) and the solution mixed gently by vortexing. Further 1: 100 dilutions were made by successively transferring 10 µl from the -2 to the -4 dilution, and then again from the -4 to the -6 dilution. The next two dilutions were 10-fold steps, so 100 µl aliquots were transferred from the -6 to the -7 tube, and then from the -7 to the -8. All dilutions were performed with either Gilson P20 or P100 'pipetman' pipettes, using a new pipette tip (Finntip Labsystems) at each stage, ensuring complete mixing between transfers.

The maintenance medium was aspirated from the cultures to be used and 0.25 ml of the three highest dilutions (-8, -7 and -6) added to three dishes each. In addition, 0.25 ml PBS was dispensed to a further set of dishes as a control. All cultures were incubated at 37 °C for 1 hr, during which time the indicator overlay was prepared.

50 ml of molten 1.0% agarose was equilibrated at 45 °C. This was prepared at the time

of use by autoclaving 1 g of agarose (Oxoid) in 50 ml distilled water. 2 x MEM concentrate was made up by diluting the 10 x medium concentrate (Gibco) 1: 5 in distilled water, and this then equilibrated at 37 °C. Immediately before adding to the plates, equal volumes of the 2 x MEM and 1% agarose were combined and FCS added to a 5% final concentration. The virus inocula were removed from the culture dishes and 7 ml of agarose overlayed quickly to avoid gelling of the agarose stock. The plates were left at RT for 10 min to allow the agarose to solidify, when they were placed inverted in a CO_2 incubator for 2 d for Semliki Forest virus plaques to develop.

A stock solution of 3% neutral red (Gurr, BDH) was diluted 1: 10 in PBS and each plate stained by the addition of 2 ml of 0.03% dye solution on top of the agarose and incubating at 37 °C in a CO_2-enriched atmosphere for 2 hr. Plaques were then visible as clear areas in the red-stained viable L-929 fibroblast monolayer. The number of plaques on dishes where individual plaques could clearly be distinguished was counted (usually -8 and -7 plates only). Enumeration was achieved by holding each dish to an anglepoise lamp and spotting each plaque with a marker pen as it was counted. The arithmetical mean number of plaques (N) for each quantifiable dilution was calculated. The virus titre was given by:

Titre = N x dilution factor x 4.

From this, the number of p.f.u./ml could be determined and the concentration of virus stock adjusted accordingly prior to assaying IFN-γ levels.

(d) Assay

L-929 indicator cells were seeded at 3 $x10^5$ cells in 1.0 ml aliquots in plastic scintillation vial inserts (LKB), allowing four tubes for each dilution of culture S/N tested. As no murine IFN-γ reference standard is currently available, a set of cells was also seeded for the murine IFN international standard G-002-904-511 (Research Resources Branch, NIAID, NIH, Bethesda, MD). The cultures were grown to confluency by incubating overnight at 37 °C in a humidified atmosphere gassed with 5% CO_2. The tissue culture S/N was removed from each vial by aspiration using a Pasteur pipette attached to a vacuum line, and replaced with 200 µl aliquots of serial dilutions of the samples under test. The cultures were reincubated for 18 hr under the same conditions as used previously.

To all test vials, 200 µl of infectious challenge Semliki Forest virus was added at 1 $x10^7$ p.f.u./ml in complete RPMI 1640 medium supplemented with 2% FCS and 3 µg/ml

actinomycin D (Calbiochem) (purchased as sterile vials containing 200 µg of solid and reconstituted just before use in 0.8 ml PBS to provide a working stock of 250 µg/ml). The viral inoculum gave a relatively high m.o.i. of approximately 25-50 p.f.u./cell, empirically determined to give high levels of [^{3}H] uridine incorporation. To control vials, 200 µl of actinomycin D-containing complete medium alone (lacking virus) was added, then all cultures incubated for 3 hr at 37 °C in a humidified CO_2 incubator.

Without removing the virus inoculum, 100 µl 2% FCS RPMI 1640 medium containing 1 µg/ml actinomycin D and 5 µCi/ml (1.85 x10^5 Bq/ml) [^{3}H] uridine (20 Ci/mmol, Amersham International) was added to all the tubes and each incubated for a further 3 hr. For each vial, the radioactive medium was aspirated before washing the cell monolayer twice with 1 ml 5% ice-cold TCA (Sigma) and once with 1 ml ethanol (May & Baker Ltd.). The residual ethanol was aspirated and the vials dried for 15 min in a 60 °C dry incubator. Solubilisation of TCA-precipitated cell monolayers was effected by the addition to each insert tube of 500 µl soluene (BDH Ltd.) diluted 1: 2 in toluene (Sigma), after which 2 ml non-aqueous scintillation fluid (Optiscint 'safe', LKB) was dispensed directly to each vial. Incorporation of beta radioactivity was measured by conventional liquid scintillation counting methods.

Titration of IFN-γ was assessed by measuring the decrease in viral RNA synthesis. The mean c.p.m. for four replicate cultures was plotted against $\log_{10}$ IFN-γ dilution. The inhibition of virus proliferation, measured as inhibition of [^{3}H] uridine uptake, was proportional to IFN-γ concentration. The IFN-γ titre was the reciprocal dilution of T cell culture S/N corresponding to a 50% reduction in tritium incorporation (the midpoint value between virus and cell controls) (Meager 1987).

2.39 Helper T cell activity

The ability of T cell lines and clones to induce splenic B cells to make a specific anti-*P. c. chabaudi* Ab response *in vitro* was assayed. A modification of the method of Pearson *et al* (1983) (Phillips, R.S., personal communication) was followed. The malaria-primed Th cells were co-cultured with B cells of varying immunocompetence to examine the ability of the former to induce a humoral immune response to the erythrocytic stages of *P. c. chabaudi* , represented *in vitro* by pRBC lysate. Culture S/N were assayed for anti-malarial Ab using the IFAT (McLean *et al* 1982 a).

For assaying helper T cell function, T cells were freshly stimulated with Ag; to do this,

lysate-exhausted cultures were used (8 d after last subculture). Cells were harvested and washed twice (300 g for 5 min) in 10% FCS RPMI 1640 medium, and dead cells removed on Metrizamide. Viable T cells were resuspended in a suspension of APC (4 $x10^6$/ml) (preparation described in 2.24) in complete medium at 4 $x10^5$/ml. Spleen cell suspensions were prepared using sterile technique and enriched for B cells by nylon wool filtration. Naive or immunologically experienced B cells were taken from uninfected or primary infection-recovered mice, respectively. The prepared B cells were added to the T cell/ APC suspension to give a concentration equivalent to that of the T cells, i.e. 4 $x10^5$/ml. Antigenic stimulation was provided by lysates of either pRBC or nRBC, each at 200 µg/ml (the previously determined optimal concentration for in vitro culture). 10 ml volumes were aliquoted to 25 ml tissue culture flasks and the cultures incubated in a horizontal position in a humidified CO_2 incubator at 37 °C for 9 d. Cultures were also set up using naive and post-infective T cells in place of in vitro-maintained Th cells as controls for in vitro Ab secretion. In practice, the T cells used were obtained as the non-adherent cell filtrate from the same nylon wool separations that gave the enriched B cell suspensions.

Cultures were harvested into plastic Universal containers, centrifuged (300 g for 5 min) to pellet the cellular fraction and the S/N collected by aspiration. As preliminary investigation showed that low reciprocal Ab titres were attained with neat S/N, each sample was concentrated three times by ultrafiltration using a Centriprep-10 concentrator (Centricon, Amicon) with a 10000 MW cut-off (Blatt et al 1968). A 5 ml volume of each S/N was poured into the Centricon sample container and the concentrator centrifuged at 1000 g for 30 min at 25 °C, when the filtrate was discarded. A further 4 ml of the same S/N was added to the original 5 ml (now less) and this new volume spun as before, after which the filtrate was decanted. Further concentration was achieved, if necessary, by centrifuging again, until the retentate volume was reduced to 3 ml. Each ultrafiltrated S/N was recovered and either assayed immediately or stored frozen (-20 °C).

The immunofluorescence method used to assay all culture S/N for in vitro Ab production was identical to that described in 2.18, using S/N in place of serum for testing.

2.40 Production of MAbs for T cell subset depletion in vitro and in vivo

Rat IgG_{2b} MAbs to mouse T lymphocyte subsets were prepared by propagation of Ab-

secreting hybridoma cells as ascites tumours in (DA x LOU) F_1 rats. The hybridomas were obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire (originally deposited by Prof. H. Waldmann and Dr. S.P. Cobbold, Dept. of Pathology, University of Cambridge). The two MAbs used recognise in all strains of mice tested the two monomorphic cell surface glycoproteins Ly-2 and Ly-4, the differential expression of which separates all T cells into two subsets. YTS 169.4 binds to mouse suppressor/ cytotoxic T cells ($Ly\text{-}2^+$ = $CD8^+$ equivalent) and their precursors in vitro , but not to helper T cells. Conversely, YTS 191.1 is specific for Ly-4-bearing mouse helper/ inducer and delayed hypersensitivity T cells ($CD4^+$ equivalent), but not for cytotoxic T cells or their precursors in vitro (Aqel et al 1984).

The MAbs were selected from fusions by the protocol of Waldmann & Milstein (1982) between the rat myeloma Y3.Agl.2.3 (Galfre et al 1979) and DA rat spleen cells immunised to CBA bone marrow or thymocytes (Cobbold 1983). Recloned hybrid myeloma lines were maintained in Iscove's modified Dulbecco's medium (Gibco) (Appendix B) supplemented with 1% FCS.

MAb production in high concentration was conveniently achieved by growing the Ab-secreting hybridoma cells in vivo as ascites tumours in (DA x LOU) F_1 rats primed with mineral oil (Potter et al 1972). The agent used for priming the peritoneal cavity of rats prior to inoculation of hybridoma cells was a component of mineral oil, 2, 6, 10, 14-tetramethylpentadecane or pristane (Hoogenraad et al 1983, Brodeur et al 1984). The ascitic fluids were partially purified by precipitation with 40-45% ammonium sulphate. The precipitates were redissolved in PBS, concentrated by dialysis and sterilised by filtration. Before use in vivo , the specificity and cytotoxic potential of the MAbs were ascertained by immunofluorescence (Cobbold et al 1986) and complement-mediated cytolysis (Bruce et al 1981), respectively.

(a) In vitro culture of hybridoma cell lines

The hybridomas were received as two frozen ampoules packed in dry ice. On arrival, the contents of each cryopreservation tube was thawed rapidly in a 37 °C water bath, decanted into a 20 ml plastic Universal tube and the cells washed in excess PBS (250 g for 5 min). The pellet was resuspended in 8 ml prewarmed and gassed RPMI 1640 medium containing 10% FCS at 2 $x10^5$ cells/ml in a 25 ml culture flask and incubated overnight at 37 °C in an atmosphere of 5% CO_2 in air. When they had reached

confluency, cells were subcultured by splitting the flasks into two 8 ml volumes for reincubation. Thereafter, the hybridomas were subcultured every 2-3 d in 20 ml volumes (75 ml flasks) at 5 $x10^5$ cells/ml. Stocks of the two cell lines were cryopreserved in liquid N_2 for longterm storage. Rather than discarding culture-conditioned medium S/N at every subculture, these were kept frozen as an additional source of MAbs to be used if the tumours failed to take in recipient rats. In the event, ascitic fluid was recovered from all rats inoculated and was used for all T cell depletions.

(b) Ascites production

Adult male DA and female LOU rats purchased from Harlan Olac Ltd. were mated in the WLEP animal house. Twelve female (DA x LOU) F_1 hybrids were used at 12 weeks of age. Rats were primed with an i.p. injection of 1 ml pristane (Aldrich Chemical Co.) 7 d prior to the inoculation of 3.20 $x10^6$ anti-Ly-4 or 4.18 $x10^6$ anti-Ly-2 hybridoma cells in 0.4 ml incomplete RPMI 1640 medium. Animals were monitored daily for abdominal swelling. Ascitic fluid was collected by inserting a 21 G needle into the peritoneal cavity and allowing the ascites to drain into 20 ml sterile Universal vessels containing 0.5 ml of 5% EDTA in PBS (pH 7.2) as anticoagulant. Animals were tapped subjectively based on the degree of swelling over a period from 12-19 d after hybridoma implantation. On each day, the ascites collected were centrifuged at 500 g for 10 min, the lime green-coloured S/N harvested and stored at -20 °C.

(c) Purification of ascitic fluid

The IgG fraction of the collected ascitic fluid was isolated by salting out with a 40-45% w/v saturated solution of ammonium sulphate. 20 ml aliquots of ascitic fluid were thawed in a 25 °C water bath, 5.4 g ammonium sulphate (BDH Ltd.) added to each to make a 45% w/v solution. After stirring thoroughly to dissolve, each ascites sample was incubated for 30 min at 25 °C. The large light green precipitate was pelleted by centrifugation at 1000 g for 30 min at 25 °C. The S/N was discarded and the protein precipitate redissolved in distilled water up to 10 ml. The IgG was reprecipitated by addition of 1.35 g ammonium sulphate to make an overall 40% w/v solution. Once dissolved, each solution was incubated for 30 min at 25 °C. The white precipitate was collected by centrifugation (1000 g for 30 min at 25 °C), pooled and redissolved in distilled water up to 80 ml.

To remove final traces of the precipitating agent, ammonium sulphate, the redissolved

precipitate was dialysed against PBS (pH 7.2). This procedure also acted to concentrate the IgG present in solution. Cellulose acetate dialysis tubing (nominal MW cut-off 12000, flat width 35 mm) (Sigma) was cut into 35 cm lengths and presoaked in PBS buffer. For each tubing, one end was sealed with a Pierce dialysis clip and 20 ml of either MAb solution to be dialysed poured in using a small glass funnel. The bag was not overfilled since the volume was expected to approximately double due to the high osmolarity of the salted out S/N. After sealing the top end, each bag was placed in a 5 l glass beaker filled with PBS and agitated gently with a magnetic bar and stirrer motor. The dialysis bags were left overnight at 4 °C (in a refrigeration cabinet) to reach equilibrium. After dialysis was complete, the opalescent, partially purified IgG anti-Ly-2 and anti-Ly-4 MAb samples were each pooled to give 150 ml and 145 ml volumes, respectively, which were then filter-sterilised (0.22 μm, Millipore). The IgG concentration, determined by a modification of the Bradford dye-binding assay, was shown to be 6.4 mg/ml and 10.8 mg/ml for the anti-Ly-2 and anti-Ly-4 monoclonals, respectively. The MAb preparations were stored as either 20 ml or 5 ml aliquots at -20 °C, avoiding denaturation through repeated freeze-thawing.

2.41 *In vitro* depletion of T cell subsets

Protocols enabling the typing to the Ly-4$^+$ mouse T cell subset of helper T cell lines and clones raised *in vitro* have been described already; these were surface indirect immunofluorescence and complement-mediated cytotoxicity, both of which employed the anti-Ly-4 and anti-Ly-2 MAbs prepared from hybridoma ascites. Each of the T cell lines was characterised as being a relatively homogeneous population of Ly-4$^+$ cells (see text); however, there was a residual fraction of Ly-2$^+$ and/or non-staining (Ly-4$^-$ Ly-2$^-$) cells. To ensure that Ag-specific proliferation was not due to overgrowth of these cells, either *in vitro* or upon adoptive transfer, and that the cell-mediated immune activity did not reside within this minority of cells, two similar assays were performed. One was a proliferation assay with the cells cultured in the presence of specific Ab and complement (Cobbold, S.P. & Phillips, R.S., personal communication); the response of any cells surviving treatment was monitored by tritium incorporation in the usual way. This assay was necessary to confirm at a functional level, i.e. cellular proliferation, effective T cell subset depletion *in vitro* , and represented an extension of the cytolysis assay. Both differential and total T cell depletion had to be shown *in vitro* prior to direct *in vivo* administration of the prepared MAbs. Similar culture conditions were utilised

for the other assay, whereby the T cell lines were depleted, in part or completely, by brief incubation with either or both MAbs, respectively, together with complement. After treatment, the cell fractions were inoculated into parasite-challenged recipient animals and the course of infection followed. By this means, it was possible to determine which T cell subset(s) conferred protection and whether any immunity was transferred by the Ly-4$^-$ Ly-2$^-$ population of non-staining T cells.

(a) <u>In vitro</u> T cell subset depletion for proliferation assay

In preparation for assay, flask cultures of T cell lines were washed twice (300 g for 5 min) in 10% FCS RPMI 1640 medium and dead cells removed on Metrizamide. After washing the viable cell fraction, it was resuspended to 4 x10^5 cells/ml. A naive splenic T cell control was also prepared at a similar concentration, and both suspensions plated out at 100 µl/well in 96 well flat-bottomed microtitre plates. 100 µl aliquots of a suspension of APC (4 x10^6/ml) in complete medium supplemented with 200 µg/ml pRBC lysate Ag (previously determined optimal concentration) were added to each well to give a 200 µl volume. The plates were cultured in a humidified incubator gassed with 5% CO_2 at 37 °C for 48 hr.

Five serial dilutions of each MAb were made in 1 ml volumes in PBS (pH 7.2) containing a 1: 40 dilution (i.e. 25 µl) of unabsorbed guinea-pig complement, to cover the titration range 1: 50 -1: 1000 initial concentration (differential T cell subset depletion). In addition, a dilution series containing both MAbs was prepared for near total T cell depletion. A 100 µl aliquot of every MAb dilution was added to quadruplicate test wells, leaving control wells without MAb. Cells in each well were resuspended and reincubated for 45 min at 37 °C. After the depletion procedure, the 300 µl culture medium overlaying the cells in each well (now settled to the bottom) was removed by gentle aspiration using a Pasteur pipette attached to a vacuum line. Trace quantities of MAb and/or complement were removed from the culture wells by washing the cells with PBS; this was then aspirated and replaced with fresh 10% FCS RPMI 1640 medium. After reculturing the plates for 24 hr, 1.0 µCi [^{3}H-methyl] thymidine was added to each well as a 20 µl aliquot and the cultures incubated for a further 18 hr, at which time they were harvested and subjected to liquid scintillation counting (2.34, Helper T cell proliferation assay).

(b) In vitro subset depletion for adoptive transfer

T cell lines were either raised from stabilate or subcultured from existing in vitro cultures at 4 x10^5 cells/ml in a suspension of APC (4 x10^6/ml). Antigenic stimulation was provided by the addition of pRBC lysate at 200 µg/ml. 40 ml volumes were dispensed into 75 ml tissue culture flasks and these incubated for 72 hr at 37 °C in a humidified atmosphere of 5% CO_2 in air. After this time, the cells were washed twice (300 g for 5 min) in 10% FCS RPMI 1640 medium and resuspended to 40 ml in the same medium containing the appropriate MAb at a dilution of 1: 50 (i.e. 0.8 ml) (Harte et al 1985 a) with guinea-pig complement (Wellcome) diluted to 1: 40 (i.e. 1 ml). After incubating for 45 min at 37 °C in a humidified CO_2 incubator, flasks were washed twice in incomplete RPMI 1640 medium and resuspended to 2 ml. 1 ml of each sample (either depleted of Ly-4^+ or Ly-2^+ T cells, or both subsets) was kept aside for i.v. injection of complete cell suspensions (containing principally dead cells) into recipient mice; this transfer was desirable to show that only viable, and not dead, Ly-4^+ T cells could confer immunity against a challenge P. c. chabaudi infection in vivo. The remaining 1 ml volumes were separated into live and dead cells by Metrizamide gradient centrifugation and only the former viable fraction collected for i.v. inoculation.

Each mouse receiving a T cell-depleted preparation was injected with a number of cells that corresponded to the proportion of original total spleen cells which could not be accounted for as Ly-4^+, Ly-2^+ or both, depending on the sample (as determined previously by surface immunofluorescence, 2.32; and complement-mediated cytotoxicity, 2.33) (modified from Araya et al 1989). In the event, it transpired that the in vitro depletion procedure was so effective that the proportion of cells surviving each depletion treatment was close to that predicted by phenotypic characterisation for each MAb incubated with each cell line.

As for all protection tests in vivo , tail blood smears were prepared daily from mice, fixed with methanol and stained with Giemsa's stain. Parasitaemias were determined by microscopic examination of slides till clearance of infection (2.7).

2.42 In vivo depletion of T cell subsets

Rat T lymphocyte hybridomas were a kind gift of Dr. Steve Cobbold, Dept. of Pathology, University of Cambridge. The monoclonal ascitic fluids drained from tumour-bearing rats inoculated with these cell lines were purified to give preparations of high IgG_{2b} content which were specific for the T lymphocyte subset Ags Ly-2 and Ly-4 (2.40 &

2.41). These monoclonals have been shown to be remarkably effective at removing the Ly-2- and Ly-4-bearing T cell subsets in vivo (Cobbold et al 1984) and could thus be used for the selective manipulation of different aspects of the immune response. In order to dissect the host cell-mediated immune response to P. c. chabaudi infection, and to determine the T cell subset(s) mediating the protective effector mechanisms involved, the MAbs were used to deplete effectively completely in vivo either or both of the two subsets of T cells recognised. In vivo depletion was achieved by direct i.v. administration of appropriately diluted ascites containing anti-Ly-2 or anti-Ly-4 MAbs. Two different protocols were followed:

(a) Direct depletion of naive mice

T cell subsets were negatively selected through a complete course of depletion (i.e. MAb administration) during primary infection of naive animals (Cobbold et al 1984). The methodology followed was that described by Süss et al (1988); mice were injected i.v. with purified Abs diluted to the appropriate concentration in PBS (pH 7.2) and delivered as a 0.25 ml inoculum. Animals received an injection of 500 µg of purified Ab 5 d prior to infection, followed by 250 µg 4 d and 1 d before challenge (total of three pretreatments). At 7, 14, 21 and 28 d p.i., experimental mice were given a further dose of 250 µg Ab, and 100 µg on d 35 and weekly thereafter for the remainder of the experiment. A control group that received normal rat serum in place of specific Ab was also established.

Groups of experimentally infected animals were sufficiently large (10 mice/group) to allow sacrifice of individual mice at weekly intervals, at and following challenge. These mice were examined to monitor the efficiency of depletion of the relevant T cell subset(s), as determined by an immunofluorescence assay (see below).

(b) Thymectomy and T cell subset depletion

In instances where protective T cell lines were adoptively transferred into immunosuppressed mice at the time of adoptive transfer, long term depletion by MAb treatment was not possible. To deplete the host T cell complement without negating the effects conferred by the grafted T cells, adult-thymectomised mice were used so that new T cells would not be produced after MAb pretreatment (Leist et al 1987; Phillips, R.S., personal communication). Immune protection conferred by the transferred cell populations could then be measured in the absence of any short term effects of each Ab.

Mice were adult-thymectomised at five weeks of age according to the method of Monaco et al (1966) described earlier. Sham-thymectomised controls were included. The animals were allowed to recover for at least four weeks before treatment with Ab. Thymectomised mice were given two 0.25 ml i.v. injections of 250 µg purified MAb 4 d apart, the animals being infected with P. c. chabaudi 12 d after the second injection (modified from Leist et al 1987).

(c) Monitoring of T cell subsets in vivo

The specificity of each of the depletion treatments was assessed by an immunofluorescence assay on acetone-fixed material (Cobbold et al 1986). This was more convenient than using a live IFAT since slides could be pooled at -20 °C for assaying in one or two batches at a later time , usually at the end of the experiments.

Mice were exsanguinated under terminal anaesthesia by cardiac puncture into heparinised PBS (10 i.u./ml). The peripheral blood so collected was separated by centrifugation (400 g for 15 min at 20 °C) on a discontinuous 30%, 40% and 50% Percoll column, and the lymphocyte layer drawn off from between the uppermost plasma and the 30% Percoll layer (described elsewhere). These cells were washed twice in excess PBS (300 g for 5 min) and resuspended to a 1 ml volume in 10% FCS RPMI 1640 medium. After total and viable cell counts were made, the lymphocyte concentration was adjusted to 2 $x10^7$/ml. Thin smears of each suspension of peripheral blood lymphocytes were made on glass microscope slides, dried in air and the slides stored dessicated at -20 °C until testing.

The methodology followed for the assay was essentially that described previously (2.32) but with minor modifications. Briefly, instead of titrating out the primary MAb, it was used at a predetermined optimal dilution of 1:100 in PBS. The Abs used were anti-mouse IgG_{2b} monoclonals specific for Ly-4, Ly-2 and Thy-1 T cell determinants. Rather than making use of the same MAb preparations employed for in vivo treatment to test for the efficiency of that depletion, commercially available reagents (Sera-Lab) were used. In each instance, normal rat serum was used in place of the primary MAb as a negative control, and a 1: 200 dilution in PBS of FITC-conjugated goat anti-rat IgG (Sigma) was added to detect indirect immunofluorescence.

Figure 2.1
History of *Plasmodium chabaudi chabaudi* AS strain parent populations

Derived from *Thamnomys rutilans* number 339 caught in the Central African Republic in March 1969.

Edinburgh

Stabilate 20 AS
↓ 2 BP
Cloned
↓ 1 BP
Mosquito transmitted 2/5/72
↓ 1 BP
Stabilate 25/5/72
↓ 1 BP
Glasgow
↓ 4 BP
GUP 373 25/4/73
↓ 2 BP
GUP 516
↓
Mosquito transmitted
↓
GUP 592
↓
Cloned
↓
GUP 603
↓ 2 BP
GUP 772

GUP772
↓ 2 BP
GUP 890
↓ 1 BP
Cloned
↓
GUP 1349
↓ 2 BP
GUP 1519 16/11/82
↓ 1 BP
WEP 30 27/4/84
↓ 1 BP
WEP 72 7/12/84
↓ 1 BP
WEP 583 19/10/87
↓ 1 BP
WEP 685 20/5/88
↓ 2 BP
WEP 900 14/6/89

Figure 2.2
History of Plasmodium chabaudi chabaudi AS strain recrudescent clone 10

Derived from Thamnomys rutilans number 339 caught in the Central African Republic in March 1969.

Cloned parent population (see Fig. 2.1)
GUP 1349
↓
Day 30 recrudescence
GUP 1591
↓
Cloned → 10 clones 31/12/82
↓
GUP 1619 (RC 10)
↓ 1 BP
WEP 540 27/6/87
↓ 1 BP
WEP 757 16/1/89

Figure 2.3
History of Plasmodium chabaudi chabaudi CB parent populations

Derived from Thamnomys rutilans caught in the Central African Republic in September 1970.

Tree rat
↓ 1 BP
Stabilate
↓ 1 BP
Mosquito transmitted 1972
↓
Stabilate
↓
Edinburgh

Edinburgh
Stabilate
↓ 1 BP
Cloned 24/7/79
↓
Stabilate
↓ 1 BP 7/2/89
Glasgow
↓ 1 BP
WEP 762 13/2/89

Table 2.1
Modified protocol for *in vitro* maintenance of T cell lines

Feed	1st	2nd	3rd	4th →
Cells	4 $x10^6$/ml	1 $x10^6$	1 $x10^5$	2.5 $x10^4$
APC	1 $x10^6$/ml	→	→	→
Ag	200 µg/ml	→	→	→

12-15 d between feeds.

CHAPTER THREE

ADOPTIVE TRANSFER OF IMMUNITY WITH LYMPHOCYTE SUBPOPULATIONS TAKEN AT DONOR PERIPHERAL BLOOD LYMPHOCYTOSIS

3.1 Introduction

Billingham et al (1954) referred to the transfer of immunity by lymphoid cells from animals which had previously received an antigenic stimulus to non-reactive hosts as 'adoptive' immunity. Immunity can be transferred between animals in such a way that cell-mediated and humoral immune responses can be demonstrated in recipients (Billingham et al 1954, Mitchison 1957).

The basic model for cell transfer studies in rodent malaria was developed by Stechschulte (1969), who showed that immunity to Plasmodium berghei could be transferred from a recovered rat to a syngeneic susceptible rat by the injection of splenic lymphocytes. Using the same model, Gravely & Kreier (1976) confirmed that the degree of immunity transferred with spleen cells is roughly proportional to the number of cells injected. Since the initial study, a wealth of evidence has shown that protection can be engendered by reconstitution with immune spleen and lymph node cells (Roberts & Tracy-Patte 1969, Phillips 1970, Kasper & Alger 1973, Brown et al 1976 a & b, McDonald & Phillips 1978, 1980, Fahey & Spitalny 1986, Cavacini et al 1986, Favila-Castillo et al 1990). However, in most of these studies, unfractionated spleen cells or preparations enriched for populations of either T or B lymphocytes, were prepared from donor mice which had recovered from at least one previous infection. Only in two instances have spleen cells been taken from donor animals at a range of times during primary infection; P. berghei in rats (Brown et al 1976 a) and P. yoelii 17X in mice (Fahey & Spitalny 1986), when cells were taken at a time when they had been exposed to the infecting parent parasite population but not immunologically primed to any variant types.

For the P. c. chabaudi AS system, transfer of immunity with splenic lymphocytes has been shown in both NIH (McDonald & Phillips 1978, 1980) and CBA/Ca (Favila-Castillo et al 1990) mice, but in both these investigations, only spleen cells taken from post-infective mice were used. A study was therefore undertaken to determine whether lymphocytes taken from semi-immune donors still showing a patent parasitaemia were capable of transferring immunity to naive and to sublethally irradiated recipients. In addition, as an alternative to the enlarging spleen as a source of lymphocytes, it was examined whether lymphocytes taken from the peripheral blood of donor mice early during infection had the capacity to protect. To do this, the reported phenomenon of a pronounced peripheral lymphocytosis during the remission of primary parasitaemia (Jayawardena et al 1977, Kumararatne et al 1987) was

at the time of peak primary parasitaemia.

After crisis, the number of lymphocytes observed in the peripheral blood rose sharply, climaxing in a marked lymphocytosis on d 13 p.i., when a level of 1.98 $x10^7$ cells/ml was reported. This vigorous lymphocyte response was very short-lived, being followed by a precipitous drop in values during the later stages of remission of the acute parasitaemia. Thereafter, from a low point of ~ 5 $x10^6$/ml, lymphocyte numbers increased gradually throughout the subpatent and recrudesent periods, but reached a plateau of 1.3 $x10^7$/ml by d 28 p.i., above which levels did not rise. By the time of parasite clearance, lymphocyte numbers in the blood stream had fallen to those observed at the time of challenge. In subsequent experiments, peripheral blood leucocyte counts were made from prospective donor mice up to and including the expected time of a maximal lymphocyte response. These studies confirmed that the pronounced lymphocytosis just after crisis was a consistent feature of P. c. chabaudi AS primary infection of the NIH mouse. That the increase of lymphocytes in the blood was actually an immune response to infection was shown by comparing the levels of circulatory lymphocytes in infected and uninfected mice (Fig. 3.2.2). It can be seen that in normal mice, under no immunological pressure, the numbers of blood-borne lymphocytes is reasonably constant at ~ 5 $x10^6$/ml, though quite variable about this mean. This variation may reflect a flux in the natural lymphocyte exchange between the blood and lymphoid organs, or may alternatively be due to an inherent error in the method by which lymphocyte levels were counted. In either case, during the proposed enhanced lymphocyte counts throughout infection, the number of cells in the peripheral blood of malarious mice was at least twice that of background levels. At the time of peak lymphocytosis, there was a 4.2-fold increase in detectable lymphocytes in the blood of malaria-infected mice compared to that of normal mice.

Although the lymphocyte is the predominant type of leucocyte in the peripheral blood generally, and certainly during P. c. chabaudi AS infection (Fig. 3.2.3), the increase in the absolute numbers of lymphocytes at the time of lymphocytosis was not reflected entirely by the differential leucocyte counts (Fig. 3.2.4). This is because the absolute neutrophil count was quite raised at this time, resulting in an increase in absolute total leucocyte levels but not an increase in the proportion of these cells that were lymphocytes. Throughout infection, lymphocytes accounted for the bulk of peripheral blood leucocytes; for the acute phase for which data is shown (Fig. 3.2.4), lymphocytes represented between 48-88% of leucocytes. The maximal proportion of

these cells was detected not on d 13 p.i. (peak lymphocytosis) but on d 18 p.i. when neutrophil levels were depressed. The lowest differential lymphocyte count arose as a result of an early peak neutrophil response to infection, when on d 5 p.i., lymphocyte and neutrophil levels were equal.

During infection, neutrophil counts fluctuated greatly, there being a 10-fold difference between minimum (1.12 $x10^6$/ml, d 18 p.i.) and maximum (1.18 $x10^7$/ml, d 36 p.i.) levels (Fig. 3.2.5). Generally, neutrophil levels were greater in infected than in uninfected mice; this was particularly evident during the recrudescence, throughout which neutrophil numbers remained elevated. At this time, neutrophils represented at least 40% of peripheral leucocytes; indeed, on d 36 p.i., neutrophils made up 56% of leucocytes in the circulation.

Despite the fact that in terms of absolute numbers of leucocytes observable in the blood stream, monocytes always made up only a small minority (Fig. 3.2.3), when the levels of monocytes were examined alone, it was found that there was a profound monocytosis during the prepatent and acute parasitaemia after which counts fell to those of uninfected mice (Fig. 3.2.6). Peak monocyte levels were attained on d 2 p.i., when a transient monocytosis of 1.78 $x10^6$ cells/ml was recorded, making up 7% of the total number of leucocytes. After this, levels dropped rapidly but rose again in parallel with primary parasitaemia. These raised levels of monocytes were not reflected in the differential cell counts as it coincided with the peak lymphocytosis of much greater magnitude.

3.3 Adoptive transfer to immunocompetent recipients of peripheral blood lymphocytes taken at lymphocytosis.

A group of 10 NIH female mice was infected with 1 $x10^5$ P. c. chabaudi AS pRBC and the course of infection and absolute lymphocyte counts determined. On d 12 p.i., the time of mean peak peripheral lymphocytosis for this group of mice, these lymphocyte donors were bled and their peripheral blood lymphocytes adoptively transferred to a group of five naive, syngeneic recipients. Each mouse was injected with 2.50 $x10^6$ leucocytes, containing 2.36 $x10^6$ lymphocytes. Uninfected blood was collected from 12 normal, age-matched mice and 4.73 $x10^6$ leucocytes (2.40 $x10^6$ lymphocytes) given to each of a group of five control mice. All these experimental mice were challenged with 1 $x10^5$ pRBC and the course of infection followed. In addition, two further mice were inoculated with a similar dose of primed leucocytes but without a

challenge infection. These animals were monitored for the presence of a patent parasitaemia and acted as a control for the possible transfer of contaminating pRBC with leucocytes separated from the peripheral blood of donor mice.

Some protective activity was transferred with pRBC-primed lymphocytes taken from semi-immune donors (Fig. 3.3.1). Each of the mice which received peripheral blood lymphocytes from infected mice had a slightly reduced primary parasitaemia compared to normal cell recipients. Furthermore, primed lymphocyte recipients were able to reduce their primary parasitaemia to subpatent levels significantly more quickly than controls (subpatency d 14 p.i. compared to d 18 p.i. for control mice; $p < 0.01$). All mice exhibited patent recrudescences, but those in recipients of semi-immune cells were of significantly shorter duration ($p < 0.01$), resulting in a final parasite clearance 6 d prior to that observed in naive lymphocyte recipients. Thus, by some of the parameters used to gauge the transfer of immunity, the enriched lymphocyte populations collected from infected mice at lymphocytosis clearly conferred anti-malarial activity. However, in both groups of mice the acute rise in parasitaemia followed a similar course. Also, although the timing of the secondary parasitaemias differed between recipients of primed and naive lymphocytes, there was no significant difference in the actual levels of recrudescent parasitaemia attained ($p > 0.05$).

pRBC were not observed in blood smears taken from mice inoculated with lymphocyte preparations alone, showing that the method used to isolate RBC from leucocytes (2.12) worked satisfactorily. Obviously, if this had not been the case, each challenged mouse would have received a far larger parasite inoculum than the10^5 pRBC dose intended. Not only would this have been of variable size, and thus invalidated the experiment, but perhaps would have led to an overwhelming infection of atypical characteristics.

3.4 Adoptive transfer to immunocompromised recipients of peripheral blood lymphocytes taken at lymphocytosis.

In order to amplify the protection given to recipient mice by the transfer of unfractionated peripheral blood lymphocytes, mice were immunodepressed by gamma irradiation shortly before adoptive transfer and challenge.

18 NIH female mice were given 4 Gy whole body irradiation 24 hr before infection. Of these, one group of six mice was injected with 2.38 $x10^6$ semi-immune

peripheral blood leucocytes (2.13 $x10^6$ lymphocytes) each, and another six mice each inoculated with 3.45 $x10^6$ naive leucocytes (2.20 $x10^6$ lymphocytes). All these recipients received 1 $x10^5$ P. c. chabaudi AS pRBC as did three other mice acting as controls of the normal course of infection upon sublethal irradiation. The remaining three irradiated animals were injected with 2.13 $x10^6$ circulatory lymphocytes as a control of pRBC contamination in the peripheral blood pool collected at lymphocytosis. Identical groups of non-irradiated, naive mice were also set up. To enable the adoptive transfer of immunologically primed lymphocytes to a total of 18 animals (six irradiated challenged mice, three irradiated controls, six naive challenged mice, three naive controls), 24 donor mice were bled on d 13 p.i., when lymphocytosis took place. 18 uninfected donor mice were used to transfer naive peripheral blood lymphocytes to groups of six irradiated and normal mice.

There was a clear demonstration of protection in irradiated recipients of parasite-exposed lymphocytes (Fig. 3.4.1). Compared to mice receiving a similar number of unprimed lymphocytes, there was a significantly reduced peak parasitaemia ($p < 0.05$), with subpatency being reached 4 d before that in naive circulatory lymphocyte recipients. The recrudescences were of a similar magnitude in both groups but for semi-immune lymphocyte recipients total parasite clearance occurred 6 d earlier than for normal lymphocyte recipients. In many respects, the differences between the courses of infection in irradiated mice receiving either normal or primed peripheral blood cells were no greater than those seen in the non-irradiated situation (Figs. 3.3.1 & 3.4.2). Immunosuppression of the host had no obvious effect on the timing of the recrudescences or the duration of the subpatencies. What can be seen, however, is that irradiation acted to amplify the magnitude of parasitaemia upon subsequent challenge. This was manifested as a greater level of recrudescence in irradiated mice (Fig. 3.4.1) compared to their non-irradiated counterparts (Fig. 3.4.2). The major effect of immunosuppression was that it was able to amplify the difference in primary parasitaemia between recipients of semi-immune lymphocytes and those of normal lymphocytes. Thus, there was a depressed peak of acute infection in recipients of lymphocytosis-derived peripheral blood cells compared to mice inoculated with naive lymphocytes.

Although there was no such divergence in the levels of the secondary parasitaemia between irradiated mice receiving one or other cell type (Fig. 3.4.1), that whole body irradiation increased host susceptibility to malaria infection was noticeable from

comparing the recrudescences of irradiated and non-irradiated recipients of either peripheral blood semi-immune lymphocytes (Fig. 3.4.3) or normal lymphocytes (Fig. 3.4.4). In both cases, the recrudescence observed in immunocompromised mice more than doubled that seen in naive recipients ($p < 0.01$). Figures 3.4.3 and 3.4.4 also illustrate that irrespective of the source of immunological activity of the cells adoptively transferred, suppression of the host's immune system changed the level, but not the pattern of infection. The only appreciable difference with regard to the course of infection in these two different immunological states was that in most irradiated mice, the onset of a patent parasitaemia occurred after that in naive mice, indicating an initial resistance to infection in irradiated hosts injected with either semi-immune (Fig. 3.4.3) or normal (Fig. 3.4.4) lymphocytes of peripheral blood origin.

3.5 Adoptive transfer of peripheral blood T & B lymphocyte populations to immunocompromised recipients

To examine whether either or both T and B lymphocyte subsets were responsible for the protection conferred by immunologically primed lymphocytes to host animals infected with P. c. chabaudi AS, subpopulations of enriched peripheral blood B and T cells, separated on nylon wool, were transferred to sublethally irradiated mice and the challenge infection followed.

Groups of six NIH female mice were irradiated with 4 Gy 24 hr before challenge with 1×10^5 P. c. chabaudi AS pRBC. Mice were infected as soon as possible after adoptive transfer of either 7.5×10^6 enriched semi-immune T or B cells, or 7.5×10^6 enriched naive T or B cells. In addition, further groups of six mice each were given similar doses of one of these treatments. Primed and normal lymphocytes were collected from the peripheral blood of a total of 41 infected and 40 naive donors, respectively (a sufficient donor size to compensate for the loss of cells during the fractionation procedure). The number of parasite-exposed peripheral blood lymphocytes collected was maximised by bleeding the donor mice at peak peripheral lymphocytosis on d 12 p.i..

The degree of enrichment of lymphocyte preparations for either B or T cells was determined by IFAT. For the semi-immune lymphocytes, 85.93% of the B-enriched fraction were Ig-bearing cells (11.27% T cells), whilst the T cell population comprised 82.14% Thy-1-bearing cells and 9.04% contaminating B cells. In the

case of the normal lymphocytes, the nylon wool-adherent population contained 79.37% B cells (12.41% T cells), whilst T & B cells made up 82.46% T and 10.35% B cells.

The enriched peripheral blood T cell population taken at lymphocytosis of infected donors gave a greater degree of protection to adoptively transferred recipients than did naive circulatory T cells. This was manifested as a subpatency lasting 11 d compared to 1 d for normal cell recipients (Fig. 3.5.1). However, the patterns of the two patencies and the levels of both were not significantly different between the two groups studied ($p > 0.05$). For the analogous situation of the transfer of peripheral blood B cell-enriched populations, irradiated semi-immune B cell recipients also showed an extended subpatent period, 11 d, compared to recipients of naive B cells (8 d) (Fig. 3.5.2). The effect of the transfer of either semi-immune T or B cells into sublethally irradiated mice was similar to that of the previously discussed transfer of unfractionated peripheral blood lymphocytes into similarly immunosuppressed recipients except in one notable respect. Recipients of enriched cells collected at lymphocytosis recrudesced after their naive lymphocyte counterparts (Figs. 3.5.1 & 2), whilst those receiving either unfractionated semi-immune cells or naive cells recrudesced at the same time (Figs. 3.4.1).

In the case of non-irradiated mice inoculated with enriched populations of semi-immune T cells, there was not such a clearly defined difference in the patterns of parasitaemia compared to that for naive T cell recipients (Fig. 3.5.3). This was to be expected since in these mice the host immune response was not dampened and thus it was difficult to distinguish the protective activity of the repopulating inoculations. The acute infections were identical for recipients of semi-immune and naive peripheral blood lymphocytes (Fig. 3.5.3) However, the course of infection for normal T cell recipients showed only a transient subpatency, whilst that for mice inoculated with immunologically primed T cells was more long lived. Similarly, in immunocompetent recipients of B lymphocytes, the protective activity conferred by enriched parasite-exposed B cells was most clearly seen after remission of the primary parasitaemia to subpatency (Fig. 3.5.4); again, for naive B cell recipients, subpatency lasted only 24 hr, compared to 6 d for semi-immune lymphocyte recipients.

The adoptive transfer of the T cell fraction taken during peripheral blood lymphocytosis of donor animals gave a greater degree of protection to sublethally

irradiated recipients than did the transfer of the B cell fraction. It has been established that both lymphocyte subsets confer greater protective activity to immunocompromised challenged mice than do naive T or B cell controls, seen as an extended subpatency. However, the enhanced protection engendered by the enriched T cell population was not observed as a difference in this parameter but in the level of recrudescence attained in infected mice. The magnitude of the recrudescence in animals reconstituted with semi-immune circulatory T lymphocytes was significantly lower than that for animals injected with enriched peripheral blood B cells ($p < 0.01$) (Fig. 3.5.5). This divergence in protective activity was also apparent in sublethally irradiated mice transferred with naive B or T cells (Fig. 3.5.6). In this instance, however, the lower recrudescence in mice receiving normal peripheral blood T cells was offset by a significantly less efficient parasite clearance after the acute phase of infection ($p < 0.05$).

The enhanced immune response by recipients of semi-immune T cells over that of B cells that was apparent in immunosuppressed mice (Fig. 3.5.5) was not observed in non-irradiated recipients, where the levels of recrudescent parasitaemia were similar (Fig. 3.5.7). In contrast, for naive recipients of non-immune peripheral blood lymphocyte subsets, the courses of infection observed were not alike (Fig. 3.5.8); although the primary parasitaemias were very similar, mice receiving naive T cells reached subpatency 4 d before recipients of B cells. However, in each case, malaria parasites reappeared in the blood stream almost immediately. This chronicity of infection was not as apparent in irradiated mice (Fig. 3.4.5) and suggested the possibility of immunosuppression by the host immune system. This effect was not so obvious in recipients of semi-immune lymphocytes, but in immunocompromised mice (Fig. 3.5.5) the subpatent period was significantly longer than for non-irradiated recipients of either semi-immune B or T cells ($p < 0.01$) (Fig. 3.5.7).

Comparing the adoptive transfer of semi-immune T- or B-enriched peripheral blood populations into irradiated and naive mice (Fig. 3.5.9), irradiated mice exhibited a delayed onset of parasitaemia, but suffered a heightened primary parasitaemia and a course of infection that extended by 12 d that in normal mice. In the analogous transfers of enriched lymphocytosis-derived B cell populations there was a similar situation (Fig. 3.4.10). If these graphs are compared with the courses of infection for irradiated and non-irradiated mice receiving an unfractionated T & B lymphocyte

population (Fig. 3.4.3), it can be seen that there was no lengthened parent parasitaemia in immunosuppressed mice. However, irradiation appeared to enhance the level of recrudescence, a feature not observed when the peripheral blood lymphocytes were separated into T and B subsets before adoptive transfer. This anomaly apart, it is clear that a greater degree of protection was conferred by a mixed population of primed T & B cells, as demonstrated by a substantially quicker clearance of parasites from the peripheral blood ($p < 0.01$). The larger recrudescence observed in irradiated recipients of unfractionated cells (Fig 3.4.3) compared to normal recipients of the same cells may have been due to the fact that all these mice received a quite small inoculum size (2.13×10^6 cells) which may not have been sufficient to counteract the effects of irradiation. In contrast, the mice injected with peripheral blood lymphocytes enriched for either B or T cell fractions received a dose of 7.5×10^6 cells; this increase in the number of repopulating cells was sufficient to control the recrudescence in irradiated mice and masked the fact that the transfer of a mixed lymphocyte population gave a greater immune response. There was a similar case for recipients of naive peripheral blood cells; irradiated mice receiving either B or T enriched fractions cleared their parasitaemias markedly later than did non-irradiated controls (Figs. 3.5.11 & 12). There was no such difference between these two states of immunocompetence when an unfractionated T & B cell population was injected into challenged mice (Fig. 3.4.4). Thus, this phenomenon is an innate characteristic of lymphoid cells of the murine immune system and not dependent upon previous exposure to P. c. chabaudi AS pRBC. However, priming did lead to a greater protective activity in irradiated and non-irradiated recipients alike. Whether immunocompromised mice received semi-immune or naive peripheral blood lymphocytes, one consistent feature observed from these studies was the delayed appearance of pRBC in the blood, compared to similarly infected non-irradiated recipients. This delay in the onset of parasitaemia was a direct result of the irradiation treatment, which caused a transiently enhanced non-specific immune response. As adoptive transfer took place only 24 hr after immunosuppression, phagocytic activity was probably heightened and it was this which led to the slow appearance of a patent parasitaemia.

3.6 Adoptive transfer of peripheral blood and splenic lymphocytes to immunocompromised recipients.

Previous results described in this chapter have shown that peripheral blood lymphocytes are capable of transferring immune protection to mice challenged with P. c. chabaudi AS. To gain a sufficient number of cells to inoculate into recipient animals, and to determine the degree of immunological priming achieved by a brief exposure to the malaria parasite, lymphocytes were derived from donor mice at a point just after crisis. Although this had proved satisfactory, it was thought desirable to compare the acquired immunity transferred by splenic lymphocytes with that given by lymphocytes extracted from the peripheral blood. This was performed with a view to using the spleen as a source of lymphocytes for future adoptive transfers since this lymphoid organ represented a far larger pool of readily available T and B cells than the circulatory system.

As the separation procedure used to isolate lymphocytes from RBC, in particular pRBC, had to be biased against the accidental collection of parasites, it was found that the yield of peripheral blood lymphocytes was far from ideal. Hence, a large number of donor animals was required for each adoptive transfer, a problem which was thought could be circumvented by the alternative use of the spleen as a source of lymphocytes.

This study was performed to examine whether similar sized inocula of lymphocytes from these two different sites gave similar or different levels of protection to immunosuppressed mice. To enable the collection of peripheral blood cells, donor mice were sacrificed at the time of lymphocytosis, when they were both bled and splenectomised.

NIH female mice were exposed to 4 Gy gamma irradiation and 24 hr later infected with 1 $x10^5$ P. c. chabaudi AS pRBC. At the time of challenge, each mouse was also inoculated with an aliquot of 7.5 $x10^6$ lymphocytes. Recipients of semi-immune lymphocytes were separated into groups of six mice each, and received either unfractionated peripheral blood lymphocytes, unfractionated splenic lymphocytes, or enriched T or B cell populations.

To enable the adoptive transfer of primed lymphocytes, 14 syngeneic donor mice had been previously infected and were bled on d 12 p.i. at peak peripheral blood lymphocytosis, when splenomegaly was evident.

Also, non-primed circulating and splenic T & B cells were inoculated into two groups

of six mice each. Eight uninfected mice were used as a source of naive lymphocytes for adoptive transfer and cells were collected immediately prior to injection.

The identity of the nylon wool-separated lymphocyte preparations was shown by immunofluorescence. The B cell- and T cell-enriched fractions exhibited 87.92% and 85.04% fluorescence with anti-κ and anti-Thy-1 markers, respectively. The enriched B cells contained 7.55% T cells, whilst the purified T population contained 8.75% B lymphocytes.

For each of the groups of mice, small volumes of serum were collected from the tails of infected animals at intervals during the course of infection. The serum Ab levels of challenged mice were then determined by IFAT.

The adoptive transfer of fractionated semi-immune spleen cell populations led to courses of infection in the two groups which were markedly different from those of mice receiving whole splenic lymphocytes (Fig. 3.6.1). Considering the course of infection of primed splenic T cell recipients with respect to B and T & B cell recipients , there was a quicker remission of the acute infection with subpatency being reached an d 21 p.i., 3 and 4 d before the B or mixed cell-transferred groups, respectively. However, unlike the other two situations, where a defined recrudescence took place only after a prolonged subpatency, for the splenic T cell recipients, subpatency was brief (3 d), after which there was a chronic period of low level parasitaemia. The pattern of parasitaemia in mice injected with enriched splenic B cells taken from infected donors resembled that of similar recipients of the complete lymphocyte suspension (Fig. 3.6.1); the lack of immunologically primed T cells did not appear to have any effect during subpatency, but the levels of both the primary recrudescent parasitaemias reached significantly higher levels ($p < 0.01$) for B cell recipients lacking a semi-immune T cell population.

That primed T cells taken from the spleen did confer some degree of protection is illustrated in Fig. 3.6.2, which compares the course of infection of semi-immune splenic T cell recipients with that of a control group receiving a naive syngeneic population of T & B spleen cells. The considerably enhanced protection conferred by the primed T cells was seen as a profound reduction of primary parasitaemia ($p < 0.01$) and subpatency reached on d 21 p.i.. Mice transferred with the unstimulated mixed lymphocyte population ultimately cleared the infection but without ever achieving a period of subpatency. Thus, the unprimed B cells inoculated into these recipients were unable to produce the same parasite clearance after the acute

infection as did the semi-immune splenic B cell population (Fig. 3.6.3).

From these studies it can be seen that some immunity to P. c. chabaudi AS could be transferred with splenic lymphocyte subsets taken from infected mice as early as d 12 after challenge. When identical numbers of cells were transferred to recipient animals, the greatest protection was achieved with a mixed T & B spleen cell inoculum, suggesting that there was a degree of synergistic activity between parasite-primed T and B lymphocytes in the immune control of infection in sublethally irradiated mice. This synergism is further supported by examination of the Ab titre profiles for recipients of semi-immune T, B, or T & B spleen cells over the course of infection (Figs. 3.6.4, 5 & 6). In each case, the mean Ab titre rose during the first wave of patent parasitaemia and plateaued during the subpatent period. Ab levels again rose either later during subpatency (B or T & B recipients; Figs. 3.6.5 & 6) or when the parasitaemia was recrudescing (T cell recipients, Fig. 3.6.4). The fluorescent Ab levels reached a peak between d 31-33 p.i. in all three groups, and thereafter declined slowly. The levels of specific anti-P. c. chabaudi AS Ab detected in the serum of mice injected with a mixed primed spleen cell population were greater than those in mice receiving an enriched suspension of either lymphocyte subset (Fig. 3.6.7). However, the highest reciprocal Ab titre attained in IFAT for sera collected from the semi-immune T & B lymphocyte recipient mice, 2024, was not significantly greater than those recorded for either splenic B (1024) or T (512) cell-transferred animals.

The enhanced ability of stimulated spleen cells over naive cells to control a primary malaria infection is further illustrated in Fig. 3.6.8, which shows the courses of infection of mice challenged with P. c. chabaudi AS after adoptive transfer of mixed lymphocyte suspensions taken from infected and naive donor mice. As for transfer of separated T or B semi-immune spleen cells (Figs. 3.6.2 & 3), transfer of unfractionated spleen cells to recipient mice resulted in remission to subpatency, an achievement not reached by recipients of naive spleen cells. Overall, the infection in mice receiving unprimed T & B lymphocytes was shorter than that exhibited by animals inoculated with primed splenic T & B cells, but this was a natural result of the 15 d subpatent period following acute infection in this latter group of mice, in which breakthrough parasitaemia occurred only on d 38 p.i.. For the analogous transfers of primed and unprimed whole lymphocyte populations derived from peripheral blood (Fig. 3.6.9), priming the immune response resulted in a better

control of challenge infection, as indicated by a subpatency of significant duration, and, in this case, a quicker parasite clearance than for mice receiving naive peripheral blood lymphocytes. In this group, the time taken for the primary parasitaemia to be resolved was 15 d compared to 8 d for recipients of semi-immune circulatory lymphocytes. However, one anomalous finding was the lower mean peak primary parasitaemia in recipients of non-immune cells (Fig. 3.6.9); this was shown only by lymphocytes taken from the peripheral blood and was not repeated by splenic cells (Fig. 3.6.8).

Comparing the patterns of parasitaemia in groups of mice inoculated with either pRBC-primed whole lymphocyte populations from the two different lymphoid sources, the acute infections had very similar characteristics (Fig. 3.6.10). Remission to subpatency was achieved for peripheral blood recipients by d 17 p.i., 6 d before that for splenic T & B cell-transferred mice. However, in mice receiving the primed spleen cells, subpatency lasted 15 d, significantly longer than the 9 d shown by mice transferred circulatory lymphocytes ($p < 0.01$). However, in the peripheral blood cell recipients, the secondary parasitaemia, although elevated, was short-lived, resulting in parasite clearance by d 37 p.i.. This contrasted with clearance of parasitaemia by only d 53 in mice injected with semi-immune T & B cells of splenic origin; however, this lag in clearance times was due, at least in part, to the extended subpatency already discussed. It appears, therefore, that syngeneic lymphocytes primed to P. c. chabaudi AS gave protection to challenged mice regardless of lymphocyte origin, but the degree of protective immunity conferred varied according to the parameters of protection used. Recipients of unprimed whole lymphocyte populations from either of the two sources used in this study gave more similar courses of infection than did mice inoculated with semi-immune cells (Fig. 3.6.11). In both cases, infection was cleared before d 40 p.i., and in this regard, transfer of naive splenic or peripheral blood T & B cells resembled transfer of circulatory T & B lymphocytes previously primed to infection (Fig. 3.6.10). However, unlike the course of infection in mice receiving primed cells, recipients of naive lymphocytes showed little or no post-primary parasitaemic subpatency.

3.7 Discussion

Haematology performed on peripheral blood taken from naive NIH mice infected with P. c. chabaudi AS showed that there was a substantial increase in the blood lymphocyte

count shortly after peak parasitaemia. This was a consistent finding both in its appearance in all mice followed within a group, and its reproducibility between various batches of mice infected at different times. This lymphocytosis had been previously reported by Kumararatne et al (1987) using this same species of malaria parasite to challenge C57BL mice. However, the increased presence of lymphocytes in the blood of mice during acute infection may not be a feature common to all parasite-host combinations of rodent malaria as both Lelchuck et al (1979) and Strickland et al (1979) did not note a pronounced lymphocytosis as a characteristic of P. yoelii infections of C57BL or C3H/HeJ mouse strains, although it has been shown for P. yoelii-challenged CBA mice (Jayawardena et al 1977).

Raised levels of lymphocytes in the bloodstream around 3 d after crisis is thought to be due not to lymphocytopoiesis, but rather to a redistribution of the lymphocyte pool (Kumararatne et al 1987). As the phenomenon of lymphocytosis is well described in splenectomised animals (Ford & Smith 1979) and in those in which lymphocyte circulation through the lymphoid tissues has been impaired, as in rats treated with lymphocytosis-promoting factor extracted from Bordetella pertussis (De Sousa 1981), it may be that in malarious mice, a decreased or altered lymphocyte traffic through the spleen may lead to a lymphocytosis. Shortly after peak primary parasitaemia, lymphocyte localisation in the spleen is reduced (Brissette et al 1978); concomitantly, there is a rise in lymphocyte migration to the blood, and to the lungs and especially the liver (Playfair & De Souza 1982), indicating a complex redistribution of the recirculating lymphocyte pool (Kumararatne et al 1987). The reason for the reduced lymphocyte migration through the spleen and the significant increase in T and B cells in the peripheral blood and liver of malaria-infected mice is not clearly understood. For P. c. chabaudi, sequestration of the parasite in deep capillary beds may affect the distribution of lymphocytes. As there is evidence for sequestration in the liver but not in the spleen (Shungu & Arnold 1972, Cox & Hommel 1984), lymphocytes may accumulate preferentially in the liver. Although it is well established that the complex migratory pathways of lymphocytes through the spleen are necessary for the cellular interactions required for a normal immune response to blood-borne Ags (Ford 1975), and that these are depressed around peak parasitaemia (Weidanz 1982), all the naive donor or recipient animals studied in this report recovered from primary parasitaemia. This suggests that the liver is important in the development of protective immunity, a finding first documented by

Taliaferro & Cannon (1936). If this is the case, this would explain the increased cell migration to the liver during malaria infection (Brissette et al 1978, Playfair & De Souza 1982, Kumararatne et al 1987). The impaired passage of lymphocytes through the spleen may actually increase the circulation of immunologically primed T and B cells to the liver where parasite destruction is taking place. This would precipitate the accumulation in the liver of substantial numbers of T cells and macrophages in a localised environment containing pRBC, which would enable the induction of cell-mediated mechanisms of immunity, by one of several possible effector metabolites.

It would appear that the peripheral blood lymphocytosis observed in this study is a consequence of the flux of lymphocytes between different lymphoid organs during acute infection. This suggested migration of lymphocytes from the spleen to other organs, notably the liver, would explain both the transient nature of the blood lymphocytosis and its timing, at the beginning of the remission of the primary parasitaemia. From the view point of the adoptive transfer experiments that followed, the occurrence of peripheral blood lymphocytosis was fortunate for it enabled the collection of a large population of committed immunologically competent cells for inoculating challenged mice. This afforded the opportunity to study whether protection was conferred by lymphocytes derived from a source other than the spleen. However, a limitation of this methodology was that it enabled cells to be taken at only one time point, relatively early in infection, due to the shortlived nature of the lymphocytosis. During the remission of the primary parasitaemia, the circulatory lymphocytes were exposed to predominantly the infecting parasite type and not the whole range of antigenic variants encountered by T and B memory cells recovered from mice after a complete course of infection. It was not possible to collect lymphocytes from the peripheral blood from immune mice that had recently cleared infection due to the low levels of cells in the blood at times other than lymphocytosis, and the vast number of donor mice that this approach would therefore demand.

Maximal neutrophil levels were recorded in the peripheral blood of infected mice on d 36 p.i., at the time of recrudescence. During the second patency, absolute neutrophil counts remained consistently higher than the background counts attained in uninfected control mice. This finding was in contrast to the only previously reported neutrophil counts during a rodent malaria infection (Jayawardena et al 1977), which showed a peak neutrophil response on d 13 p.i.. As neutrophils have an essential dependence on Abs to exert their principal anti-microbial mechanism of phagocytosis (through

opsonisation of foreign material), it would be reasonable to assume that in malaria, in which Ab levels do not rise till remission of primary parasitaemia, neutrophil levels would be greatest at or after this time. Once serum Ab titres rise, they are maintained at substantial levels throughout infection, although protective Ig levels may decline, thus facilitating the increase in neutrophil numbers observed in this study.

Monocyte levels in the peripheral blood were greatest 48 hr after challenge, at a time before pRBC were detectable in the blood. This monocytosis, which was quite shortlived, may have been due to a redistribution of these cells throughout the circulation, but is more likely to have been caused by monocyte proliferation. This is because monocytes are always detectable in the blood and because it is hard to attribute such a contrast in cell numbers over a period of 24 hr immediately upon infection solely to recruitment of monocytes to the peripheral blood. It is more likely that this effect represents one of the initial steps in the stimulation of the immune system by the very few bloodstream parasites that are present upon challenge inoculation. The fall in the levels of detectable monocytes over the next few days may, in turn, be due to the maturation of these monocytes into macrophages which then emigrate from the blood to the lymphoid tissues. The variable, but sustained high levels of monocytes in the blood during the acute phase of infection fit with this proposed process of macrophage activation following monocyte proliferation. The elevated concentration of macrophages occurs at a time when non-specific mechanisms of immunity are thought to predominate in the acquired resistance to malaria infection. The monocytosis observed during the remission of the primary parasitaemia concurs with that found by Jayawardena et al (1977) at the same stage of infection of P. yoelii-infected CBA mice.

Regarding the adoptive transfer of lymphocytes into challenged mice, the first study, which used competent recipients and transferred unfractionated peripheral blood lymphocytes, showed that a mixed lymphocyte population could transfer protective immunity to naive mice as early as 12 d after infection of the cell donors. Protection was also conferred by the transfer of such semi-immune lymphocytes to 4 Gy-irradiated mice. These observations with the P. c. chabaudi/NIH system were in accordance with those of Fahey & Spitalny (1986) using P. yoelii 17X infection of A/Tru mice. In this model, successful adoptive immunisation of naive recipients with unfractionated spleen cells, T cells or B cells was first demonstrable with

lymphocytes harvested 7 d p.i., peaked at 14 d p.i. and was no longer detectable by d 21 p.i., concomitant with the elimination of the primary infection in donor mice. This study and the results described in this chapter are, however, at variance with the recent report by Favila-Castillo et al (1990). These investigators used the same strain of parasite, P. c. chabaudi AS, as used here, but in CBA/Ca mice, and claimed that it was only possible to transfer immunity to 4.5 Gy-irradiated mice with spleen cells taken from donors which had been infected seven times prior to cell transfer. In mice inoculated with unfractionated splenic lymphocytes taken after recovery from a single infection, there was a 100% mortality rate. This discrepancy is difficult to reconcile but points to the possibility of a genetic factor involved in host immunity to P. c. chabaudi, as suggested by Stevenson et al (1982). However, neither the CBA/Ca or NIH strain of mouse was employed in this study. Alternatively, the non-transfer of immunity with lymphocytes other than those taken from superinfected donors (Favila-Castillo et al 1990) could be due to the immune response by memory T and B cells requiring in vivo stimulation of the immune donor cells before adoptive transfer to recipients. This would be necessary to boost the proliferative response of these primed, aged lymphocytes which would not normally be stimulated to replicate. The failure to transfer protection with P. c. chabaudi AS-specific lymphocytes taken from donor mice after the elimination of pRBC may be due to their reduced proliferation. As it is reasonable to hypothesise that the transfer of immunity against challenge infection requires T and B cells harvested during that phase of the primary immune response to the malaria parasite when the host is actively expressing immunity to an ongoing infection, protective lymphocytes tend to be cells capable of rapid replication. In none of the experiments performed herein did the adoptive transfer of lymphocytes, regardless of source or state of immunological commitment, suppress the ascending acute blood infection. Instead, protection was indicated by the subsequent suppression of parasitaemia. This was in keeping with previous studies of adoptive immunity to rodent malarias (Brown et al 1976 a & b, Gravely & Kreier 1976, McDonald & Phillips 1978, 1980, Brinkmann et al 1985, Cavacini et al 1986) who showed that in various levels of immunological suppression, infected mice and rats inoculated with immune lymphocytes exhibited a protective response demonstrable by reduced parasitaemia, quickened pRBC elimination and/or host survival.

In sublethally irradiated mice, the protective effect of unfractionated lymphocytes

was greater than that of either enriched semi-immune T or B cells, although both of these subsets did give effective immunity. This maximal protection was observed as a combination of the immune responses conferred by either lymphocyte subset when transferred alone, and is suggestive of a synergistic effect between the inoculated cells of mixed phenotype with each other, and also possibly with the radioresistant lymphocytes circulating in the irradiated recipient. Working with the same host-parasite combination, McDonald & Phillips (1978) also showed that for the most efficient protection of irradiated mice against P. c. chabaudi infection with immune syngeneic cells (from donor mice infected at least twice), a population containing both T and B cells, such as unfractionated spleen cells, was required. However, they failed to show unequivocally synergistic activity between primed lymphocyte subsets in irradiated mice. The fact that enriched subpopulations of both T and B lymphocytes taken from donor mice during resolution of primary acute infection imparted some protection to recipients, as shown here, implies that both lymphocyte subsets may be required for the elaboration of maximal acquired immunity. This finding is in concurrence with those of McDonald & Phillips (1978, 1980) and of Favila-Castillo et al (1990). McDonald & Phillips (1978) have shown that most but not all of the protective activity on adoptive transfer of P. c. chabaudi immune spleen cells lay in the B cell-enriched fraction; Ferraroni & Speer (1982) had a similar result with P. berghei-primed mouse splenic lymphocytes. Brown et al (1976 a) demonstrated protective activity of T-enriched populations from the spleen of P. berghei infected rats upon adoptive transfer to naive, syngeneic recipients, but there was increased protection on the addition of B cells. Moreover, in this study, it was reported that immunity could be transferred with lymphocytes taken as early as 11 d after infection of the cell donors, although greater levels of protective activity were achieved using spleen cells collected later in the infection.

In a similar study, Gravely & Kreier (1976) concluded that the bulk of the protection conferred to rats infected with P. berghei resided in the differentiated splenic B cell population. The enriched splenic T cell fraction was generally less protective than the B cells or the mixed T & B lymphocyte pool, and they considered that the primed T cells were acting as helper cells in the production of specific anti-P. berghei protective Ab. This suggested role for T cells was first proposed by Brown (1974). He reported that lymphocyte suspensions from rats immune to P. berghei and depleted of the B cell fraction were capable of conferring immunity to subsequent challenge of

naive recipients, and interpreted this as meaning that the inoculated immune T cells were acting as helpers in the humoral response driven by the B cell complement of the infected host. For P. berghei and P. c. chabaudi, which are known to undergo antigenic variation, it can be argued that the enriched B cell population recovered from mice which have cleared at least one infection contains B memory cells which are already primed to new variant-specific Ags as they emerge during recrudescent parasitaemias, as well as to determinants common to all parasite variants, and are thus equipped to synthesise specific protective Abs. Th memory cells, in contrast, may only respond to Ags common to all plasmodial variants but characteristic of the strain producing infection. As B cells are more radiosensitive than T cells (Janeway 1975 b), irradiation treatment may lead to adoptive transfer recipients having insufficient B cells, virgin or primed, to cooperate with to generate variant-specific protective Abs rapidly. This was suggested by McDonald & Phillips (1978) as the reason for their finding that recrudescences occurred most frequently in 6 or 8 Gy-irradiated mice receiving an enriched immune T cell population. In the studies described herein, all patent parasitaemias recrudesced in sublethally irradiated mice adoptively transferred with lymphocyte populations, regardless of phenotype, and this indicates that this may have been caused by something other than a specific defect in the T cell repertoire. That recrudescent parasitaemias were a consistent feature of infection of mice infused with either peripheral blood or splenic lymphocytes, regardless of origin or type, may have been due to the fact that the heterogeneous populations of lymphocytes transferred presumably had a range of different specificities. It is, however, more probably a reflection of the fact that the cells transferred were immunologically not fully mature. This perhaps was to be expected as the lymphocytes were collected from donor animals on d 12-13 p.i., when it was likely that only the predominant antigenic type, that of the infecting parasite population, had been encountered. Thus, neither B or T cells were primed to variant-specific Ags present on the surface of pRBC emerging in the bloodstream under immunological pressure. However, an Ab-mediated response was clearly detectable in recipients of adoptively transferred cells; that there was no significant difference in the specific anti-P. c. chabaudi Ab profiles and endpoint titres for recipients of semi-immune T, B or T & B lymphocytes does indeed suggest that the B cells present failed to recognise a wider antigenic repertoire that did the T cells, hence the similar kinetics with which a protective response arose. Indeed, in sublethally irradiated

mice, recipients of splenic B cells alone exhibited a recrudescence of larger magnitude, if lesser duration, than mice reconstituted with splenic T cells. It can be inferred from these collective results that protection to P. c. chabaudi is predominantly due to a humoral response, although other mechanisms may play a part. After all, although mice receiving primed lymphocytes taken on d 12-13 p.i. showed patent recrudescences, infection was cleared effectively. As these donor cells were collected from mice at a time when it is thought that non-specific mechanisms of immunity, such as the release of toxic mediators by activated macrophages, predominate in the immune response to P. c. chabaudi (Langhorne 1989), lymphocytes, in particular the T cell subset, taken at this time from donor mice may engender protection by this pathway.

In other rodent models of malaria, immune lymphocytes appear to resolve primary infection by non-humoral mechanisms. This has been shown for P. yoelii 17X in CBA/Ca or C57BL mice (Jayawardena et al 1982, Brinkmann et al 1985), an Ab-dependent system, and also in P. chabaudi adami in BALB/c mice (Cavacini et al 1986), an Ab-independent system. Brinkmann et al (1985) concluded that resident T cells of recipient animals were required for expression of adoptively transferred anti-P. yoelii immunity and that the failure to confer protection to nude mice was due to their congenital T cell depletion. By reconstituting with naive T cells, but not with B cells, before adoptive transfer, a protective immune response could be restored to the recipient animals. These findings contrast with the results of Jayawardena et al (1982), who transferred protection to mice which were selectively T cell depleted by adult thymectomy, irradiation and bone marrow reconstitution prior to adoptive transfer. This discrepancy may have been due to nude mice lacking a cell population relevant to the development of protection, which is present in adult-thymectomised and normal uninfected mice alike. In the case of athymic mice infected with P. c. adami, a high-grade, fulminating infection developed; however, T lymphocytes were shown to mediate protection in recipient mice, since T cell-, but not B cell-enriched spleen cell fractions suppressed challenge infection in otherwise susceptible nude mice (Cavacini et al 1986). In these cases, protection was achieved by activating as yet unidentified inhibiting cell systems; these may well be the same as those non-Ab mediated responses that may account for the immune reactivity of semi-immune T cells that protect against a primary challenge of P. c. chabaudi. Certainly, in this study, in which T and B cells were collected at the time of lymphocytosis, transfer of

an enriched T cell population led to a similar pattern of primary parasitaemia in recipient mice as in those inoculated with a mixed unfractionated preparation. Furthermore, animals reconstituted with T cells alone had a significantly lower peak parasitaemia than did recipients of an enriched B cell population, and cleared the acute infection to subpatency far more rapidly. These findings implicate a role for cell-mediated immunity other than T cell help, in protection of NIH mice against P. c. chabaudi AS.

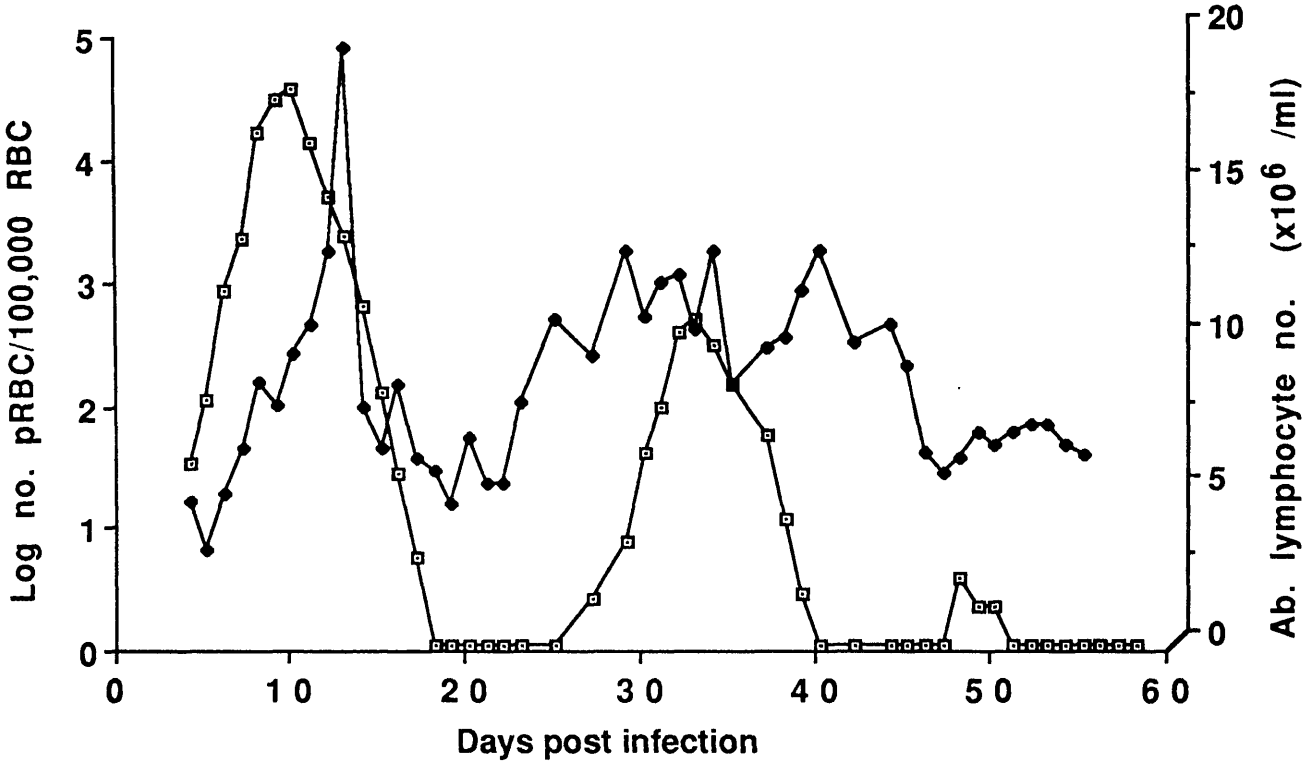


Fig. 3.2.1

Parasitaemia

Absolute lymphocyte count ($x10^6$/ml)

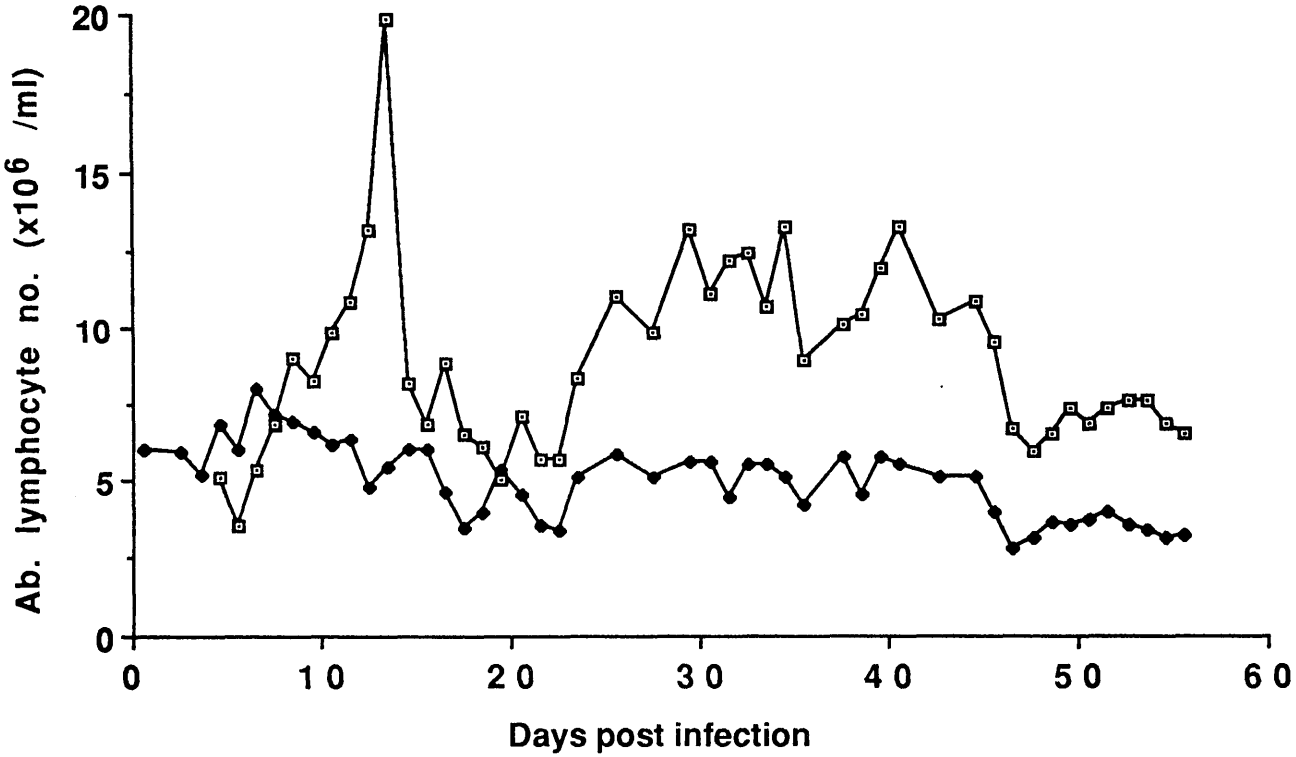


Fig. 3.2.2

Absolute lymphocyte count in infected mice

Absolute lymphocyte count in naive control mice

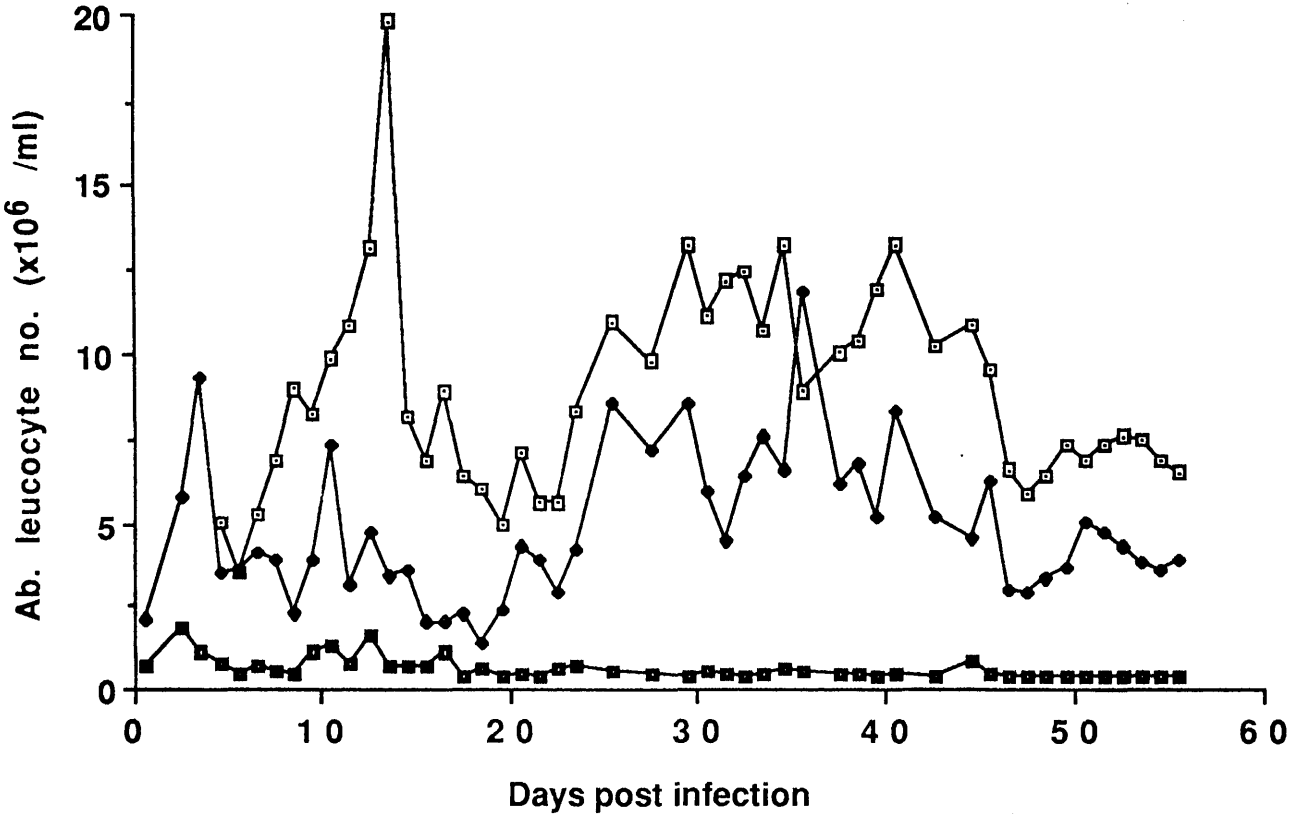


Fig. 3.2.3

Absolute lymphocyte count (x10^6/ml)

Absolute neutrophil count (x10^6/ml)

Absolute monocyte count (x10^6/ml)

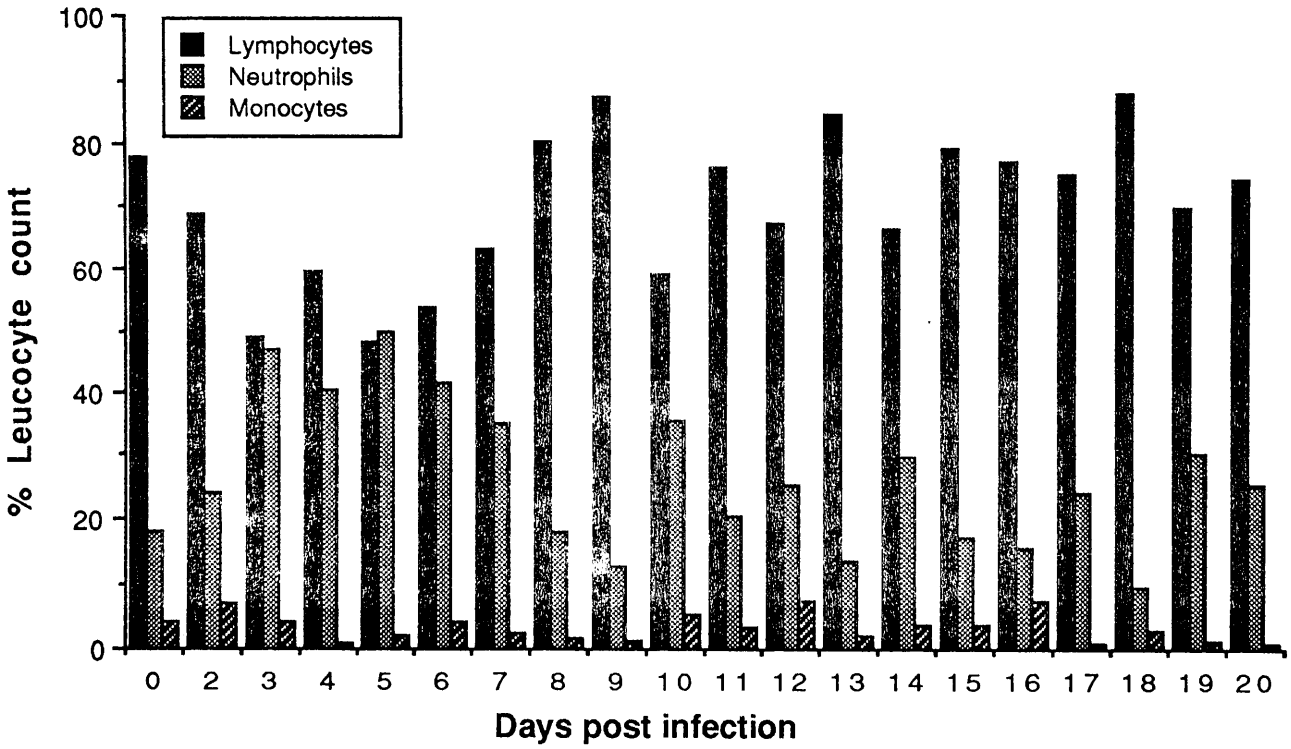


Fig. 3.2.4 Relative leucocyte counts in the peripheral blood of P. c. chabaudi-infected NIH mice.

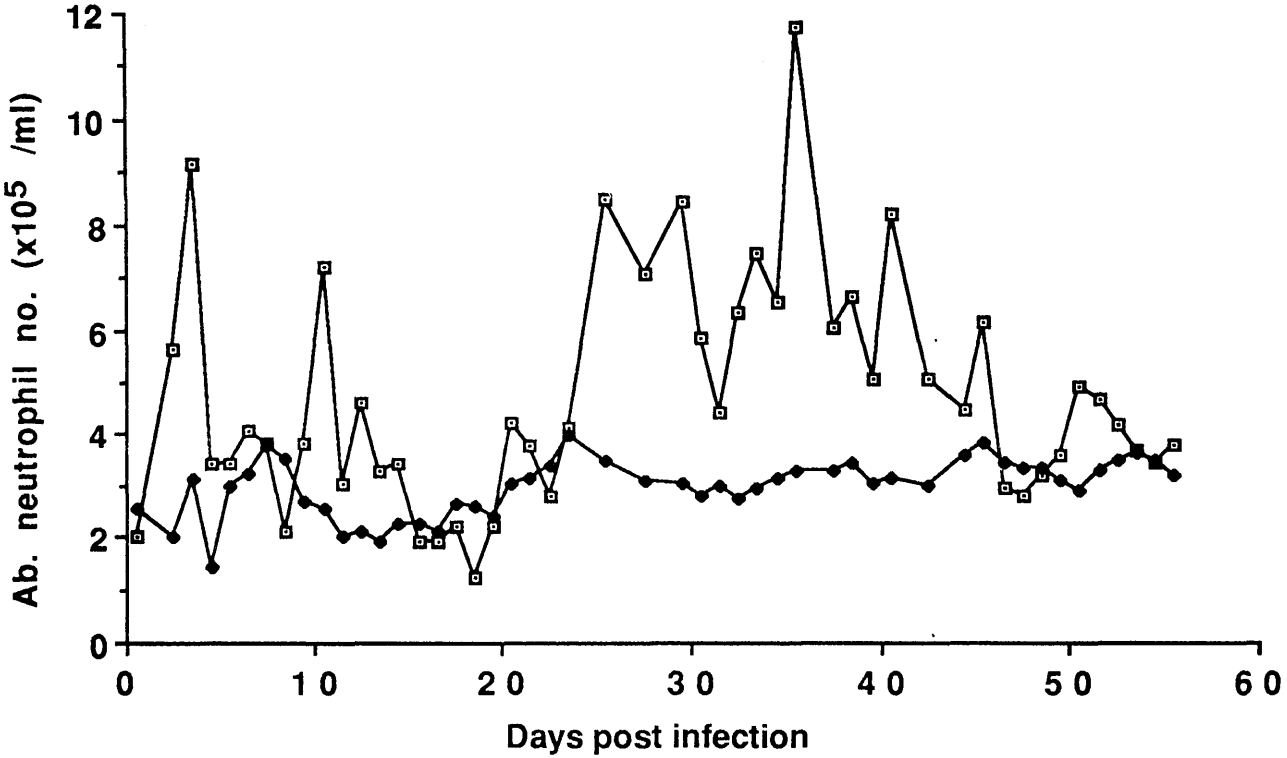


Fig. 3.2.5

Absolute neutrophil count in infected mice

Absolute neutrophil count in naive control mice

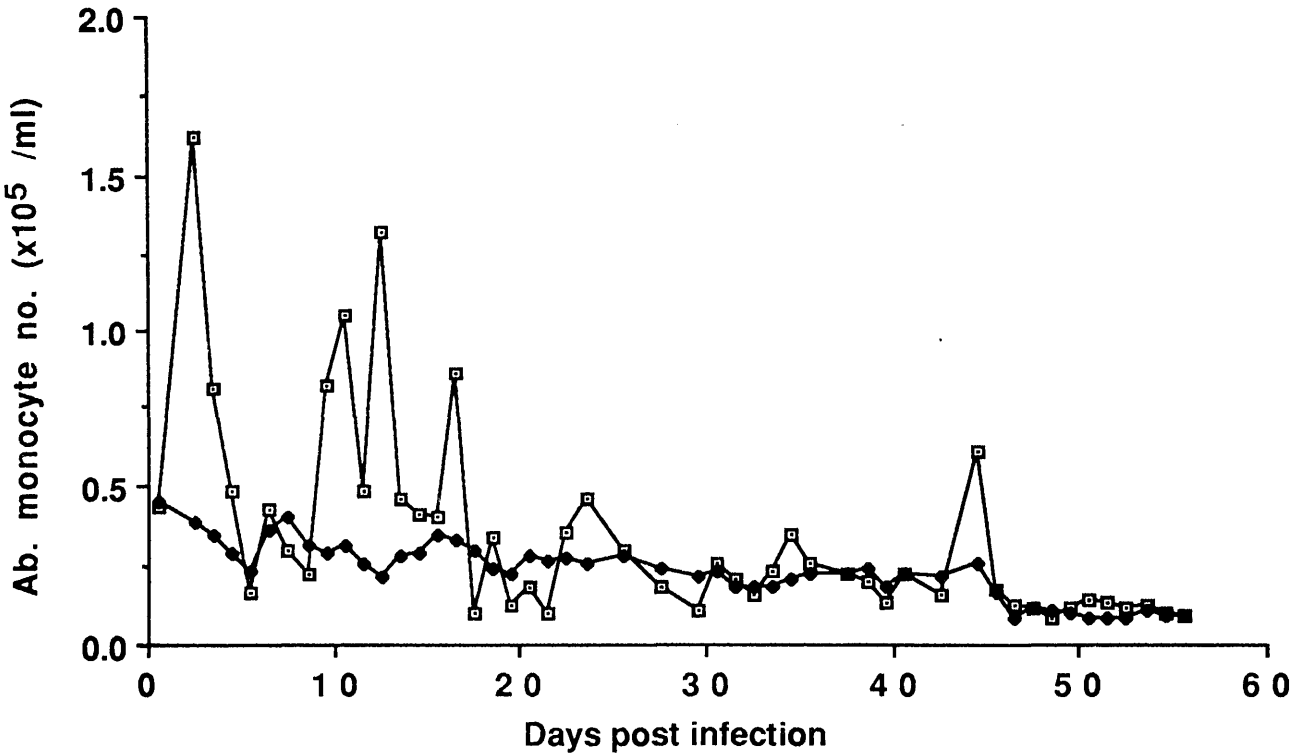


Fig. 3.2.6

Absolute monocyte count in infected mice

Absolute monocyte count in naive control mice

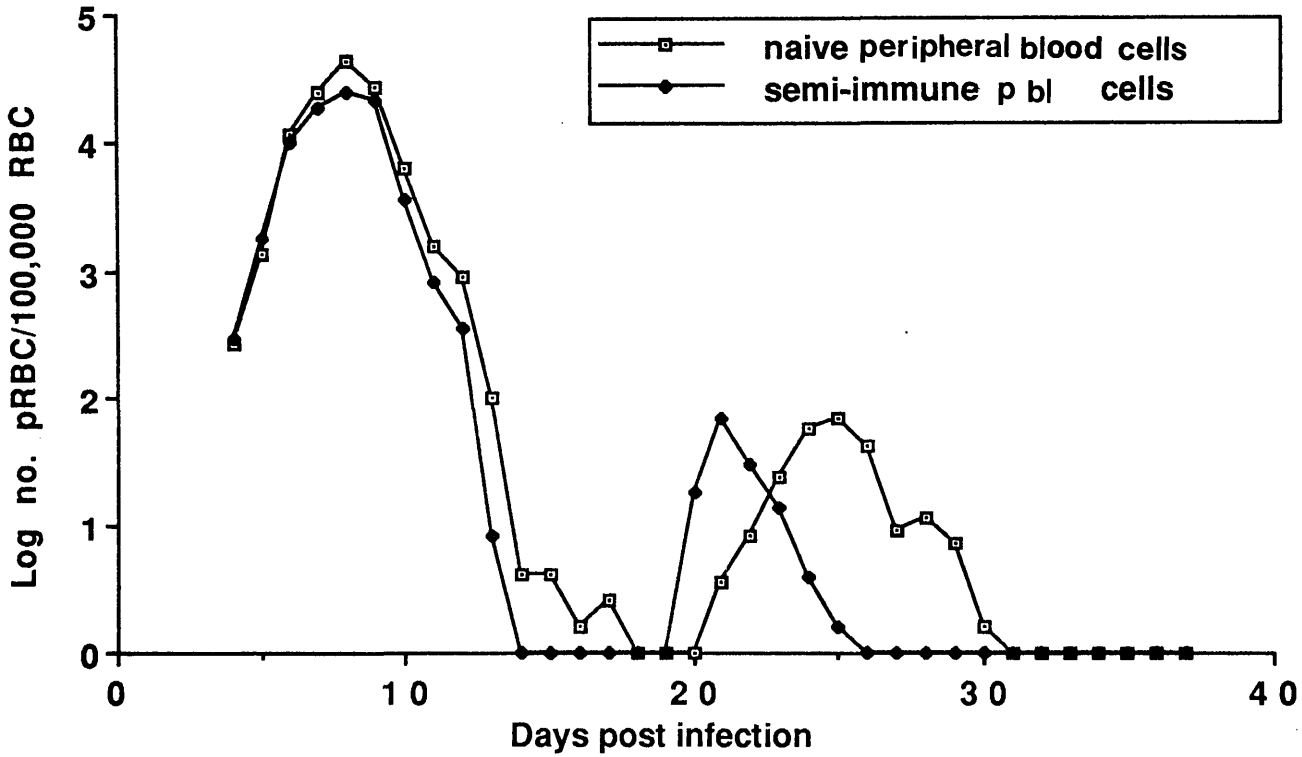


Fig. 3.3.1 Course of infection in naive NIH recipients of peripheral blood lymphocytes challenged with 1 x10^5 pRBC i.v..

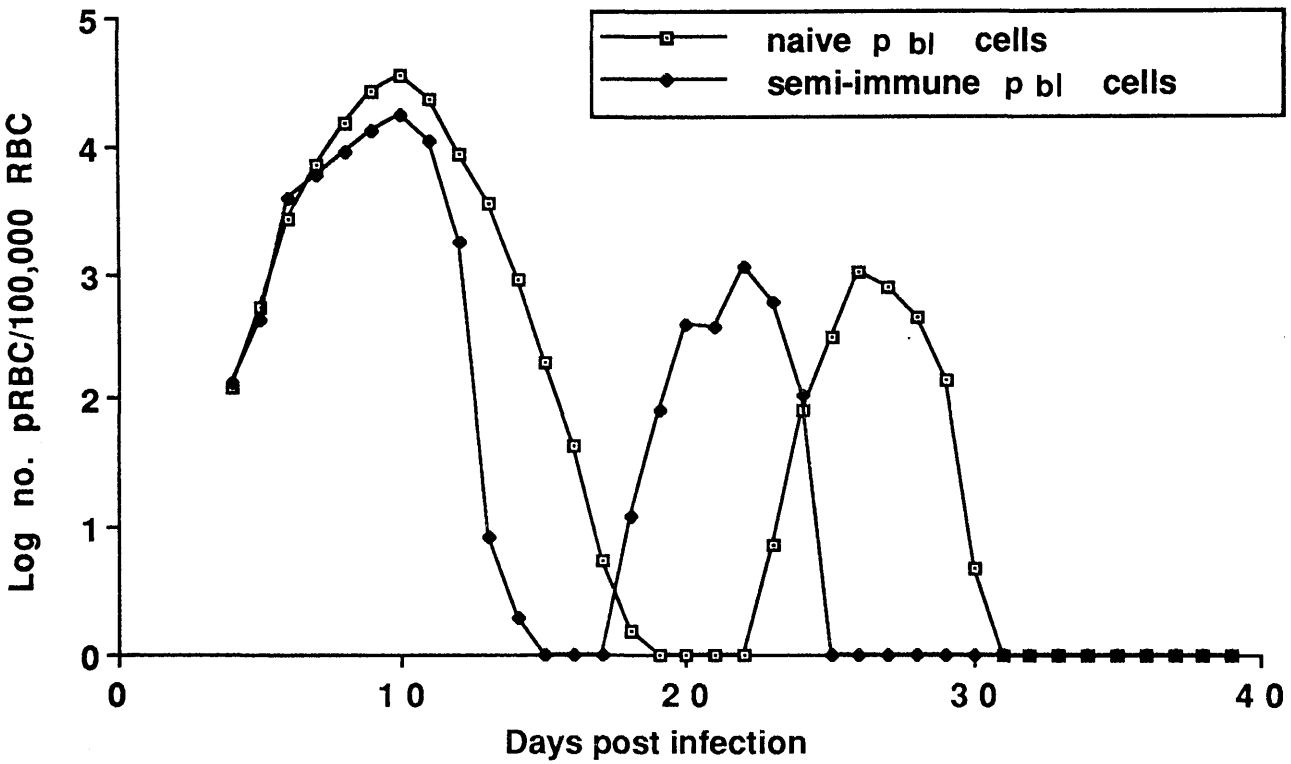


Fig. 3.4.1 Course of infection in sublethally irradiated NIH recipients of periph bld lymphocytes challenged with 1 x10^5 pRBC i.v..

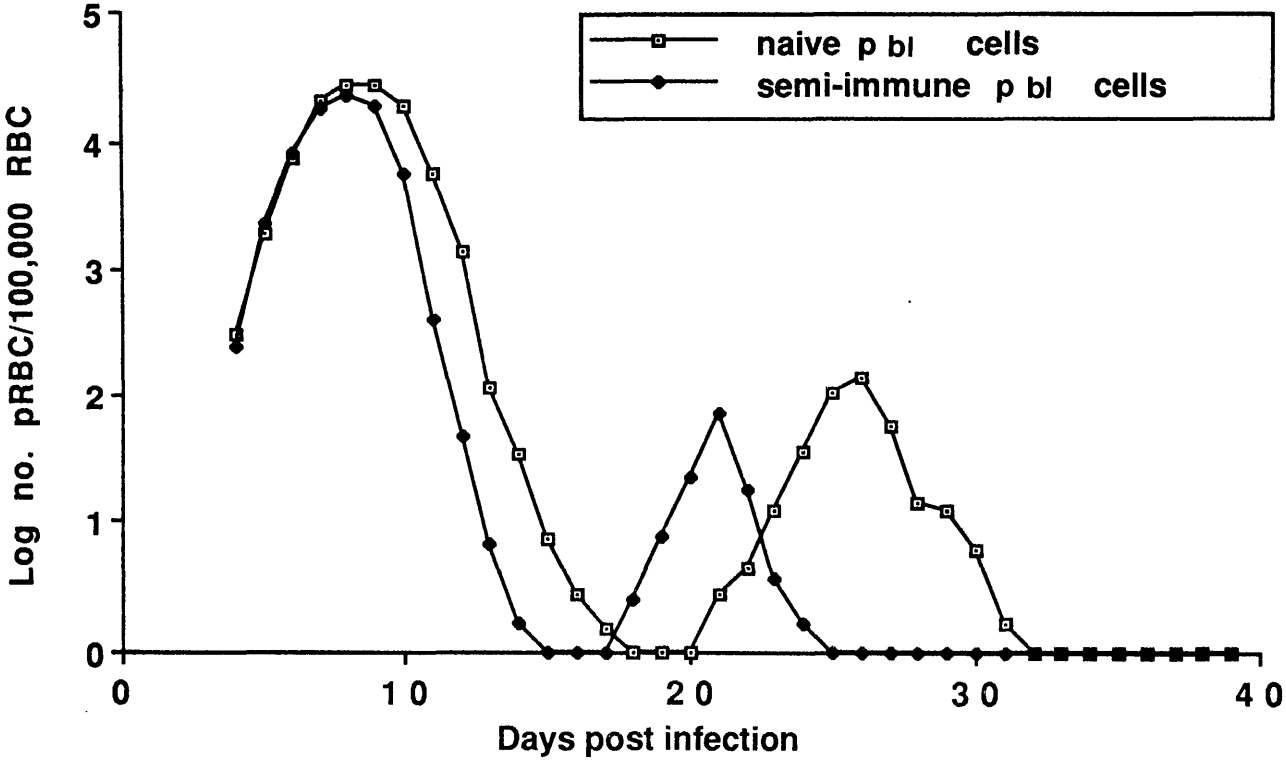


Fig. 3.4.2 Course of infection in naive NIH recipients of peripheral blood lymphocytes challenged with 1 x10^5 pRBC i.v..

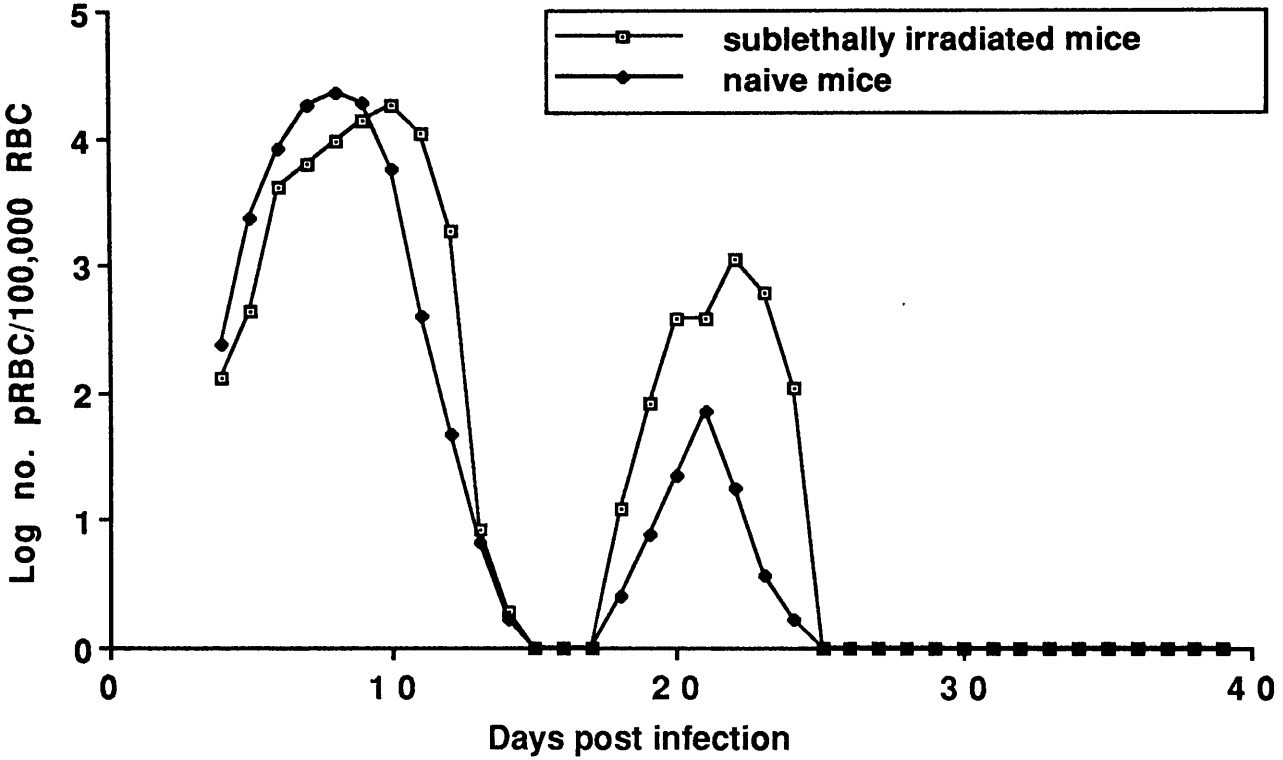


Fig. 3.4.3 Course of infection in NIH recipients of semi-immune peripheral blood lymphocytes challenged with 1 x10^5 pRBC i.v..

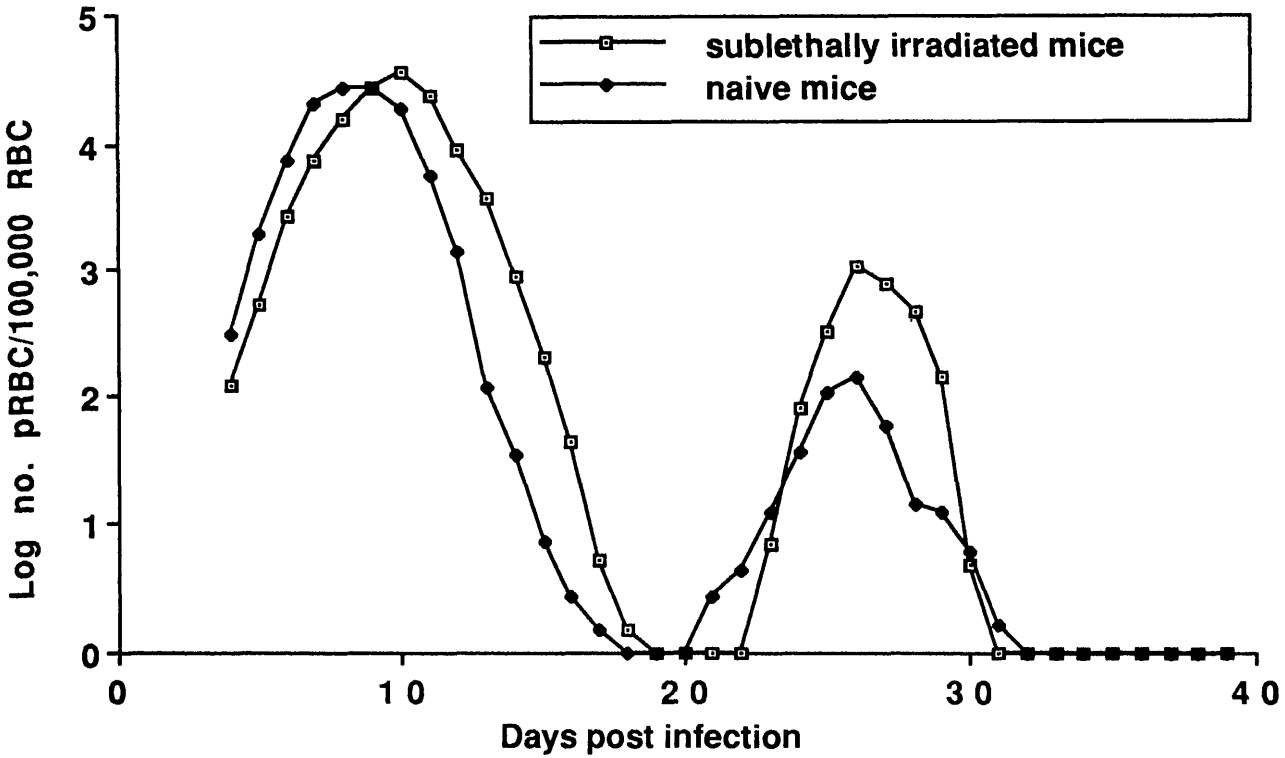


Fig. 3.4.4 Course of infection in NIH recipients of naive peripheral blood lymphocytes challenged with 1 x10^5 pRBC i.v..

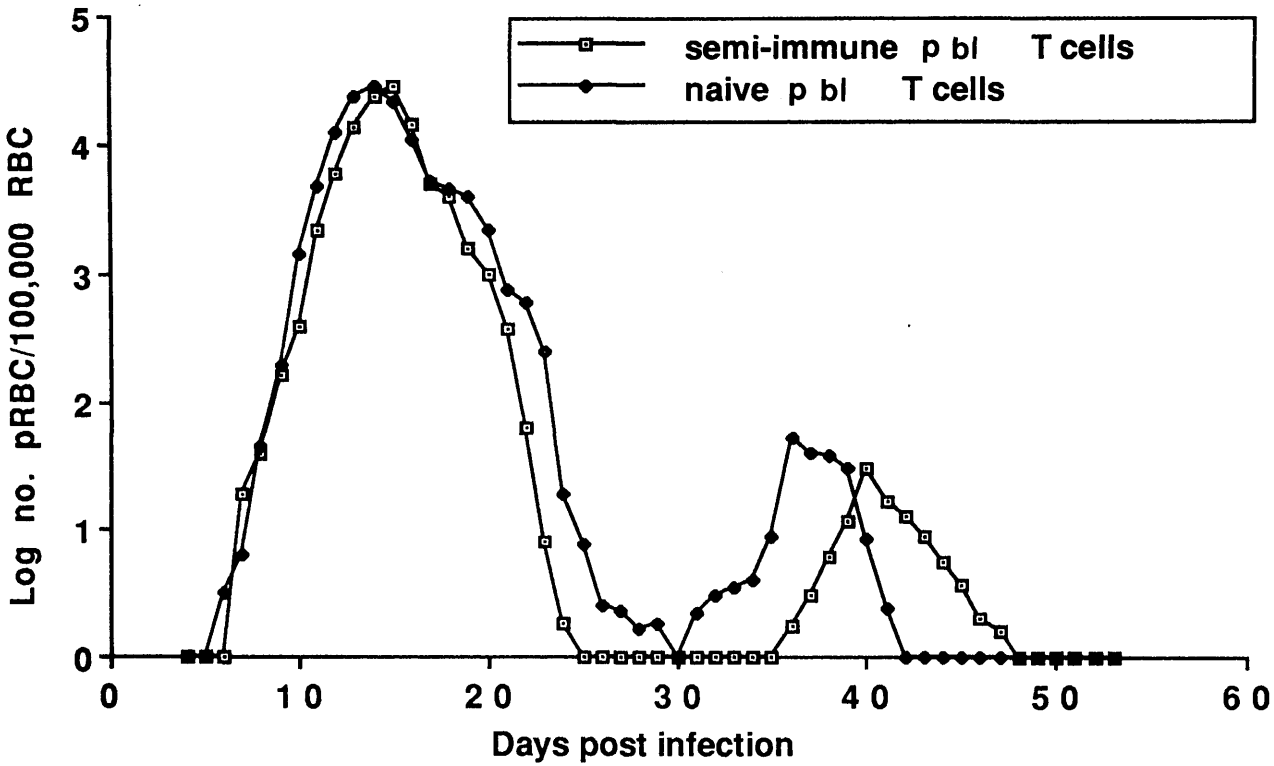


Fig. 3.5.1 Course of infection in sublethally irradiated NIH recipients of periph bld T lymphocytes challenged with 1 x10^5 pRBC i.v..

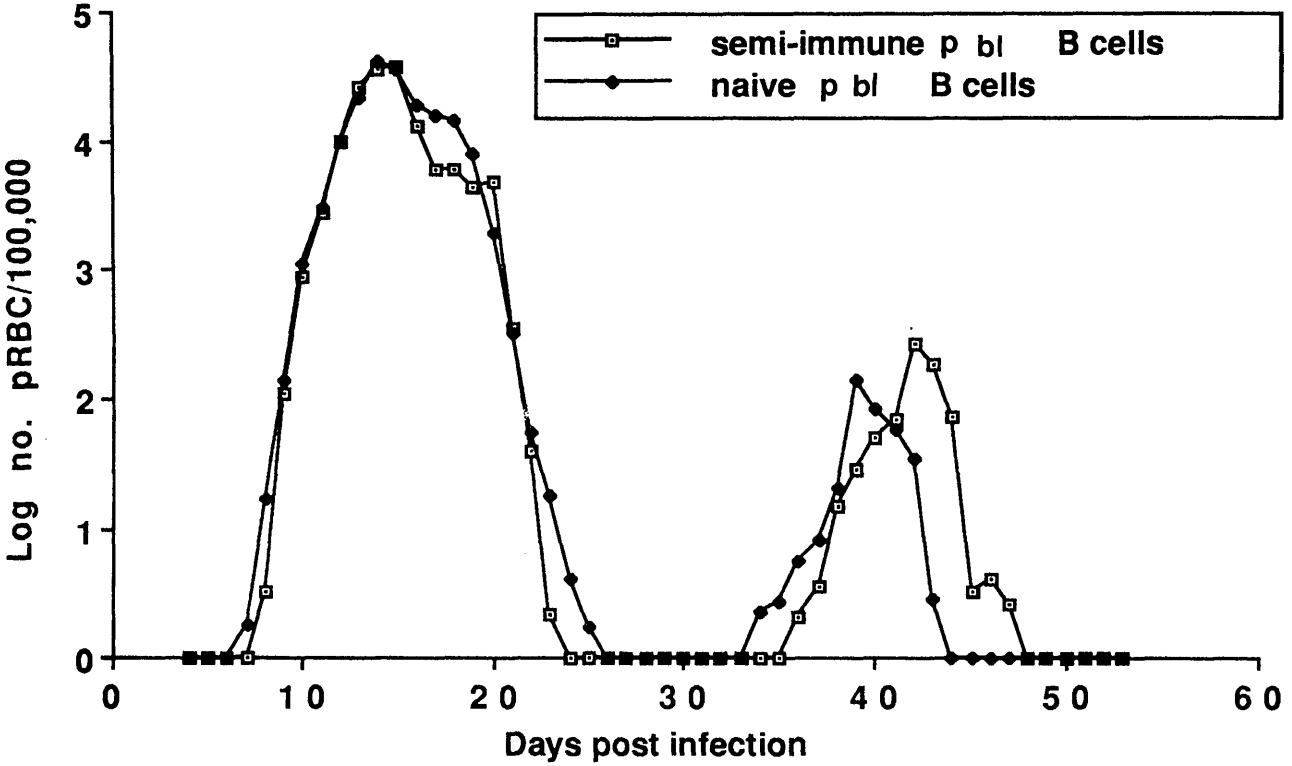


Fig. 3.5.2 Course of infection in sublethally irradiated NIH recipients of periph bld B lymphocytes challenged with 1 x10^5 pRBC i.v..

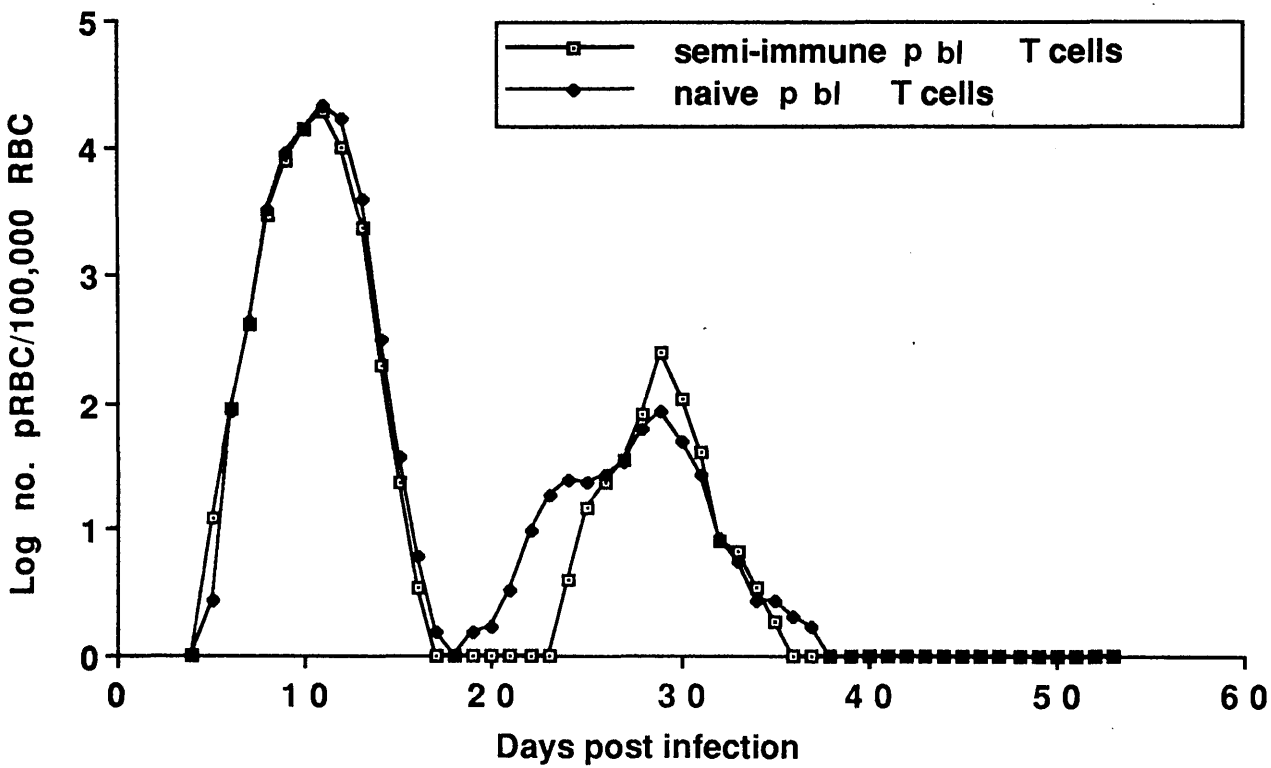


Fig. 3.5.3 Course of infection in naive NIH recipients of periph bld T lymphocytes challenged with 1 x10^5 pRBC i.v..

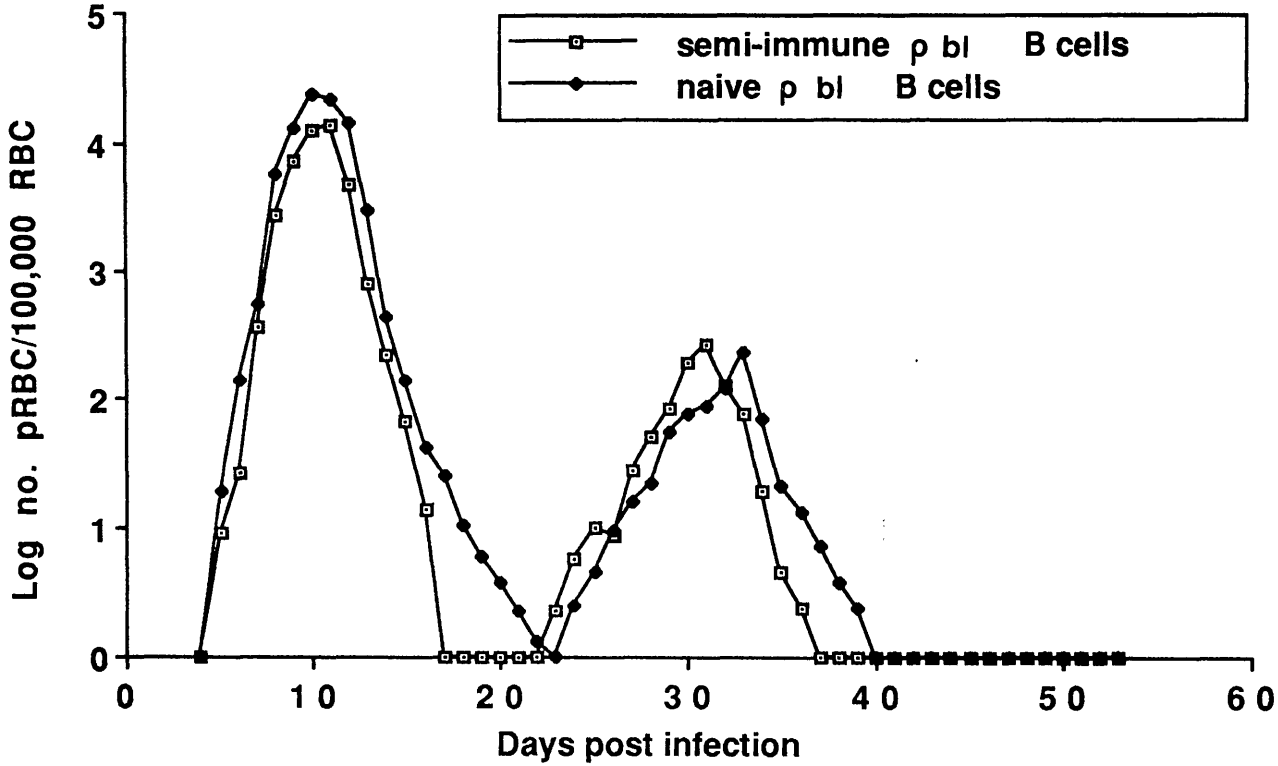


Fig. 3.5.4 Course of infection in naive NIH recipients of periph bld B lymphocytes challenged with 1 x10^5 pRBC i.v..

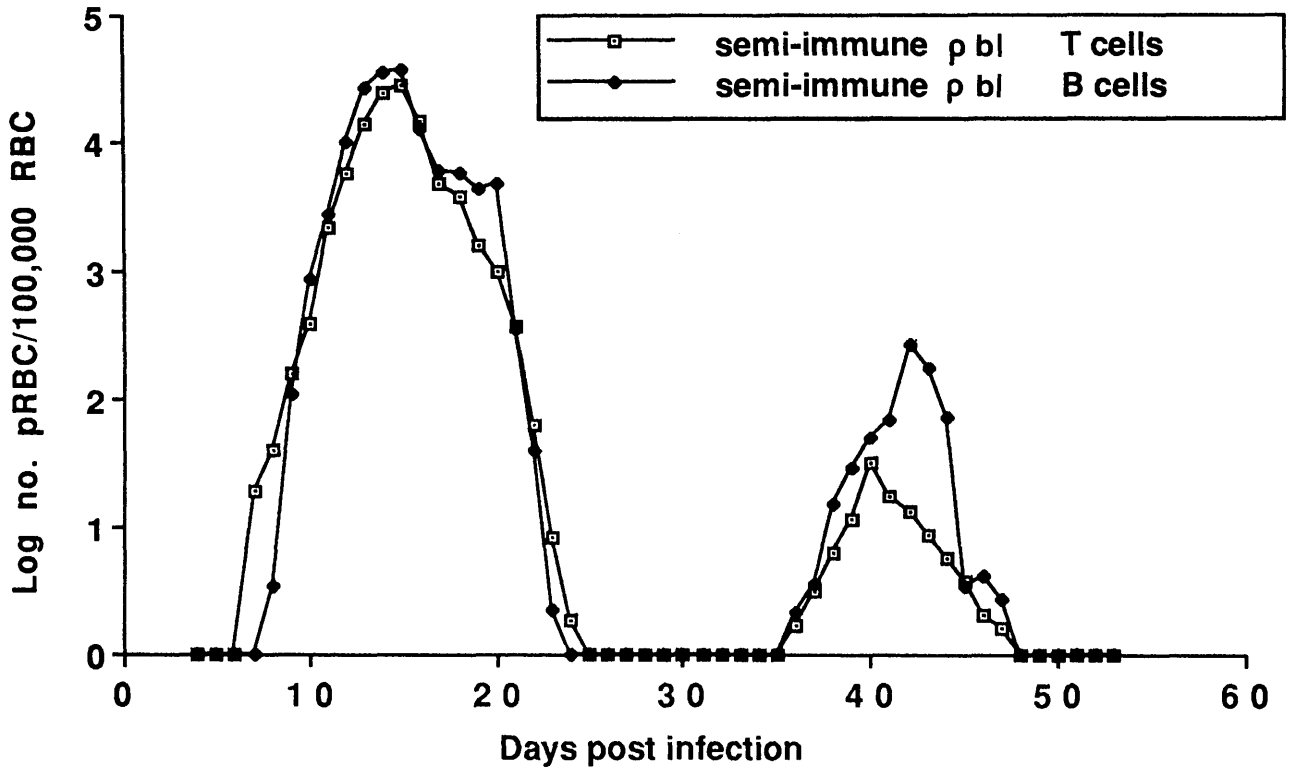


Fig. 3.5.5 Course of infection in sublethally irradiated NIH recipients of semi-immune periph bld lymphocytes challenged with 1 x10^5 pRBC i.v..

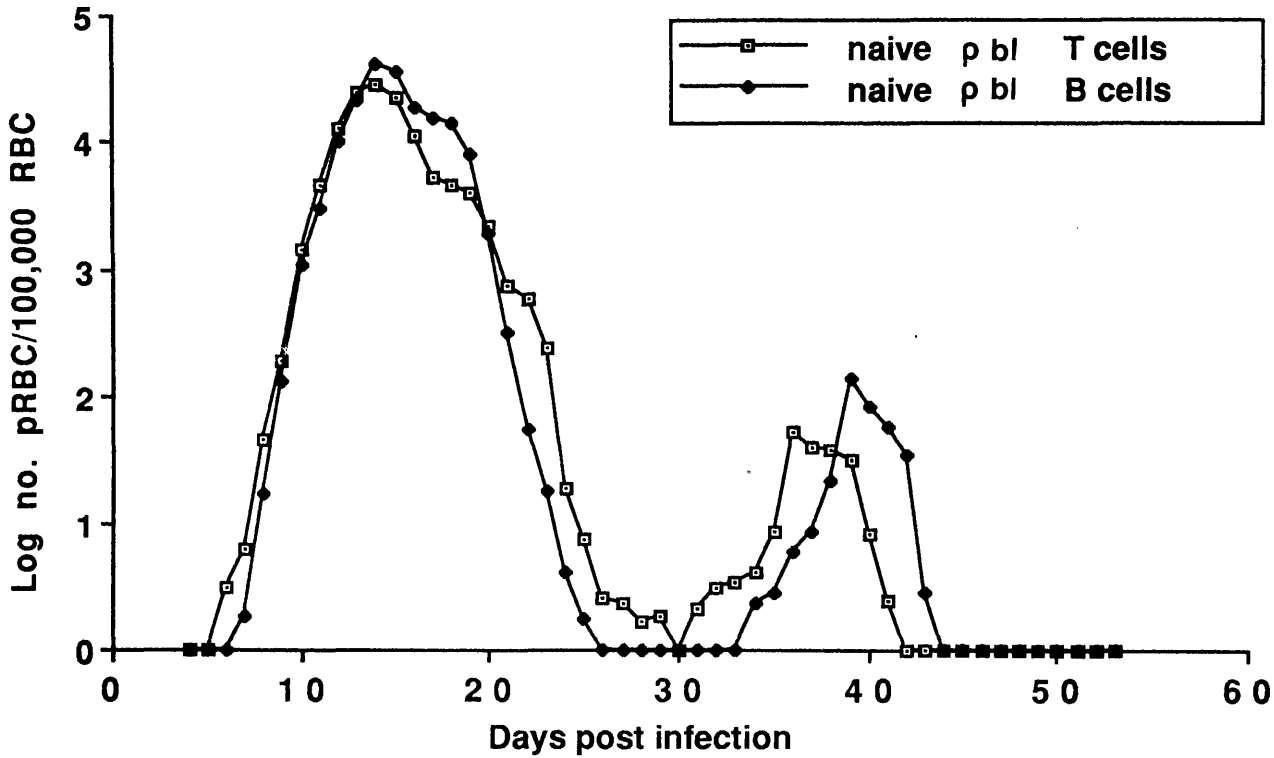


Fig. 3.5.6 Course of infection in sublethally irradiated NIH recipients of naive periph bld lymphocytes challenged with 1 x10^5 pRBC i.v..

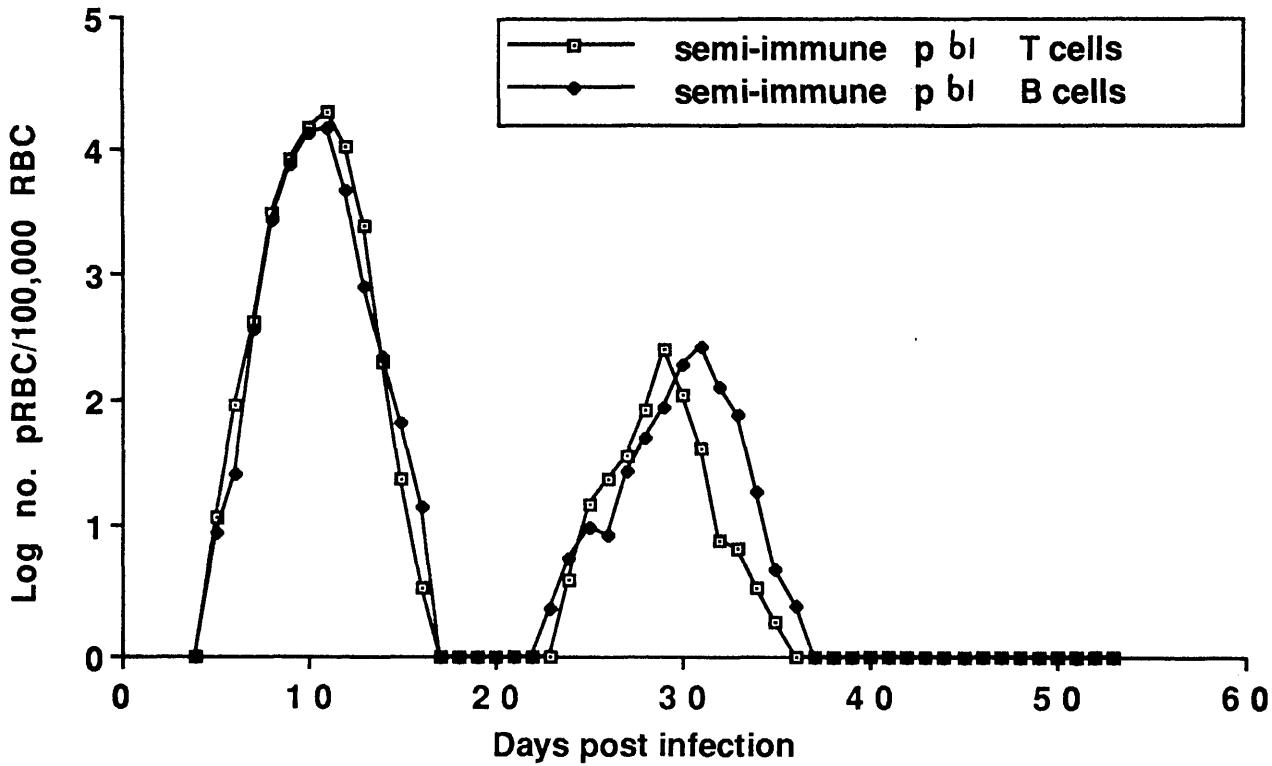


Fig. 3.5.7 Course of infection in naive NIH recipients of semi-immune periph bld lymphocytes challenged with 1 x10^5 pRBC i.v..

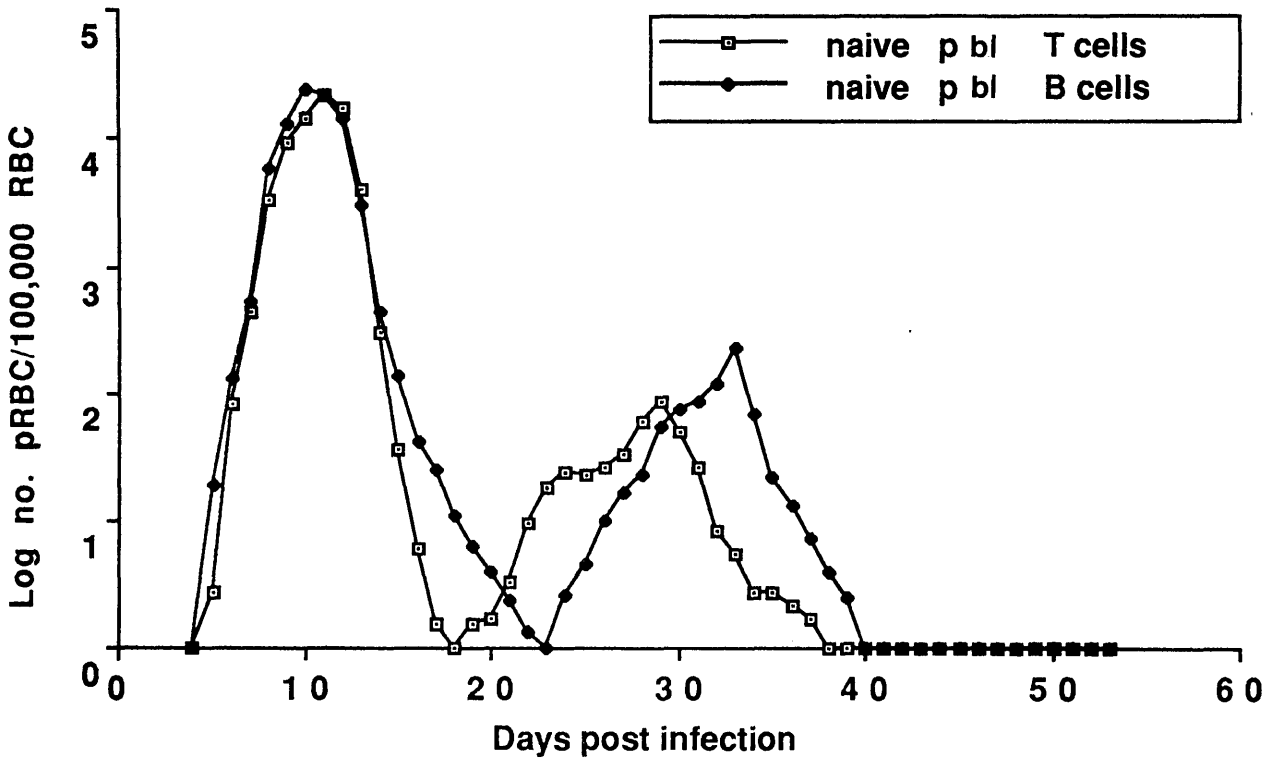


Fig. 3.5.8 Course of infection in naive NIH recipients of naive peripheral lymphocytes challenged with 1 x10^5 pRBC i.v..

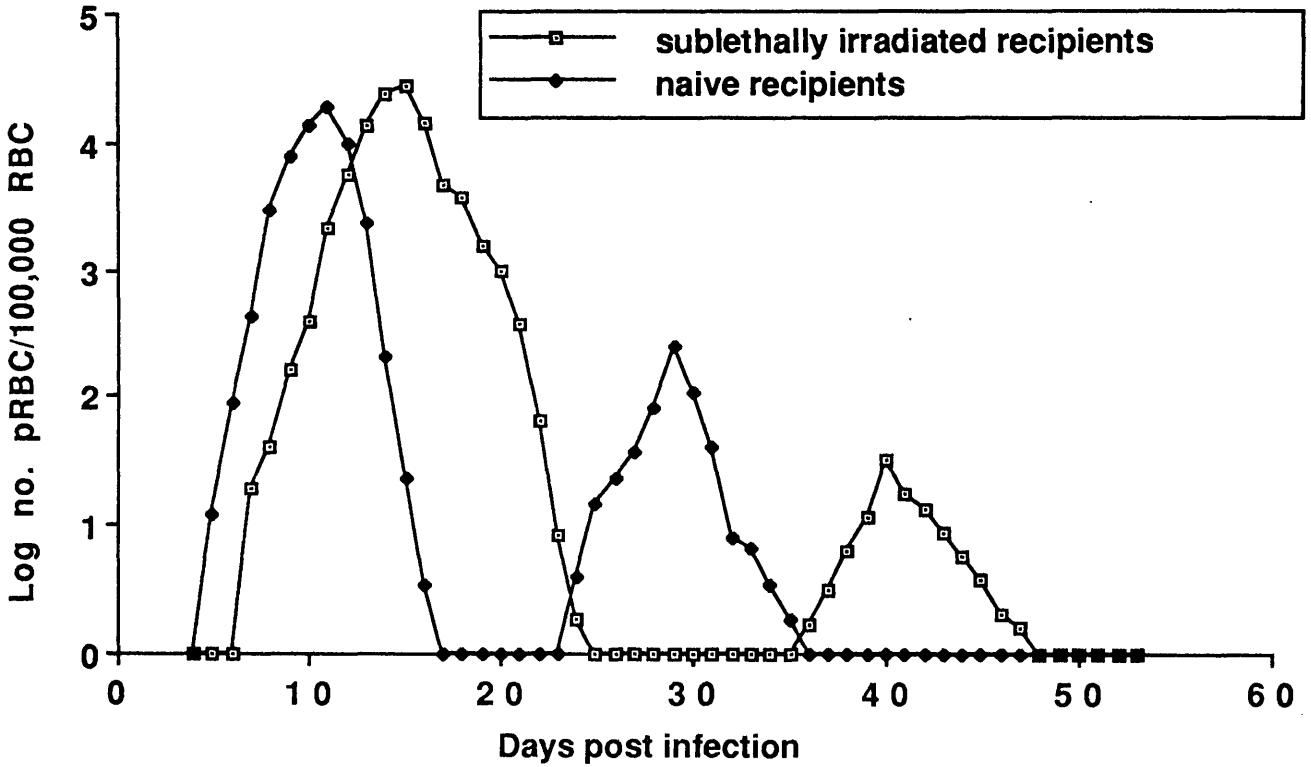


Fig. 3.5.9 Course of infection in NIH recipients of semi-immune peripheral blood T lymphocytes challenged with 1 x10^5 pRBC i.v..

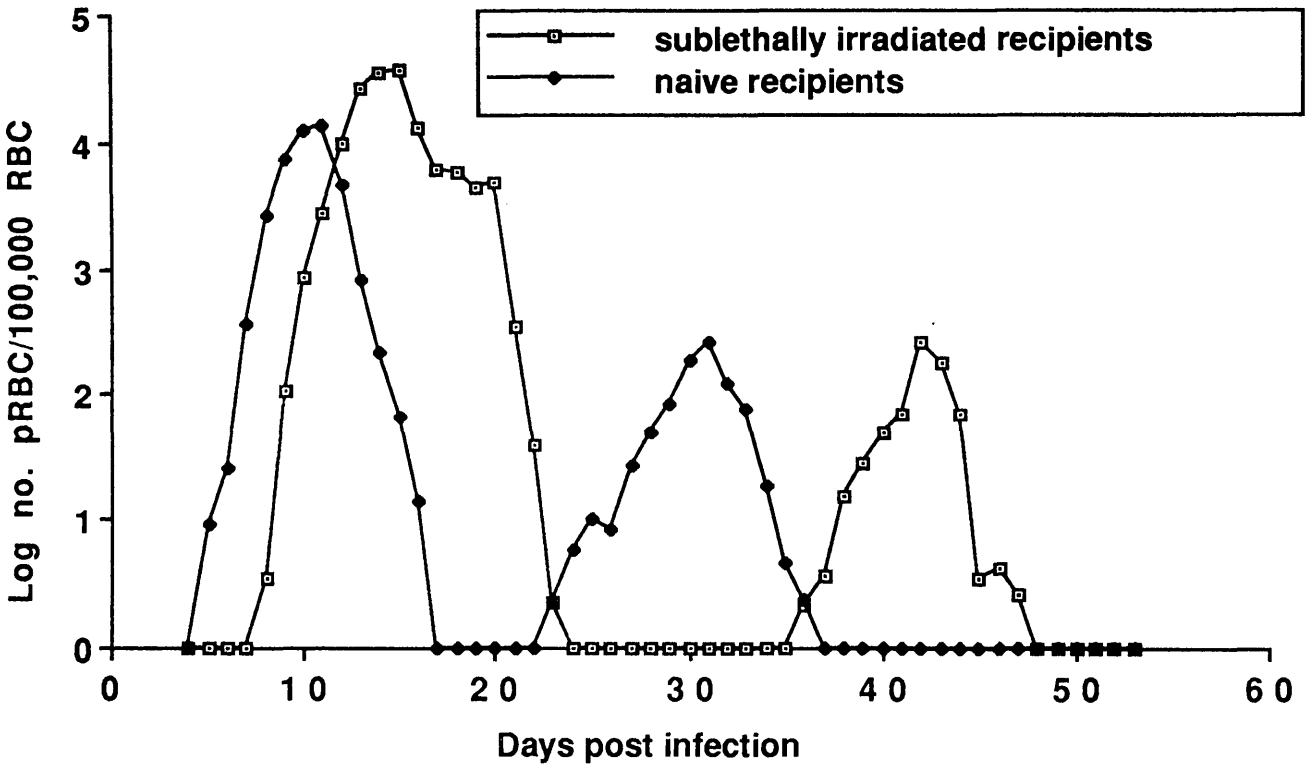


Fig. 3.5.10 Course of infection in NIH recipients of semi-immune peripheral blood B lymphocytes challenged with 1 x10^5 pRBC i.v..

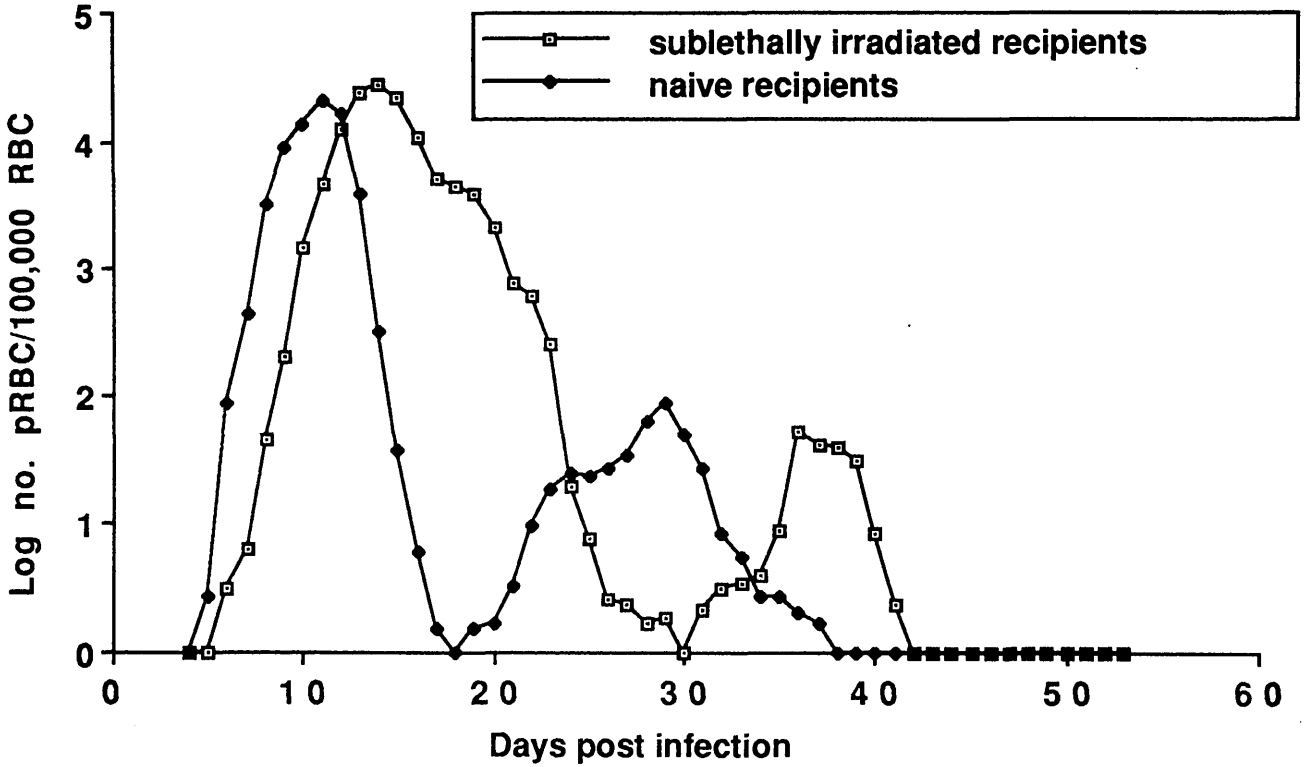


Fig. 3.5.11 Course of infection in NIH recipients of naive peripheral blood T lymphocytes challenged with 1 x10^5 pRBC i.v..

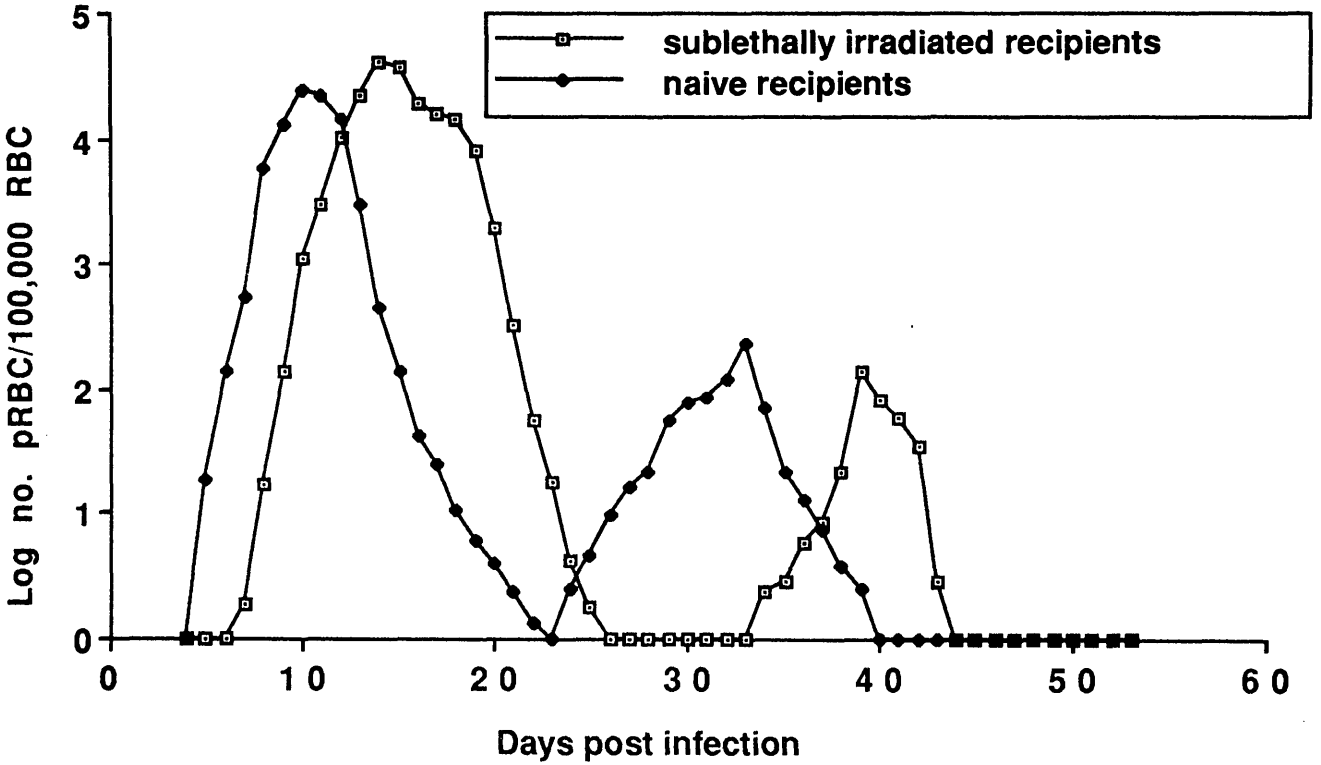


Fig. 3.5.12 Course of infection in NIH recipients of naive peripheral blood B lymphocytes challenged with 1 x10^5 pRBC i.v..

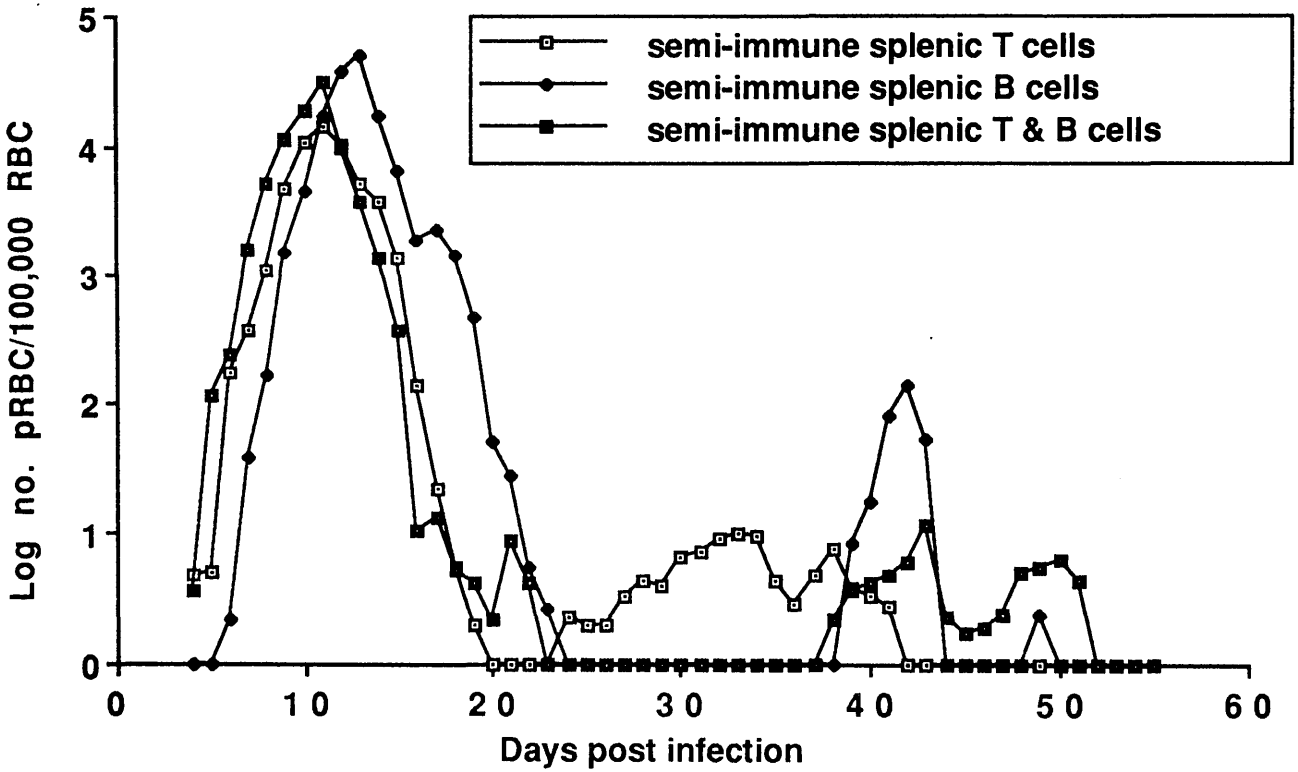


Fig. 3.6.1 Course of infection in sublethally irradiated NIH recipients of semi-immune splenic lymphocytes challenged with 1 x10^5 pRBC i.v..

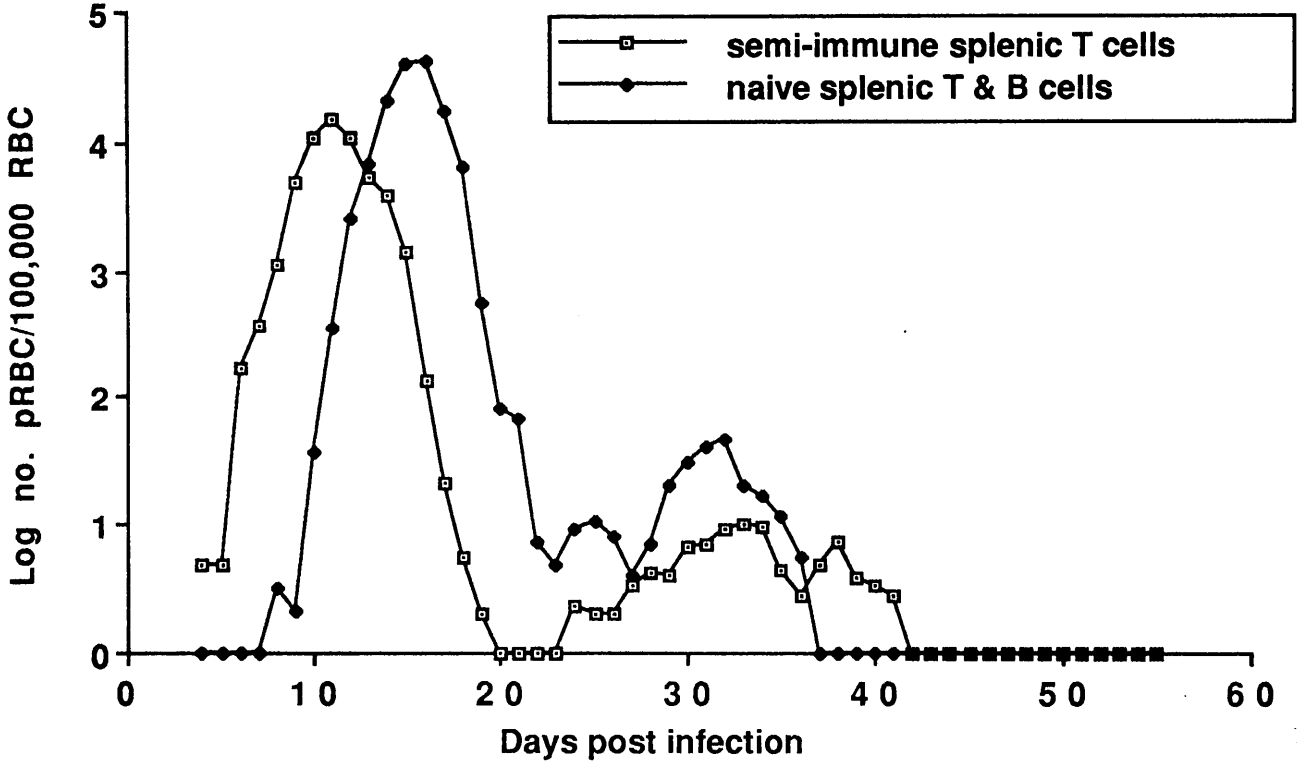


Fig. 3.6.2 Course of infection in sublethally irradiated NIH recipients of splenic lymphocytes challenged with 1 x10^5 pRBC i.v..

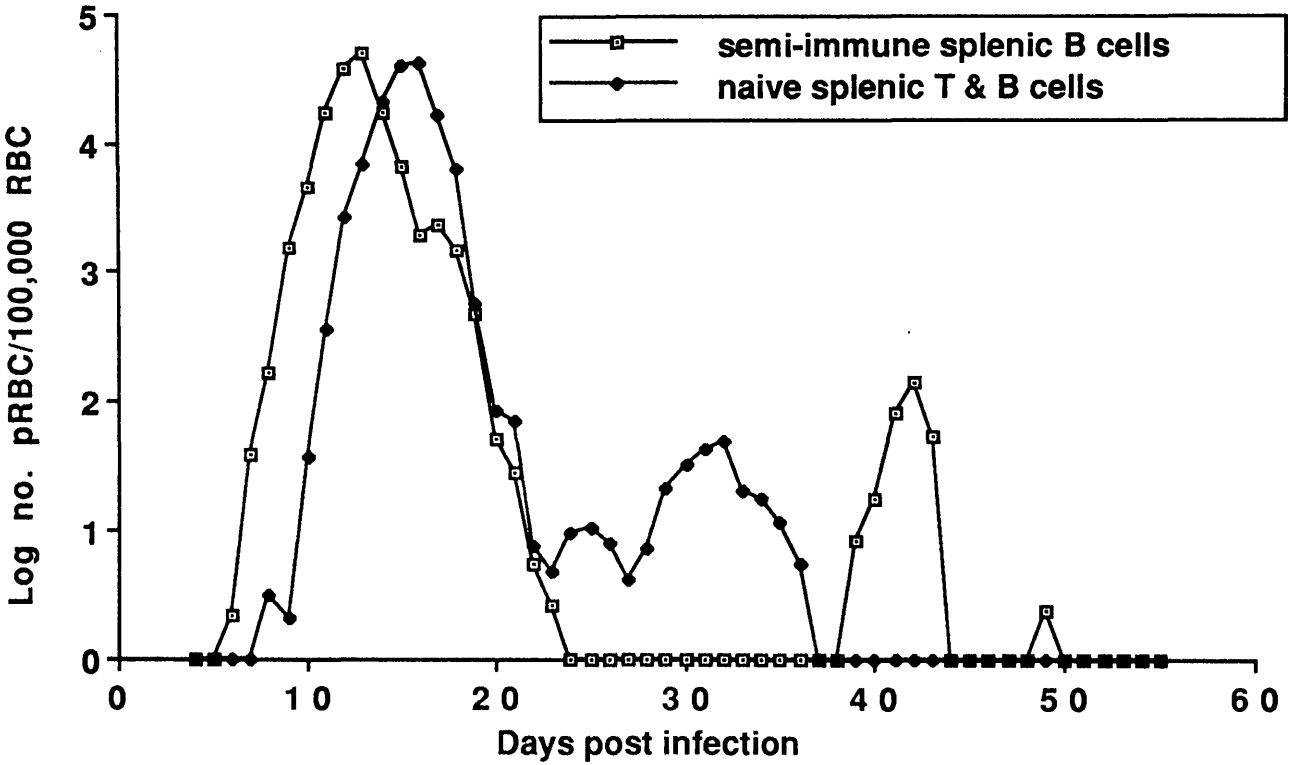


Fig. 3.6.3 Course of infection in sublethally irradiated NIH recipients of splenic lymphocytes challenged with 1 x10^5 pRBC i.v..

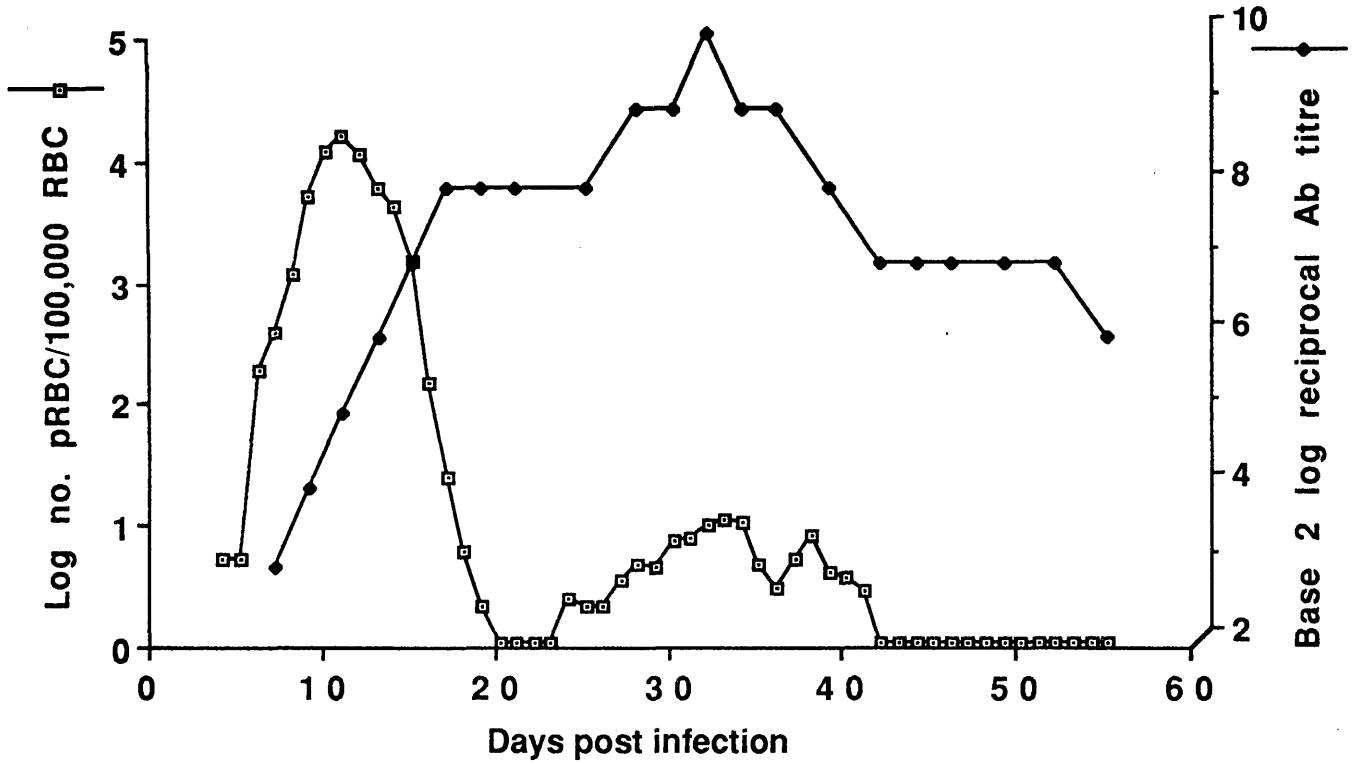


Fig. 3.6.4 The serum antibody titre during the course of infection in sublethally irradiated NIH recipients of semi-immune splenic T lymphocytes challenged with 1 x10^5 pRBC i.v..

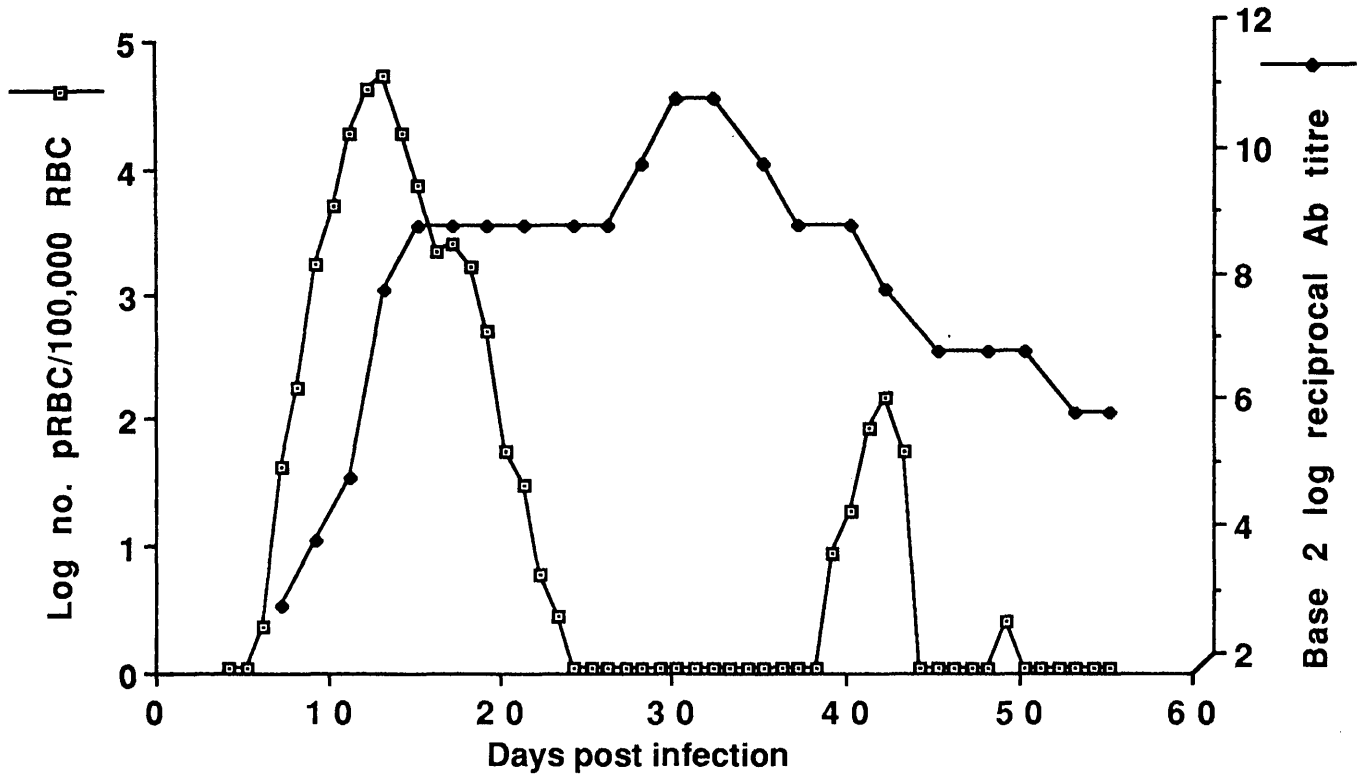


Fig. 3.6.5 The serum antibody titre during the course of infection in sublethally irradiated NIH recipients of semi-immune splenic B lymphocytes challenged with 1 x10^5 pRBC i.v.

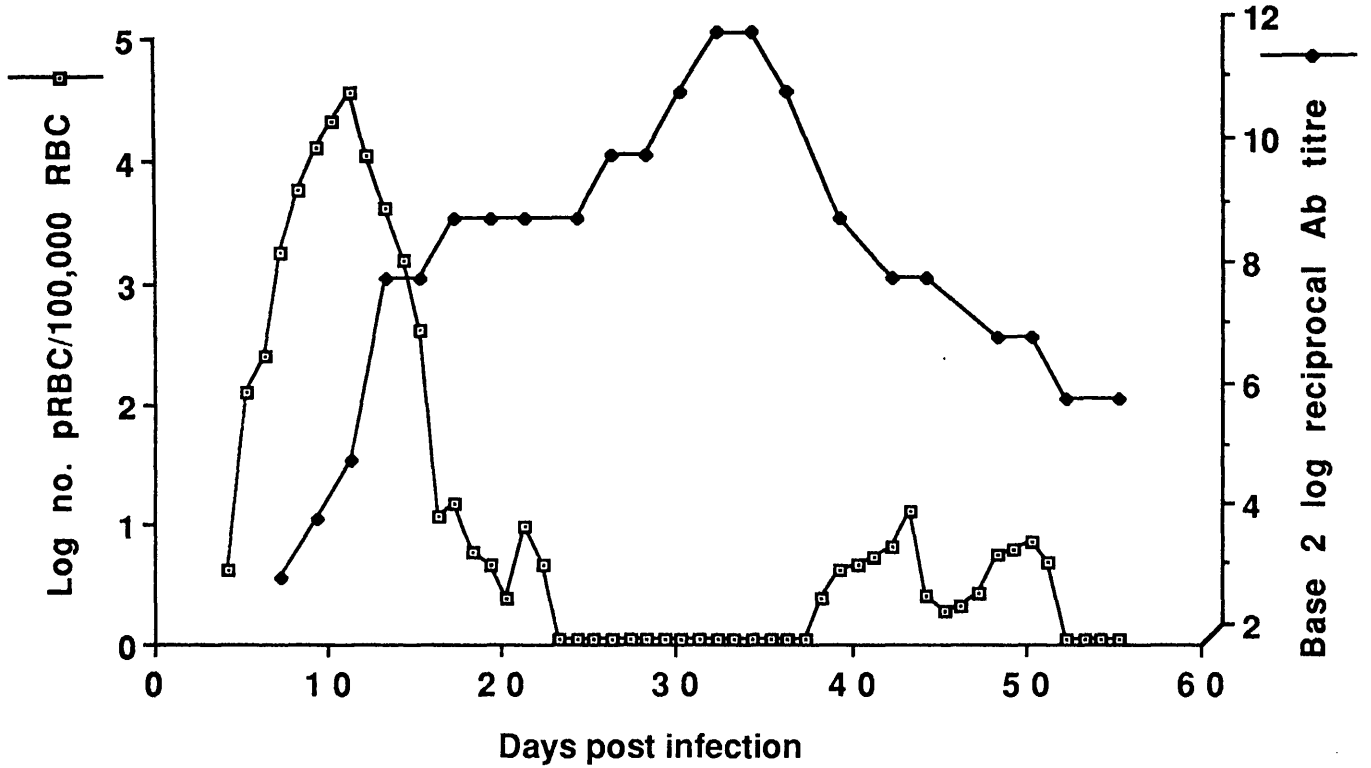


Fig. 3.6.6 The serum antibody titre during the course of infection in sublethally irradiated NIH recipients of semi-immune splenic T & B lymphocytes challenged with 1 x10^5 pRBC i.v..

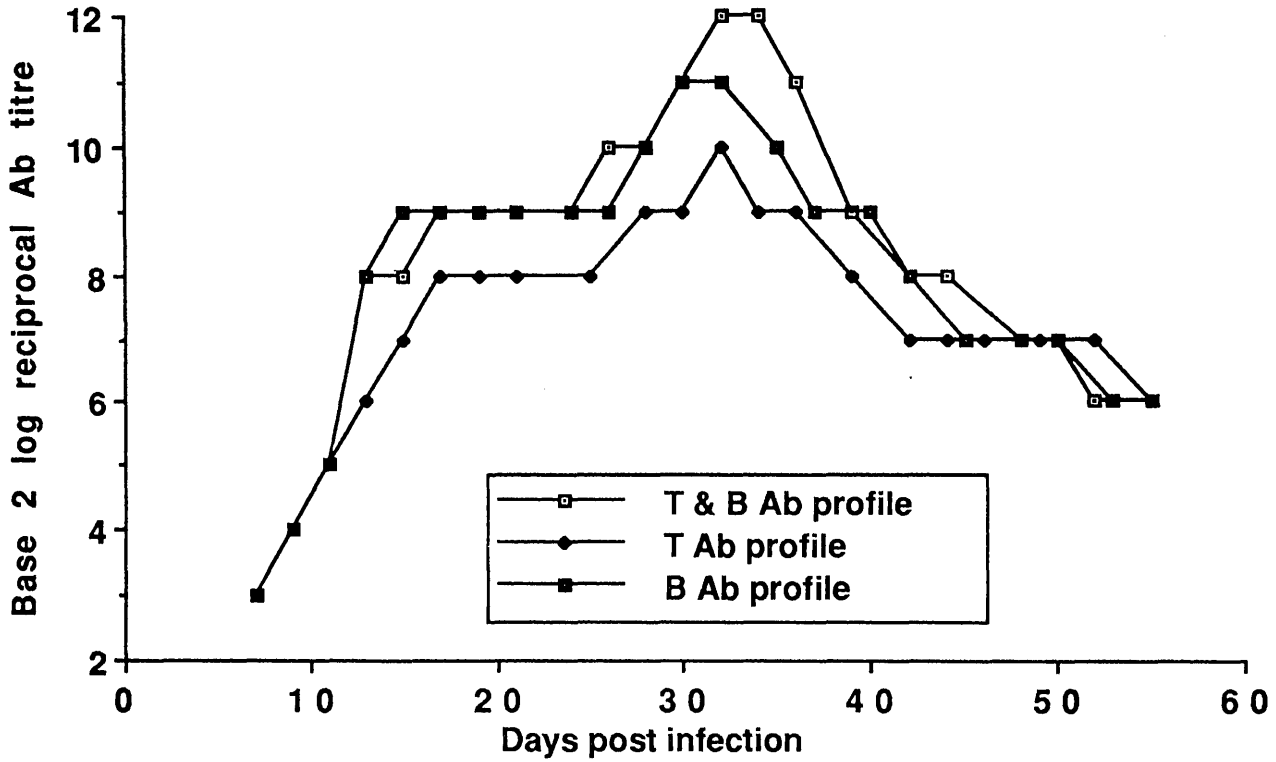


Fig. 3.6.7 Comparison of the serum antibody titres during the course of infection in sublethally irradiated NIH recipients of semi-immune splenic lymphocytes challenged with 1 x10^5 pRBC i.v..

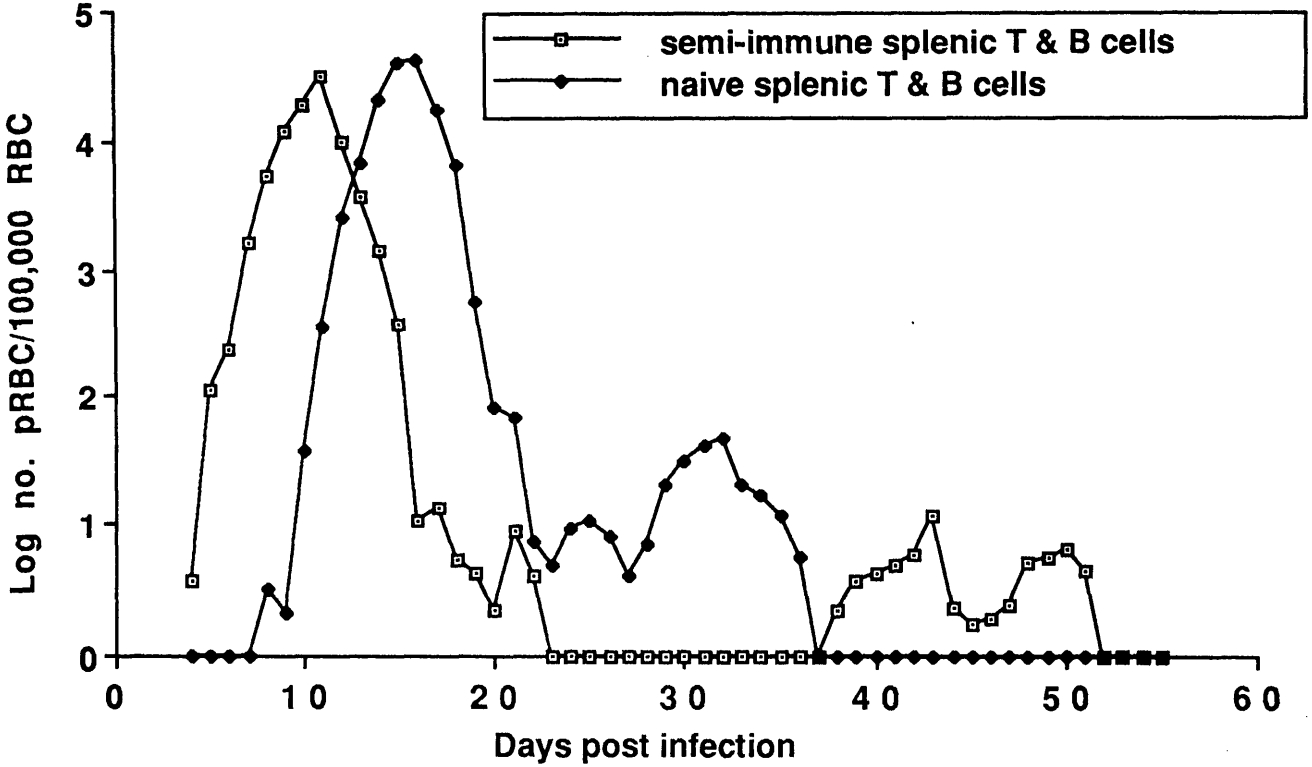


Fig. 3.6.8 Course of infection in sublethally irradiated NIH recipients of splenic T & B lymphocytes challenged with 1 x10^5 pRBC i.v..

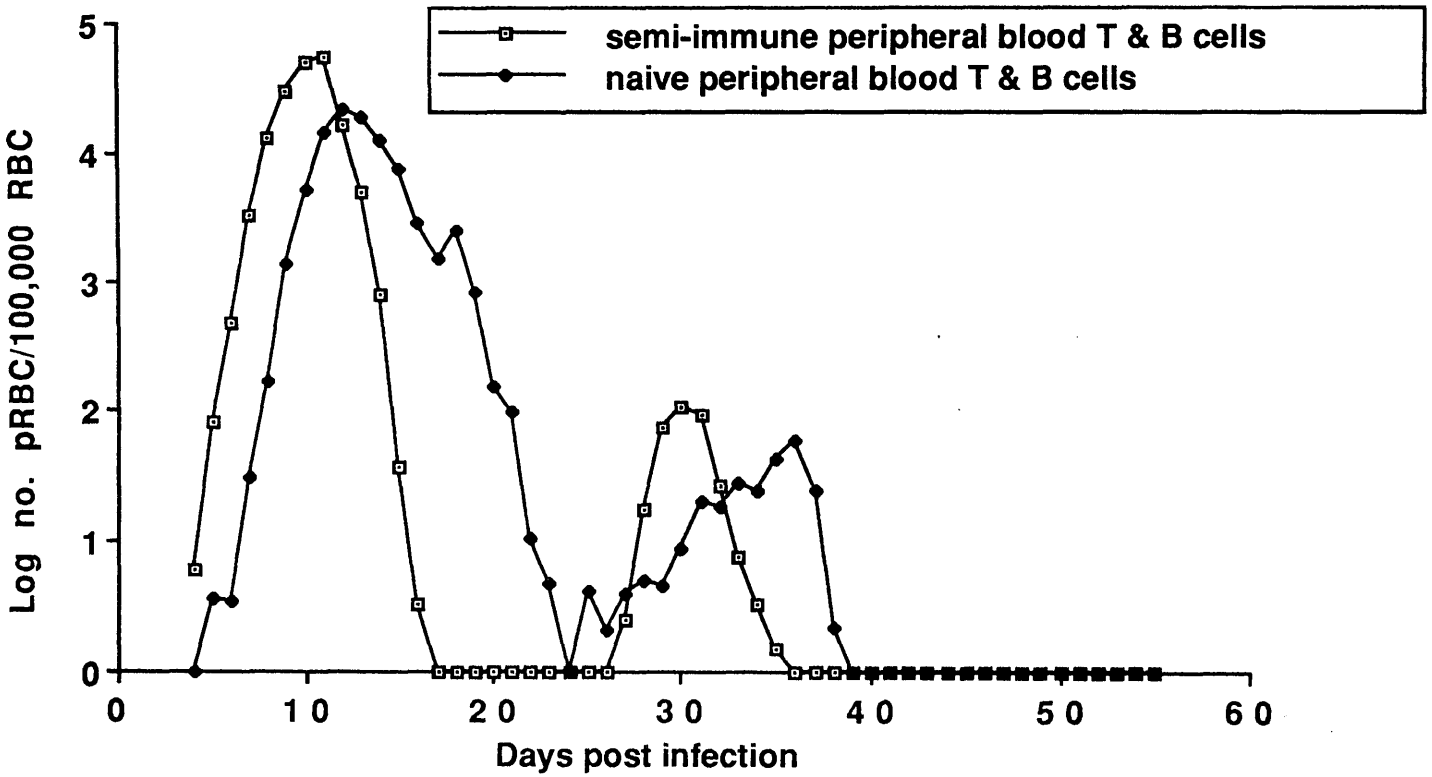


Fig. 3.6.9 Course of infection in sublethally irradiated NIH recipients of peripheral blood T & B lymphocytes challenged with 1 x10^5 pRBC i.v..

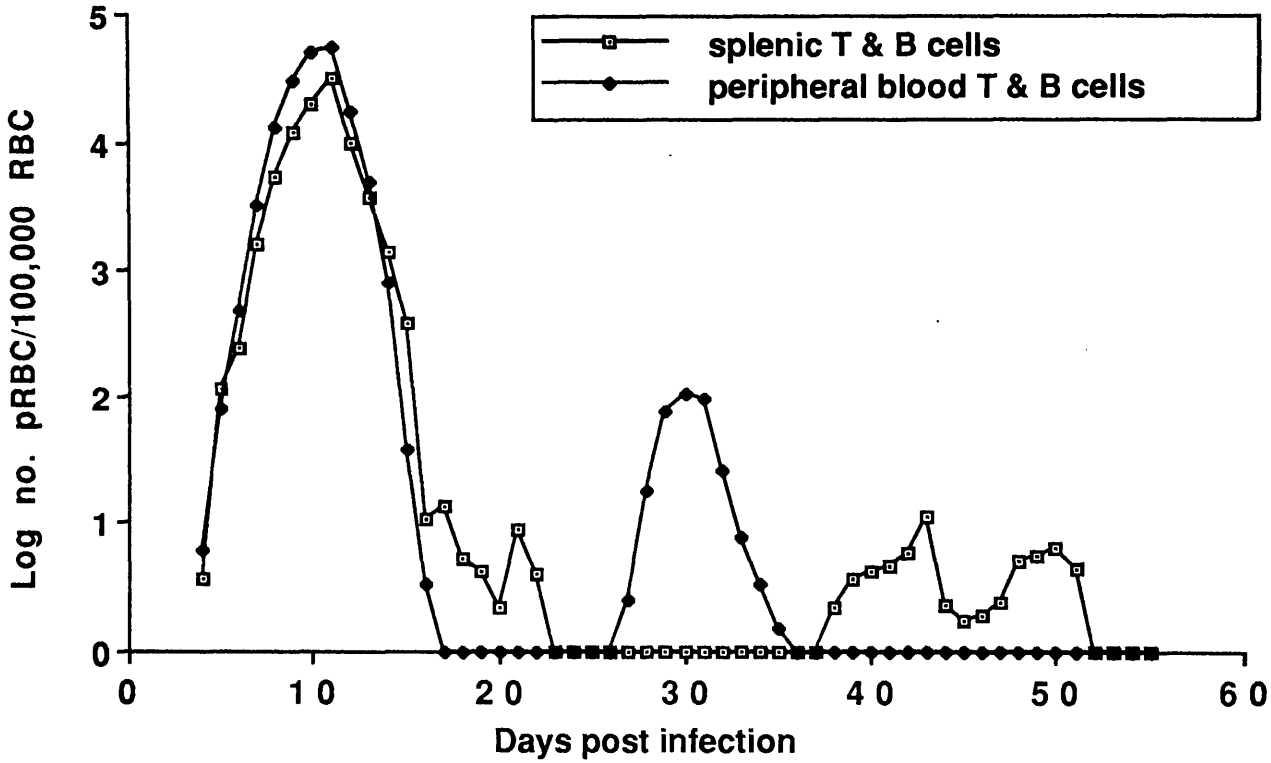


Fig. 3.6.10 Course of infection in sublethally irradiated NIH recipients of semi-immune T & B lymphocytes challenged with 1 x10^5 pRBC i.v..

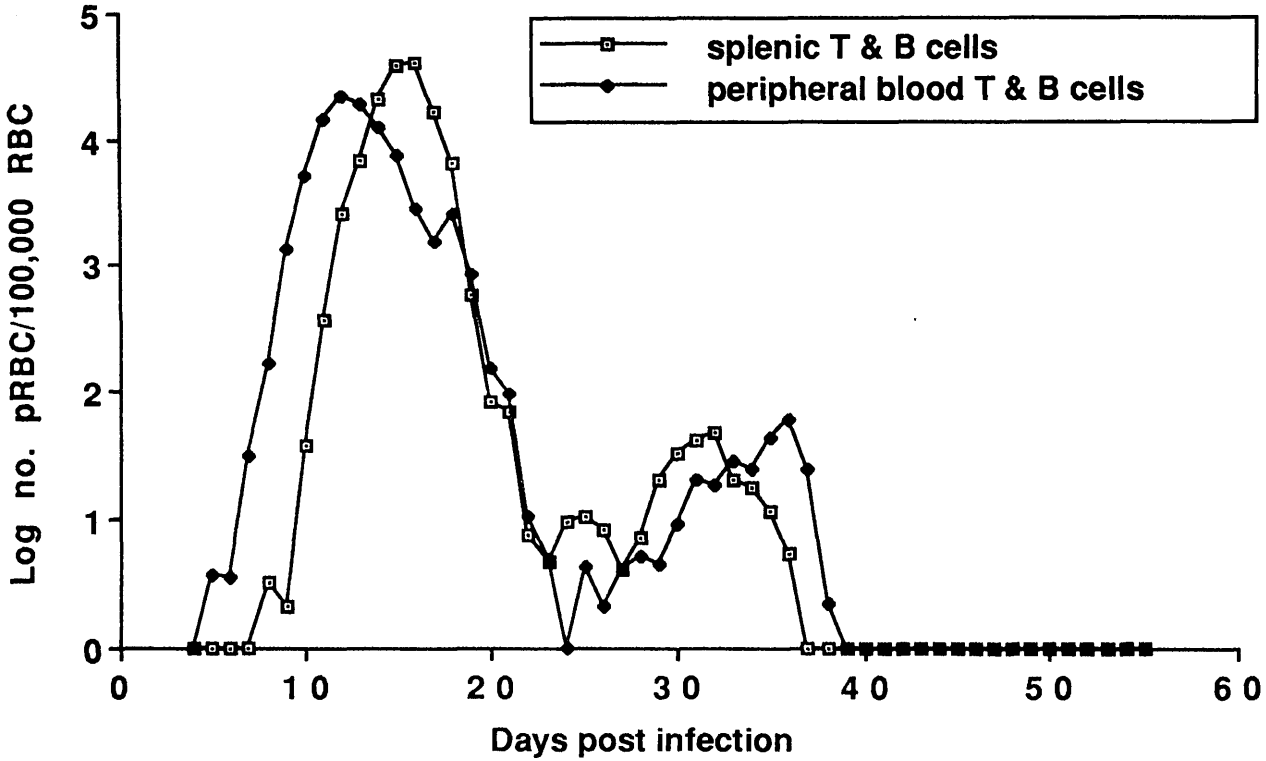


Fig. 3.6.11 Course of infection in sublethally irradiated NIH recipients of naive T & B lymphocytes challenged with 1 x10^5 pRBC i.v..

CHAPTER FOUR

IN VITRO CULTURE OF T CELL LINES AND CLONES

4.1 Introduction

Recent studies using mice depleted of T cells in vivo and in adoptive transfer experiments have demonstrated that the protective immune response to the asexual erythrocytic stages of Plasmodium chabaudi chabaudi and P. yoelii is absolutely dependent on the presence of Ly-4-bearing T cells (Süss et al 1988, Vinetz et al 1990). These reports supported the pioneering work of Jayawardena et al (1982), who implicated a role for Ly-4^+ but not Ly-2^+ T cells in acquired resistance to blood stage malaria parasites, a view later contradicted by Mogil et al (1987) working with the same 17X strain of P. yoelii. The discrepancy in the results obtained was thought to be due to attempts to dissect the contributions of the two subsets of T lymphocytes using enriched, but nevertheless heterogeneous populations of cells for adoptive transfer. This problem has been overcome through the recent advance in T cell biology of the generation and use of Ag-specific T cell lines and clones (Gillis & Smith 1977). It is now known that following Ag recognition, T cells secrete IL-2 and express high affinity IL-2 receptors at their cell surface. The combination of IL-2 with its receptor and subsequent signal transduction allows Ag-stimulated T cells to undergo clonal expansion (Smith 1984). With this knowledge, it has proved possible to enrich for Ag-specific T cells of either Ly-2^+ or Ly-4^+ phenotype by repeated cycles of Ag stimulation followed by expansion with IL-2, or alternatively MAb against CD3 (Van Wauve et al 1980). These T cells may be maintained as a polyclonal population, or, following cloning, as cells with a unique Ag specificity. Recognition of Ag by T lymphocytes may then be assayed either by lymphokine release (Kappler et al 1980) or by T cell proliferation (Seeger & Oppenheim 1970). Using this technology, malaria-specific T cell lines have been raised, the transfer of which has been demonstrated to provide protection against P. berghei infections in rats (Gross et al 1984) and P. c. adami infections in BALB/c mice (Brake et al 1986). In this latter study, one of the Ly-4^+ cell lines was cloned without loss of adoptive protection in this Ab-independent rodent malaria system (Brake et al 1988).

The work described in this chapter outlines the preparation of Ly-4^+ T cell lines and daughter clones from different time points of a primary infection of NIH mice with P. c. chabaudi AS. The initiation and maintenance of long term cultured spleen cells is detailed, as is the adaptation of established T lymphocyte limiting dilution cloning techniques employed successfully to attain homogeneous populations of cells. The results of the routine assaying of Ly-4-bearing lymphocytes by the measure of a proliferative

response to semi-defined P. c. chabaudi AS pRBC Ags is also described, but the further characterisation of each of the lines or clones used in adoptive transfer experiments (Chapters 5-9) is detailed separately in Chapter 9 of this thesis.

Before any attempt was made to raise malaria-specific T cell lines in vitro, each of the variable culture conditions was examined. This necessitated the defining of in vitro culture conditions optimal for growth of Ly-4$^+$ cells; variables included concentrations of IL-2, pRBC lysate Ag, responder T cells, APC and FCS supplements to RPMI 1640 medium, as well as batch analysis of serum to test for support of cell growth, but lack of non-specific proliferation. For this preliminary work, spleen cells were taken from either naive donors or from mice recently recovered from a primary infection. However, as the adoptive transfer experiments described in Chapter 3 showed that some protection to P. c. chabaudi AS could be give to naive mice with enriched T cells from at least as early as d 12 of infection when the acute primary parasitaemia was in decline, it was decided to attempt to raise lines with spleen cells isolated from donor animals before they had cleared primary infection. These lines would have a similar immunological status as the lymphocytosis-derived T cells used previously, but would have the advantage of being cultivated in vitro as relatively homogeneous populations suitable for assaying lymphokine secretion and B cell helper activity. In addition, these lines could be cultured in bulk to provide sufficient cell numbers to facilitate adoptive transfer into syngeneic recipient mice of differing states of immunological competence. As well as culturing spleen cells taken from infected donor mice, animals that had cleared multiple infections with the same P. c. chabaudi AS parasite clone were splenectomised. Thus, lines of T cells were initiated using lymphocytes primed to different degrees to the infecting malaria parasite and, therefore, presumably of varying immunological competence. This would allow the study of specific cellular responses both during and after malaria infection in this immune system, results of which would bear comparison with similar experiments using human T cells primed to P. falciparum (e.g. Troye-Blomberg et al 1983 b, 1984, Riley et al 1988 a & b) and to P. vivax (Goonewardena et al 1990).

4.2 Assay of IL-2 on murine T cell blasts and murine spleen cells.

The survival of T lymphocytes in vitro is critically dependent on IL-2, a tissue-specific growth factor synthesised and secreted by helper T cells (CD4$^+$ or Ly-4$^+$ in man and mouse, respectively) following their activation with Ags or polyclonal mitogens (Smith

1989). For the in vitro culture of T cell lines and clones, three different sources of IL-2 were used in all, namely Con A S/N (2.21), autocrine IL-2 (2.22) and recombinant murine IL-2. As a prerequisite for routine long term culture, each batch of these various sources was tested for its ability to support the growth of polyclonally-activated murine T cell blasts (2.23). After establishing the growth-supporting ability of each IL-2 stock, they were then tested against spleen cells ranging in their states of immunological commitment. This was done in order to discover whether this lymphokine would maintain each cell type equally well in vitro.

Figure 4.2.1 shows a typical result of a blast cell assay. Each of the three sources of IL-2 maintained the growth of the PHA-R-stimulated peripheral blood blast cells, seen as a significantly higher rate of DNA synthesis over that of the negative controls ($p < 0.01$). This support of lymphocyte proliferation occurred at relatively high levels for all concentrations of IL-2 used, but was maximal at 10% v/v for each sample; this was equivalent to 10 i.u./ml for the recombinant murine IL-2. Thus, it was decided to use IL-2 stock at 10% v/v in the culturing of T cells, unless conditions dictated otherwise.

Having assayed the IL-2 content of each sample by measurement of the proliferation of a specialised IL-2-dependent cell line, the samples were then assayed in a similar manner against NIH mouse spleen cells taken from naive animals, mice recently recovered from a primary infection, or from mice sacrificed on d 16 of a primary infection. The dose-response curves of tritium uptake (Figs. 4.2.2-4) indicate that Con A S/N, auto IL-2 and rIL-2 all supported the growth of the specific cell types that would be used in future studies. Moreover, the IL-2-dependent proliferation did not appear to be dependent upon the state of commitment or competence of the cells tested, as unprimed naive spleen cells and P. c. chabaudi AS-primed memory spleen cells proliferated at approximately the same rate in each of the assays. Lymphocytes taken from mice during the subpatent period of primary infection with P. c. chabaudi AS, equivalent to the semi-immune cells used in Chapter 3, also grew in vitro in an IL-2-dependent manner (Figs. 4.2.2-4), if at a slightly lesser rate. However, the levels of tritium incorporation for each spleen cell sample cultured with each IL-2 stock at a given concentration were not significantly different ($p > 0.05$).

An additional point to make is that in all three proliferation profiles, maximal growth was attained when IL-2 was used as a 10% v/v supplement to RPMI 1640 medium. This observation underlines the validity of the original IL-2 concentration assay (Fig. 4.2.1) and stresses that IL-2 supports the proliferation of all T lymphocytes, regardless of cell

lineage. Furthermore, it confirms that in the spleen cell proliferation assays, the lymphocytes were responding to the IL-2 present in solution and were not stimulated by any other factor in the growth medium.

Regardless of the proliferative response of each cell type, within each group the stimulation given by auto IL-2 was significantly less than that induced by either Con A S/N or r IL-2 ($p < 0.01$) (Figs. 4.2.5-7).

4.3 Assay of anti-CD3 MAb on naive spleen cells.

For use in cloning by limiting dilution, the anti-CD3 MAb was used as an alternative to auto IL-2. This was obtained as a cell culture S/N from the 145-2C11 hybridoma, and prior to use in stimulating T cell clone expansion, the S/N stock was assayed for quantification of cellular proliferation against naive splenic lymphocytes (2.28).

Results showed that Ab against CD3 was able to induce a very high proliferative response from the cultured test cells (Fig. 4.3.1). Each of the S/N dilutions assayed gave an equal or greater stimulation to T lymphocyte growth than did 10% auto IL-2 run in parallel. Thus, any of the MAb titres used was deemed suitable for use in cloning (2.29); in the event, 20% S/N v/v was employed in Ly-4^+ cell expansion, as recommended by Wasik & Morimoto (1990).

4.4 Batch testing of FCS for use in vitro.

It is known that FCS may induce non-specific mitogenic stimulation of lymphocytes which may obscure the effects of Ag-specific T cell responses in vitro (Hudson & Hay 1989). The incidence of mitogenic activity in random test samples of FCS may be as high as 85% (Shiigi & Mishell 1975). It was therefore necessary to pretest FCS for its supportive activity for an immune response in vitro. This was performed initially by assaying proliferation of naive spleen cells cultured in RPMI 1640 medium supplemented with 5-20% v/v additions of FCS made available by different suppliers (Fig. 4.4.1). Once batches of a certain commercially available FCS had shown a demonstrable lack of mitogenic stimulation, they were tested further for support of IL-2-promoted lymphocyte proliferation (Fig. 4.4.2).

It was found that most sera tested induced cell proliferation to a considerable degree (Fig. 4.4.1) and proved, therefore, unacceptable for the purposes of in vitro culture. Of FCS obtained from four different companies, only batches from Flow Laboratories Ltd. routinely gave minimal support to lymphocyte growth. On this basis, it was decided to

use FCS from this company in all subsequent in vitro cultivation procedures, and each batch purchased throughout the course of this study was screened for mitogenicity prior to use; most samples proved very satisfactory.

When assaying for the support of spleen cell growth in the presence of Con A S/N, the FCS was added to RPMI 1640 medium at 10% v/v, a concentration used standardly for supplementing media for the culture of T cells (Taylor et al 1987). Figure 4.4.2 shows a typical profile of Con A S/N-stimulated lymphocyte proliferation. At a concentration of 10% v/v, the selected batch of FCS gave acceptable cell survival without a high background of incorporation of the DNA analogue.

4.5 Assay of responder cell concentration

To determine the optimal concentration of responder cells for the Ag-specific proliferation assay and for bulk T cell cultures, a test system was set up using a fixed concentration of 10% v/v Con A S/N in 10% FCS RPMI 1640 medium, and titrating out the proliferative response of spleen cells over the starting concentration range 0.1 - 10 $x10^5$ cells/ml. As for 4.2, lymphocytes from three different sources were tested.

This assay showed that the proliferation of activated lymphocytes was dependent upon an adequate initial concentration of cells in the microtitre environment. A responder cell concentration of < 1 $x10^5$/ml was insufficient to support spleen cell growth (Fig. 4.5.1). Above this level, all inocula enabled a reasonable rate of [^{3}H]thymidine incorporation, maximal values being attained when the cultures were started at 2 $x10^5$ cells/ml. When the spleen cells were plated out at a high density, 0.5-1 $x10^6$ cells/ml, the degree of incorporation of the radiolabel fell away, presumably because these cells were reaching the plateau phase of their growth by the time of addition of the titrated thymidine. Therefore, a concentration of 2 $x10^5$/ml was chosen for all further proliferation assays, this value enabling the responder cells to survive initial plating and to proliferate logarithmically by 72 hr when the DNA nucleotide analogue was most usually added.

A further point of interest from this experiment was the significantly lower proliferative response at all starting concentrations of the splenic lymphocytes taken from infected mice after clearance of the acute infection ($p < 0.01$) (Fig. 4.5.1). For the other two sources of spleen cells, uninfected or post-infective mice, the proliferation profiles were not significantly different from each other. These two observations will be raised again later (4.6 & 4.8).

4.6 Assay of APC concentration

Another variable in the in vitro culture of T cells lines and clones that needed to be optimised was the concentration of APC. These cells were required to process native pRBC lysate Ag and present it to the Ly-4^+ T cells in an MHC class II-restricted manner (reviewed by Unanue 1984, Schwartz 1986). Without the processing function of the syngeneic APC, the primed T cells would not be able to recognise the antigenic determinant(s) to which they specifically respond. A co-stimulatory activity for T lymphocyte stimulation has also been advocated (reviewed by Weaver & Unanue 1990). The easiest available source of APC for T cell culture was the spleens of uninfected NIH mice; splenic B cells, macrophages and dendritic cells would all perform the task of Ag presentation. Each spleen cell suspension was subjected to gamma irradiation prior to culturing to block the background growth of these cells rather than that of the desired T cells.

APC concentration was assayed over the range 0.1-10 $x10^6$/ml using fixed concentrations of 2 $x10^5$/ml responder cells and 10% v/v IL-2. Con A S/N, auto IL-2 and rIL-2 were all tested against each of post-infection, post-primary parasitaemia and naive spleen cell preparations.

For each source of responder cells, maximal proliferation was attained when the APC concentration was 2 $x10^6$/ml, i.e. 10 times that of the responder cells themselves (Figs. 4.6.1-3). This was regardless of which stock of IL-2 was used to stimulate T cell activation (Figs. 4.6.4-6). Using Con A S/N at 10% v/v to stimulate cellular proliferation (Fig. 4.6.4), there was a similar finding to that seen in Fig. 4.5.1, namely, the same level of growth by post-infection and naive spleen cells, but a significantly lower level for lymphocytes taken on d 16 p.i. ($p < 0.01$). This observation was repeated using auto IL-2 and r IL-2 (Figs. 4.6.5 & 6). Also, for each splenic lymphocyte type, regardless of its intrinsic ability to proliferate in response to IL-2, the response at each APC concentration upon activation with auto IL-2 was always less, and significantly so ($p < 0.01$), than the responses to Con A S/N or r IL-2 (Figs. 4.6.1-3). This supported the observations made during the IL-2 proliferation assays (Figs. 4.2.5-7). Only in the case of post-acute infection spleen cells did the proliferative activity induced by auto IL-2 come within 2 SD of that of r IL-2, and then only at the optimal APC concentration, 2 $x10^6$/ml (Fig. 4.6.3). This was due, in all probability, not to the enhanced proliferation of d 16 splenic lymphocytes in the presence of auto IL-2 but because of the generally depressed activity of these cells in

4.7 Assay of inhibition of APC proliferation

To inhibit the potential proliferation under culture conditions of the freshly prepared naive spleen cell preparations which were routinely used as a source of APC during this project, each sample was subjected to a dose of gamma irradiation sufficient to block cellular proliferation. This treatment had no adverse effects on the capacity of such cells to present Ag in a suitable manner for responder cell recognition and activation. To ascertain the minimum exposure to ^{60}Co required to prevent spleen cell proliferation, naive NIH splenic preparations were assayed for growth (2.34) after receiving different levels of irradiation.

Figure 4.7.1 shows that a dose of 20 Gy immediately prior to culture was necessary to abrogate cellular proliferation; lesser levels of irradiation failed to reduce to background values the degree of tritium incorporation. In the event, a dose of 30 Gy was adopted as the standard gamma irradiation treatment for preparation of APC for all subsequent T lymphocyte in vitro culture.

response to stimulation of any kind.

4.8 Assay of Ag concentration

The T cell lines raised in vitro were primed to P. c. chabaudi AS in vivo and thereafter stimulated to proliferate under culture conditions upon exposure to a crude lysate of pRBC of the same parasite. To elucidate whether a lysate of RBC was a suitable source of antigenic determinants against which a cellular response could be mounted, an Ag concentration assay was performed. This also would determine the optimum concentration of protein necessary to elicit the maximal level of proliferation, and, by using lysates of both pRBC and nRBC, show whether lymphocyte growth was in response to recognition of parasite-specific Ag(s).

Initial studies were performed using naive spleen cells or those taken from mice primed to P. c. chabaudi AS through either complete or partial courses of infection. These were cultured at 2 $x10^5$/ml with 2 $x10^6$/ml APC in microtitre wells and assayed for proliferation by [^{3}H]thymidine incorporation.

The proliferative response of immune or semi-immune splenic T cells to P. c. chabaudi AS Ags in the form of a pRBC lysate was dose-dependent (Figs. 4.8.1 & 2). In each case, the activation induced by pRBC Ag was considerably greater than that induced by nRBC Ag ($p < 0.01$), indicating that the stimulus for cell growth was parasite-specific and not a common determinant on all RBC. For lymphocytes prepared from the spleens of uninfected mice, there was no significant difference between the proliferation profiles of the responder cells to the two different lysates over the concentration range tested (Fig. 4.8.3). This observation implied that previous exposure to the parasite Ags, in this case by in vivo priming, was a necessary prerequisite for the P. c. chabaudi AS-specific proliferative response by Ly-4^+ T cells in the presence of syngeneic accessory cells.

Another finding from this experiment was that where a dose-dependent response to plasmodial Ags did occur, the maximal proliferation was in cultures containing 200 µg/ml pRBC lysate (Figs 4.8.1 & 2). For these in vivo primed spleen cells, the nRBC-stimulated cell growth peaked at a concentration of 150 µg/ml protein lysate. This again implied that the response to pRBC was activated by one or more plasmodial determinants. Moreover, the response of naive splenic lymphocytes to both pRBC and nRBC lysates showed optimum levels at 150 µg/ml lysate concentration (Fig. 4.8.3); this suggests that the non-specific proliferation by these unprimed cells to the pRBC Ag was because the same or similar non-plasmodial epitopes were recognised in all RBC lysate

preparations.

When the proliferation of each of the spleen cell populations upon activation by pRBC lysate is considered, it can be seen that at each concentration of lysate assayed the response by post-infection lymphocytes was significantly greater than that by lymphocytes taken during infection ($p < 0.01$) (Fig. 4.8.4). For the response to the nRBC control, the response of d 16 p.i. spleen cells was again significantly depressed (Fig. 4.8.5). This was similar to the results of assays for both responder cell concentration (Fig. 4.5.1) and APC concentration (Figs. 4.6.4-6). The reduced proliferative response of these semi-immune lynphocytes was therefore observed upon activation by IL-2 and by lysates of parasitised and uninfected RBC.

In the case of naive splenic lymphocytes, the lack of response by these cells when cultured with pRBC lysate (Fig. 4.8.4) was due to a specific non-recognition of parasite Ags. Only under these circumstances did the growth of naive cells fall below that of lymphocytes taken from infected mice. Tritium incorporation was similar with both lysate samples used and was not parasite-specific (Fig. 4.8.3). That cells from uninfected donor mice were capable of mounting a proliferative response had been shown previously (Figs. 4.5.1 and 4.6.4-6), when the growth recorded upon activation with IL-2 was of the same magnitude as that exhibited by primed spleen cells.

4.9 Initiation and maintenance of helper T cell lines

Having standardised the variable factors present in the *in vitro* cultivation of T cells by preliminary studies using unfractionated spleen cell preparations, it was now possible to attempt to generate helper T cell lines *in vitro*. These could then be analysed both *in vitro* and *in vivo* for an anti-*P. c. chabaudi* AS immune response.

All Ly-4^+ cell lines established *in vitro* were raised by priming to *P. c. chabaudi* AS *in vivo* and then by following either the original or modified protocols for the generation of helper T cells *in vitro* (2.26). However, for later use, these lines were recovered from cryopreservation and propagated exclusively by the modified protocol outlined in 2.26. Although the classical feed-starve-feed methodology of Kimoto & Fathman (1980) proved successful in generating Ly-4^+ cell lines by selective pRBC Ag-induced clonal expansion *in vitro*, it was considered that this regimen was too cumbersome for the purposes of routine culture of well-established T cell lines. Specifically, the starve period, in which responder cells were cultured with Con A S/N and APC but without antigenic stimulation, was omitted and replaced by a longer time between successive

feeds. This alteration of the in vitro culture technique had no apparent effect on the composition or character of each of the lines propagated; indeed, all the Ly-4$^+$ lines and daughter clones described in this thesis maintained their Ag-specificity and function upon long term growth in vitro (this chapter and Chapter 9).

It was noted that with the modified cultivation protocol, in which dead cells and debris were not removed at every subculture, there was no drop in the viability of the cultured helper T cells. As the use of Metrizamide to remove the dead cell fraction had been effective but had always resulted in an unnecessary loss of viable responder lymphocytes, the dropping of this procedure resulted in a relative enrichment of Ly-4$^+$ cells at every subculture, compared to the yields attained previously.

Several splenic lymphocyte lines were raised during this study but only four were used in further experiments. The details of the generation of these cell lines is shown in Table 4.9.1. All donor mice used for in vivo priming were infected with the same parent P. c. chabaudi AS clone, WEP 685 (Fig. 2.1), with which the mice bled to prepare the pRBC lysate stocks had been challenged.

4.10 Generation of helper T cell clones by limiting dilution

The initial attempt at single cell cloning of an established Ly-4$^+$ cell line, WEP 737, proved unsuccessful. Lymphocytes were collected 3 d after the fifth antigenic stimulation with pRBC lysate and diluted out in microtitre plates. A 20% auto IL-2 v/v supplement to RPMI 1640 medium was intended to promote single cell growth. This procedure, however, failed to yield any clones of a sufficient size to expand from plate to flask culture. Clones were detected routinely upon screening 12-16 d after cloning, and were identified by having a larger, more amorphous shape than background APC and appearing quite bright under phase contrast microscopy. Unfortunately, the expected cell growth did not occur, all remaining a regularly small appearance. Instead of expanding such small cell clusters, the microtitre plates were maintained by replacing 20% auto IL-2 and 200 µg/ml pRBC Ag in 10% FCS PRMI 1640 medium every 3-4 d. Despite these efforts to trigger continuous clonal proliferation, all clones failed to grow from their initially detected size.

Due to this lack of success, subsequent adoptive transfer experiments were performed with each of the four IL-2-dependent Ag-specific lymphocyte lines described in Table 4.9.1. Results showed that these lines were relatively homogeneous in their expression of the Ly-4 surface Ag (Chapter 9), and, furthermore, that they could adoptively

transfer protection to both immunocompetent and immunosuppressed mice challenged with P. c. chabaudi AS (Chapters 5-8). Since they could confer a protective immune response to the parasite line to which they had been raised, these lines were ideal candidates for consideration with regard to cloning. Thus, a further attempt at cloning was made considerably later in this project, using the Ly-4$^+$ lines WEP 737 and WEP 775 as the basis for development of cloned cells. The methodology followed was similar to that first used (2.29), but replacing auto IL-2 with anti-CD3 MAb as a 20% supplement to complete RPMI 1640 medium. Plates were scored for the presence of clones on d 10-14 and microtitre wells supporting positive colony growth were expanded on d 14-15 following initial cloning (2.30). Of 384 wells plated at the three lowest limiting dilution densities used, 10-1 cell/ml (1-0.1 cell/well), only 10 and 11 wells exhibited positive cell growth for WEP 737 and WEP 775, respectively. However, all these T cell clones were successfully expanded in vitro with the use of anti-CD3 to stimulate clonal proliferation. Between 16-18 d after the clones had been transferred from 96 to 24 well plates, each had grown to confluency and was transferred to flask culture, first as 10 ml volumes in 25 ml flasks and then as 30-40 ml volumes in 75 ml flasks. Once established in bulk culture, each Ly-4$^+$ clone was maintained by the modified protocol for T cell propagation (2.29). Except in one or two instances where 20% anti-CD3 was continued as the supplement to the main culture medium, no difficulties were encountered in switching the newly established clonal populations from stimulation with anti-CD3 used for clonal expansion to auto IL-2 used for routine maintenance.

Table 4.10.1 shows the history of each of the T lymphocyte clones isolated by limiting dilution. For both sets of clones, only those derived by diluting the parent lymphocyte line to either 0.5 or 0.1 cells/well (5 or 1 cells/ml) were used for characterisation and functional assays in vitro and for adoptive transfer in vivo (Chapter 9). This was to ensure the absolute clonality of the populations used in subsequent analyses.

4.11 Assay of Ag-specific proliferation

The proliferative responsiveness of Ly-4$^+$ T cell populations to a soluble lysate of pRBC Ags was routinely tested as described in 2.34. This assay determined the antigenic specificity of various lines and clones cultured in vitro and thereby demonstrated whether or not these preparations were immunologically primed to the blood stages of P. c. chabaudi AS against which the original cell lines used in this study had been raised. As

such, this assay was used not only to confirm the antigenic reactivity of newly generated T cell lines and clones but also to examine periodically the maintenance of this specificity by long term cultures.

Figure 4.11.1 shows the proliferative responses of the four cultured IL-2-propagated T lymphocyte lines used in this study. Each was assayed initially 7 d after the fourth round of antigenic stimulation with solubilised pRBC lysate to enable comparison of the respective growth rates. All the lines derived from semi-immune or immune mice showed a significant proliferation when cultured with P. c. chabaudi AS Ag over the entire range of Ag lysate concentrations assayed. For each lymphocyte line, the maximal cellular growth was achieved by activation with 200 μg/ml pRBC lysate Ag, the same concentration already demonstrated to give optimal proliferations of unfractionated primed spleen cells (Fig. 4.8.4). Although the patterns of the dose-response curves for all the lines were essentially similar, the actual levels of tritium incorporation attained varied markedly. For WEP 737 and WEP 723, the cell lines established by culturing splenic lymphocytes taken from mice recovered from multiple infections, the degree of proliferation over the range 150-400 μg/ml pRBC lysate was significantly increased ($p < 0.01$) over that exhibited by either WEP 775 or WEP 779, derived from mice during primary infection (Fig. 4.11.1). There were, however, no significant differences ($p < 0.05$) between the proliferative responses between WEP 737 and WEP 723 or between WEP 775 and WEP 779 at any lysate dilution tested. This reduced cellular growth pattern of the splenic lymphocyte lines taken from mice during the remission or subpatent periods of a primary P. c. chabaudi AS infection was similar to that observed previously for unfractionated post-primary parasitaemia spleen cells (Fig. 4.8.4). However, unlike the Ag concentration assays already described, where the response of d 16 p.i. freshly cultured spleen cells to uninfected RBC was also significantly less than that of post-infection splenic lymphocytes (Fig. 4.8.5), the level of proliferation of WEP 775 and of WEP 779 to nRBC lysate Ag was similar to that of WEP 737 and of WEP 723 (Fig. 4.11.2). There was no significant difference in the degree of [^{3}H]thymidine uptake between any of the four Ly-4$^+$ lines upon stimulation with uninfected RBC in vitro. Moreover, each population showed a peak proliferation when cultured with 150 μg/ml nRBC lysate Ag. Incidentally, this was the same nRBC lysate concentration observed to stimulate maximal growth of heterogeneous spleen cell populations (Fig. 4.8.5). The dose-response profiles upon activation by nRBC Ag (Fig. 4.11.2) were similar to those for pRBC Ag-stimulated proliferation (Fig. 4.11.1);

however, the peak responses for the former did not exceed 4,000 c.p.m. whereas those for the latter nearly reached a mean c.p.m. value of 30,000. Thus, the ability of pRBC lysate to induce lymphocyte proliferation by each of the P. c. chabaudi AS-primed cell lines was well in excess of that of the lysate preparation of uninfected RBC. This suggests strongly that the proliferation of each established Ly-4$^+$ line was largely in response to stimulation by cocultured parasite-determined antigenic determinants. This response was dependent on the availability of APC, since no proliferation was observed when T cells were stimulated with pRBC lysate alone. That the lymphocyte lines were not activated by APC themselves was showed by a similar lack of growth in the absence of pRBC Ag (Fig. 4.11.1). Thus, the proliferation assay controls showed that APC were necessary in the in vitro culture system to process and present P. c. chabaudi AS Ags in a form recognised by the Ly-4$^+$ T cell-APC complex.

In order to assess whether each of the in vitro-propagated lymphocyte lines varied in its Ag specificity upon continuous culture or after cryopreservation, the cell lines were assayed at different times throughout this study. This was necessary to ensure that the in vivo-primed and in vitro-cultivated spleen cell populations still recognised pRBC Ags before commencing an adoptive transfer experiment. That the lines did not drift in their Ag-specific proliferative response is shown in Figures 4.11.3 & 4, for WEP 775 and WEP 737, respectively. These were the two lines used for cell cloning. Each figure compares the dose-response curve of the originally assayed population (i.e. 7 d after the fourth feed) with that of the same stabilate after prolonged in vitro cultivation (cells assayed 7 d after the ninth feed; cultures were cryopreserved after the sixth stimulation and recovered from frozen to initiate the seventh feed). For both WEP 775 and WEP 737, the tritium incorporation by the subcultured cell preparation was higher than that of the originally established population. This indicated that the lymphocyte lines not only maintained their Ag specificity in long term culture but actually exhibited an enhanced proliferative response after repeated subculture in vitro.

The clones derived by limiting dilution culture were also subjected to an Ag-specific proliferation assay; this was performed, as for the T cell lines, 7 d after the fourth feed with pRBC lysate, i.e. soon after the cloned populations had become established. Each batch of clones exhibited the same P. c. chabaudi AS pRBC-dependent cellular proliferation as that exhibited by the parent cell line (Figs. 4.11.5 & 6). For each cloned parent line, the pRBC lysate-stimulated proliferation at each Ag concentration for most of the daughter clones was within 2 S.D. of each other. However, each clone did

have a recognisably distinct proliferation pattern and differences did occur, most notably the lower growth rates of WEP 985 and WEP 986 compared to similar clones and to the parent WEP 737 (Fig. 4.11.6). In general, however, the growth rate characteristics of the homogeneous lymphocyte populations were similar to those of the line from which they were cloned, in terms of both specificity and magnitude. There was no difference in the Ag specificity of lymphocytes cloned from cell lines derived either from semi-immune or immune mice (for daughter clones of WEP 775 or WEP 737, respectively). This suggests that in vivo exposure to P. c. chabaudi AS for the relatively short period of 16 d was adequate to prime the splenic T cell population sufficiently to give a parasite-specific proliferative response that was maintained not only upon long term culture but also after cloning.

4.12 Assay of MHC restriction of Ag-specific proliferation

This assay was identical to that described previously to determine whether or not the proliferative response of Ly-4$^+$ T cells was specific towards P. c. chabaudi AS Ag, save that instead of using only syngeneic NIH irradiated spleen cells as APC, allogeneic APC were also assayed (2.35). Various strains of inbred (NIH, C57BL/10) and H-2 congenic (B10.AKM, B10.AQR, B10.BR, B10.D2, B10.G, B10.HTT, B10.R111, B10.S, B10.S(9R) & B10.6(TR)) mice were used to cover a wide range of MHC haplotypes (Table 4.12.1). All congenic mice used were on the B10 genetic background so that non-H-2 genes were identical; thus, any differences between the proliferation of Ly-4$^+$ cells in response to presentation of pRBC Ag by APC of varying haplotype would be due to the influence of the H-2 complex alone. By using recombinant haplotypes, in which a genetic crossover has occurred within the MHC, it was possible to map, to a limited degree, the locus at which restriction resides.

As for 4.11, assays were performed on either Ly-4$^+$ T cell lines or clones that had been established long term in vitro. This circumvented the problem of using non-selected T cells in such a system. If freshly primed Ly-4$^+$ lymphocytes had been used, the assay would have been complicated by the presence of T cells with specificity for allogeneic H-2 determinants, thereby inducing a mixed lymphocyte reaction. The two cell lines tested, WEP 775 and WEP 737, were those that had been cloned by limiting dilution (4.10), and were taken 7 d after the seventh antigenic feed. The two representative clones investigated were WEP 999 and WEP 988, the highest dilution (0.1 cell/well) clones of WEP 775 and WEP 737, respectively. These were assayed 7 d after the fifth

stimulation with pRBC lysate.

The results of these studies showed that the interaction of Ly-4^+ T cells with accessory cells exhibited demonstrable MHC restriction in the recognition of P. c. chabaudi AS Ag. This was manifested by substantial proliferation of responder cells upon Ag presentation by syngeneic APC (NIH, B10.G strains) but not by allogeneic APC (Figs. 4.12.1-4). The dose-response curves of proliferation over the concentration range of pRBC lysate assayed were essentially alike for Ly-4^+ cells presented Ag by H-2^q haplotype of either NIH or B10.G origin, and were similar to those attained previously (4.11). Save for one exception, the proliferation by the responder cells cultured with allogeneic APC did not show dose-response kinetics, recording levels of tritium incorporation at all lysate concentrations barely greater than for negative controls (Figs. 4.12.1-4). There was no significant difference between the proliferation profiles of Ly-4^+ lines and their daughter clones; this not only demonstrated the similarity in the proliferative response of cells before and after cloning, as shown earlier (4.11) but confirmed the enrichment of long term-cultivated T cells for malaria parasite antigenic specificity. If the proliferation of these lines in response to allogeneic APC function had been higher than that of background or had shown a response that varied with lysate concentration, it could be concluded that these responder cell preparations were not sufficiently pure to react in a unique manner in vitro. Fortunately, the homogeneity of the in vitro maintained lymphocyte populations was very high (Chapter 9), and thus these lines showed the same proliferation kinetics as did the daughter preparations for which clonality is assured.

A further insight into the basis of this genetically restricted proliferative response was gained by using APC of recombinant haplotype, in particular y_1 and y_2. APC from the B10.6 (TR) strain, which has the H-2^d allele at the H-2^D locus, but for which all other loci are filled by H-2^q alleles, supported a P. c. chabaudi AS-specific proliferation of the same intensity as that attained using syngeneic APC (Figs. 4.12.1-4). It would appear from this that the D region of the H-2 complex is not involved in the control of immune responsiveness to P. c. chabaudi AS. This finding was substantiated further by the use of B10.AQR strain mice as the source of splenic lymphocytes for Ag presentation. In this haplotype, none of the loci are occupied by H-2^q alleles except for the K locus (Table 4.12.1). APC from this mouse strain could not induce a proliferative response by the H-2^q Ly-4^+ lymphocytes under test. Furthermore, the loss of the H-2^q allele at the H-2 I-A and I-E loci when changing APC recombinants from B10.6(TR) to B10.AQR

resulted in the absence of a pRBC-specific cellular response. These data would therefore also occlude the D region of the MHC from a role in the control of T cell immunity to P. c. chabaudi AS. Further attempts to assign those H-2 genes influencing anti-malarial resistance to the different subregions of the MHC complex were not possible as only a limited number of H-2 recombinant mouse strains carrying $H\text{-}2^q$ alleles are available at present. The results of this study do, however, indicate that malaria protective Ags can be presented in context with one or more molecules encoded by the I-A or I-E subregions of the H-2 complex.

4.13 Discussion

The results of the experiments detailed in this chapter describe the initiation and maintenance of anti-malaria helper T cell lines. This involved preliminary investigations to determine the optimum in vitro conditions suitable for specific $Ly\text{-}4^+$ T cell enrichment by selective P. c. chabaudi AS-induced clonal expansion. The established pRBC Ag-dependent, IL-2-propagated lines were then subjected to single-cell cloning. Both daughter clones and parent lymphocyte lines retained Ag specificity in vitro, as evidenced by the fact that they could proliferate in response to a crude soluble lysate of P. c. chabaudi AS pRBC but not to a similar preparation made from nRBC. These results concur with previous findings using T cell lines raised against P. berghei (Gross et al 1984, Gross & Frankenburg 1988) and P. c. adami (Brake et al 1986, 1988). However, in both these sets of experiments, helper T cell populations were isolated only from the spleens of mice recovered from two full courses of blood stage malaria infection and not from mice at any earlier time. The data presented in this chapter indicates that anti-malaria T cell lines could be raised as soon as d 16 after challenge of the donor animals. Previously, Chemtai et al (1984 a) had raised lymph node cell lines specific for the virulent IPPCI strain of P. c. chabaudi. These preparations were made using cells taken from naive mice primed for either 8 or 14 d. The proliferative response of these established lines was parasite-specific with similar dose-response characteristics. Recently, it has been reported that a specific response from splenic T cells could be measured as early as 7 d p.i. (Langhorne & Simon 1989). This investigation employed a limiting dilution assay of undefined splenic lymphocytes. Interestingly, Chemtai et al (1984 a) found no difference between the ability of whole or lysed pRBC to induce lymphocyte activation. Unlike the results presented, however, these workers failed to establish pRBC-specific lines in vitro using splenic

lymphocytes. They did succeed in attaining a P. c. chabaudi-specific T cell line by infecting donor BALB/c mice with P. c. chabaudi AS and rechallenging after recovery with the virulent IPPCI strain of the parasite. Thus, in all reported cases, it was possible to generate splenic T cell lines exhibiting parasite-specific responsiveness after priming by at least one in vivo infection. Therefore, in vivo-primed T cells cultured in vitro retain their capacity to respond to plasmodial Ags in vitro. Furthermore, De Souza & Playfair (1988) demonstrated that there was a significant correlation between priming in vivo for helper T cells against blood stage P. yoelii malaria and the immune protection conferred by the in vitro cultured cells upon subsequent immunisation. The effectiveness of in vivo activation of splenic T cells to malarial Ags, however, contradicts the report of Bandeira et al (1987) who showed that in vivo primed splenic lymphocytes were refractory to IL-2-dependent growth in vitro. It is thought that this divergence may be due to the varying states of differentiation of the T cell populations involved.

Throughout this study, naive spleen cells were irradiated and these then used as the regular source of APC for in vitro culture purposes. These preparations proved satisfactory in the presentation of plasmodial Ags, so enabling clonal expansion of the responder cell population. However, Brake et al (1988) found that coculturing APC with the same soluble Ag as that used to stimulate T cell proliferation for 3 hr before irradiation gave the highest cell growth response. This was presumably because the macrophages and B cells present in the spleen cell preparation were sensitised to the pRBC lysate Ags prior to cultivation. Furthermore, Chemtai et al (1984 a & b) showed an increased proliferative response of peripheral lymph node cells to P. c. chabaudi pRBC lysate using lysate-fed macrophages. One drawback of APC sensitisation, however, is the reduced radiosensitivity at 30 Gy (Ashwell et al 1988).

The preliminary standardisation of the in vitro culture conditions resulted in the use of these variables at values similar to those employed in previous studies. The responder Ly-4^+ cells were cultured at 2 x10^5/ml, a concentration also used by Gross et al (1984) and by Brake et al (1986, 1988) whilst Chemtai et al (1984 a) put lines up at 5 x10^5 cells/ml. As the pRBC Ag used took the form of a lysate, its concentration was measured in terms of protein concentration. It was thus difficult to determine the amount of antigenic stimulation provided with that of other experimenters, who used whole, unlysed pRBC at 1 x10^6 pRBC/ml (Gross et al 1984, Brake et al 1986, 1988). Where Chemtai et al (1984 a) did not use a lysate preparation, they quantified the

volume added as a whole pRBC equivalent (2 x10^6/ml). The only study which can be compared directly to this one showed that the response of normal and P. yoelii-immune freshly cultured BALB/c splenic lymphocytes was optimal with a dose of 400 µg/ml soluble Ag preparation (Weinbaum et al 1976 a). This is twice the concentration of antigenic material used to stimulate T cell proliferation in this culture system, but this variance has no significance. In terms of APC concentration, there was a considerable difference between the various culture protocols. Gross et al (1984) used 6 x10^6 spleen cells/ml, whereas Brake et al (1986, 1988) only 2 x10^5 spleen cells/ml. In the experiment described herein, splenic APC were cultured in vitro at 2 x10^6/ml, a value close to the 1 x10^6/ml peritoneal macrophages quoted by Chemtai et al (1984 a). This variation in numbers is hard to explain, but is probably a reflection of the fact that spleen cell concentration is not a direct measure of APC concentration. Rather, the functional frequency of cells which fulfill the role of APC to different types of responder cell may vary according to such factors as the state of purification or differentiation of the T lymphocyte population. For instance, it has been demonstrated that the frequency of APC in unfractionated spleen cells is ~ 1:7000 but this increases markedly to 1:15 when enriched populations consisting of 70-95% dendritic cells are used as stimulators (Goodacre et al 1987). Despite this possibility of a large discrepancy in actual APC numbers used in different anti-malaria T cell culture systems, this variation is probably not critical as Goodacre et al (1987) showed that APC frequency did not reflect directly the magnitude of Ag-induced T cell proliferation.

The in vitro proliferative response of immune spleen cells taken from mice recently recovered from a P. yoelii infection was maximal on d 3 when cultured with 10^6 pRBC (Weinbaum et al 1976 a). Similarly, Chemtai et al (1984 a) demonstrated in time-response experiments a maximal proliferation 3 d after initiation of cultures. These findings showed that in the T cell proliferation assays employed as a measure of pRBC Ag-specific cell growth, the [^{3}H]thymidine alternative nucleotide precursor was added to the microtitre test plates when the lymphocytes were growing exponentially. Hence, the c.p.m. values obtained were likely to correlate well with the actual rate of induction of P. c. chabaudi AS-specific proliferation encountered after recent subculture of each of the Ly-4$^+$ lines or clones.

Continuous T cell lines were propagated in culture by repetitive stimulation in the presence of P. c. chabaudi AS Ag and accessory cells. This technique of repeated cycles of antigenic stimulation and rest selects for a subset of T cells capable of supporting their

own growth. This is because the continuous activation of lymphocytes in vitro by specific parasite Ags requires the presence of IL-2. As the exogenous supply of this lymphokine to cultured T cells would either be consumed rapidly under physiological conditions or otherwise denatured, the only lymphocytes that survive in vitro are those that produce their own autocrine growth factor. This selection mechanism, therefore, automatically favours the growth of Ly-4^+ rather than Ly-2^+ T cells as the latter have an absolute requirement for exogenous IL-2 for in vitro propagation (Taylor et al 1987). It is now known that the two Ly-4^+ T cell subsets, T_H1 and T_H2 differ in their autocrine growth factor secretion and growth response (Greenbaum et al 1988). The inflammatory subset, T_H1, produces IL-2 as its autocrine growth factor, proliferates in response to IL-2 and, in the presence of limiting amounts of IL-2, shows increased proliferation to IL-4. In contrast, the T_H2 helper subset secretes IL-4 and proliferates in response to IL-2 or IL-4 in the presence of IL-1. It would appear that freshly cultured Ly-4^+ lymphocytes of splenic origin would contain proportions of both functionally distinct subsets but that upon long term in vitro cultivation, established Ly-4^+ lines contain predominantly cells of one or other type, but not both (Chapter 9). Although the reason for this differential selection of Ly-4^+ subsets is not substantiated unequivocally, Greenbaum et al (1988) have suggested that IL-1 may play a controlling role in the clonal expansion of Ly-4^+ T cells of different functional types. This is because only T_H2 lymphocytes grow in response to IL-4 and this is dependent on the addition of IL-1 (IL-1 is constitutively secreted by all Ly-4^+ cells). This, in turn, suggests means by which the Ly-4^+ T cell immune response could be directed into Ab-mediated or Ab-independent pathways.

Another aspect of the initiation and maintenance of T cell lines by the protocols used in this study is that after five cycles of Ag stimulation, the resulting Ly-4^+ lymphocyte populations are comprised almost exclusively of pRBC lysate-specific cells so that cloning from these highly P. c. chabaudi AS Ag-specific lines can yield, with reasonable assurance, clones of predefined specificity. Indeed, this was the case when the WEP 775 and WEP 737 lines were cloned by the modified methodology. The failure of the first attempt at cloning to yield any clones of sufficient size to propagate further may have been due, in part, to a low specific precursor frequency. The subsequent limiting dilution procedure employed lines that had been established long term in culture, and were, therefore, presumably of a more homogeneous nature. However, the difference in the success rates of the two cloning attempts was also attributable to the switch from

auto IL-2 to anti-CD3 Abs to activate polyclonally T cells in vitro. Whereas virgin T lymphocytes remain relatively quiescent with regard to cell growth in the presence of anti-CD3, it has been shown that the Ab to CD3 induces IL-2 production and cellular proliferation of primed or memory T cells in vitro (Byrne et al 1988). Thus, the use of this MAb to trigger clonal expansion acts to bias single cell proliferation in favour of those cells which are malaria parasite-specific. Although clonal activation and proliferation by such means can occur in vitro, it remains to be seen whether such expansion can be driven regularly by Ag under anything approaching physiological conditions.

The response of Ly-4-bearing T cells, both before and after cloning, was specific to the AS strain of P. c. chabaudi against which the lines had been previously primed in vivo. The proliferative response, as measured by tritium incorporation, was consistently found to be specific for pRBC, compared to an nRBC control. In another study, Brake et al (1986) showed that Ly-4^+ cell lines raised to P. c. adami pRBC could proliferate in response to cells infected with this species, but not to a similar preparation made from RBC infected with P. berghei. This highly specific proliferation is not that surprising in view of the fact that in vitro propagation selects for populations of cells with greater reactivity to the original priming Ags (Hom et al 1986).

The process of plasmodial Ag recognition by cultured T cells has an absolute requirement for APC, since no proliferation was detected in the absence of such cells. These findings are consistent with previous observations that showed Ag-specific proliferation to parasite Ags only in the presence of additional accessory cells (Louis et al 1979, 1981). In particular, other studies have reported the necessity for APC in anti-malaria T cell cultures (Chemtai et al 1984 a & b, Gross et al 1984, Gross & Frankenburg 1988, Brake et al 1986, 1988). Furthermore, MHC compatibility between APC and responder T lymphocytes was required for P. c. chabaudi AS-induced T cell proliferation, since only syngeneic splenic APC were effective in presenting pRBC Ag. In the system studied, APC of the same H-2^q haplotype as the NIH murine Ly-4^+ cells used were capable of participating in the APC-T cell complex. However, allogeneic APC failed to participate in any cognate T cell-APC reaction. These data suggest that splenic T cells derived from P. c. chabaudi AS-immune or semi-immune NIH mice maintain plasmodial Ag specificity in an H-2-restricted manner when cultured in vitro. A similar finding was reported by Brake et al (1986), who showed MHC restriction in the presentation of Ags to P. c. adami-specific T cells. In this case, BALB/c mouse strain APC of the same H-2^d

haplotype as the responder cells could effectively present lysate, but allogeneic H-2^k haplotype APC (CBA/J mice) were unable to induce a proliferative response in the presence of homologous pRBC. The mechanism of resistance to P. c. chabaudi AS has been examined, first by Stevenson et al (1982) and then in more detail by Wunderlich et al (1988). These investigations indicated that murine resistance to P. c. chabaudi AS is under complex polygenic control involving one or more non-H-2 genes as well as genes in both I-A and I-E subregions of the H-2 complex. With regard to parasite-specific T cell proliferation, malaria-protective Ags were thought to be presented in context with I-A^b molecules but not in context with I-A^k molecules. The data presented, though less complete, do support these findings. Using strains of mice recombinant at the H-2 complex, preliminary investigations implicated the I-A and/or I-E regions of the MHC in the determination of parasite-specific immune reactivity. That I-A^b-encoded molecules present Ag could not be discounted from the results. Moreover, the observation by Wunderlich et al (1988) that the D MHC region lies outwith genetic control of anti-P. c. chabaudi immunity was confirmed. Together, both sets of MHC restriction analyses indicate that the proteins encoded by the K and D regions of the H-2 complex do not interact in Ag recognition and presentation. This is not surprising since the K and D loci contain class I genes which code for transplantation Ags, the presence of which on cytotoxic Ly-2^+ T cells plays a role in killing target cells. This fits with other line of investigation which tend against Ly-2^+ T cells conferring protection against blood stage malaria parasites.

As genes in the I region of the H-2 complex encode class II molecules which play a dominant role in Ag presentation to Ly-4^+ cells (Klein 1975), it was originally presumed that Ag presentation and/or T cells were defective with respect to pRBC Ags in P. c. chabaudi AS-susceptible strains of mouse. This, however, proved not to be the case (Wunderlich et al 1988) as no general defect in APC presentation or in the ability of Ly-4^+ T cells to be stimulated by P. c. chabaudi AS amongst various murine strains has been found. Rather, it has been demonstrated that host survival after infection with this malaria parasite may be determined by the ability to replace destroyed RBC quickly and efficiently (Stevenson et al 1982). It appears that it is the rate of erythropoiesis that is genetically controlled. Moreover, information is available that murine resistance to Plasmodium is sex-dependent in that female mice are more resistant than male mice; this has been ascribed to a superior erythropoietic system in females (Stevenson et al 1982). Recently, the sex hormone testosterone, the regulation of which is H-2-linked

(Klein 1975), has been implicated in susceptibility to malaria. Wunderlich et al (1988) described experiments in which the I-A^b-controlled resistance to P. c. chabaudi AS was abrogated by treatment of mice with testosterone prior to challenge. The mechanism by which testosterone exerts its negative effect is unknown, but whatever this may be, the fact that this sex hormone interferes with the development of protective immune mechanisms against malaria parasites has an important implication for vaccine development against human malaria. Thus far, studies of natural P. falciparum infections in man have indicated, but not proven, that the apparent non-responsiveness to the malaria-specific Ag Pf155/RESA of certain blood donors is due to genetic restriction, in this case of the HLA complex (e.g. Troye-Blomberg et al 1988). The uncertainty with which results have been viewed reflects the fact that in most field studies in endemic regions, very few of the infected individuals examined had a similar match of MHC class II haplotypes, making evaluation of the relationship between MHC type and malaria T cell responsiveness very difficult. It should be stressed that genetic control of immunity to malaria is not unique to the asexual erythrocytic stages, for it has also been shown that protection to the exoerythrocytic stages of P. yoelii is controlled by H-2 and non-H-2 genes (Weiss et al 1989). If similar complex regulation of immunity to these stages also occurs in the human malarias, this too will be a major hurdle for vaccine production.

Finally, the results of this chapter show the occurrence of immunodepressed lymphocyte responses to P. c. chabaudi AS Ags. This was observed both with unfractionated spleen cell preparations, and, more significantly, with established lines and clones of the Ly-4^+ phenotype. With all these sources of cells, the Ag-specific proliferative response was significantly lower for lymphocytes taken during acute infection than for those taken from mice convalescing from infection. This transient immune non-responsiveness of the cell-mediated immune system has been little studied in murine models of malaria and has been described previously only for P. yoelii-infected mice (Weinbaum et al 1976 a, 1978). In the experiments performed, the cellular growth of all assayed cells, irrespective of the degree of response, was pRBC lysate-specific. This finding is in line with those reported by others working on P. falciparum infection (Kass et al 1971, Wyler & Oppenheim 1974, Bygbjerg et al 1981, Troye-Blomberg et al 1983 b). Further, responsiveness of T cells from patients with acute P. falciparum malaria gave a weak but specific proliferation, peaking after 3-4 d of incubation, but waning within 5-6 d (Troye-Blomberg et al 1983 b, 1984). In contrast, T cells from

a group of apparently immune donors living in highly malaria endemic areas developed strong and long-lasting proliferative responses to P. falciparum AS with a peak on d 5-6 of in vitro culture. A very similar response pattern was also obtained with spleen cells from malarious mice (Weinbaum et al 1976 a). The experiments herein were also very similar, for spleen cells taken on d 16 of a primary infection of P. c. chabaudi AS or Ly-4$^+$ lines or clones generated from spleens of mice sacrificed on d 16 or d 20 p.i. showed low proliferative responses compared to spleen cell preparations or lines derived from multiply-infected mice. The finding of reduced proliferation of lymphocytes only during the primary parasitaemia or subpatency indicates the occurrence of immune suppression. Like the P. falciparum system of Troye-Blomberg et al (1983 b), this suppression could not be overcome by increasing the pRBC Ag dose.

There are conflicting reports on the proliferation of lymphocytes of non-sensitised individuals upon exposure in vitro to P. falciparum Ags. It has been reported that plasmodial extracts have non-specific mitogenic effects on human lymphocytes in vitro (Wyler & Oppenheim 1974, Greenwood & Vick 1975, Greenwood et al 1979, Druilhe et al 1980, Ballet et al 1981, Gabrielsen & Jensen 1982). However, Bygbjerg et al (1981, 1985) indicated that P. falciparum Ags had a seemingly specific stimulating effect on parasite-primed human lymphocytes. The results described in this chapter support this latter view, for the response of uninfected spleen cells to P. c. chabaudi AS pRBC Ags was markedly less than that of primed lymphocytes, even those taken on d 16 p.i.. This difference may be due to the difficulties encountered by many authors in distinguishing between Ag-specific and polyclonal stimulatory effects of malaria blood stage extracts. Recently, Riley et al (1988 a) have shown that lymphocytes of malaria-immune adults respond in an Ag-specific manner to purified soluble malaria Ags but that crude P. falciparum schizont sonic extract is non-specifically mitogenic for both immune and non-immune cells. Thus, the different results described previously may be due to the use of pRBC Ag preparations of varying definition.

A preliminary investigation into the mitogenic reactivity of spleen cells primed to varying degrees to P. c. chabaudi AS suggested that the response of semi-immune lymphocytes to the T cell mitogen Con A was less than that of either fully immune or naive spleen cells. This would indicate a general suppression to all sources of stimulation on behalf of lymphocytes taken during the acute phase of a primary infection. This finding contradicts that of others using P. falciparum (Brasseur et al 1983, Troye-Blomberg et al 1984). This observation may have reflected the activation

of suppressor mechanisms by other splenic cell types. Indeed, a major suppressor cell of adherent macrophage origin has been suggested to be important in animal malaria (Strambachova-McBride & Micklem 1978, Corrêa et al 1980). As such, this experiment most likely highlighted the feature of non-specific immunosuppression that occurs in acute falciparum malaria infections in man (Weidanz 1982) and presumably also during murine malaria. Recent evidence suggests that there is also specific suppression of T cell-mediated responses to malaria Ags during acute infection, but that cellular responses to many other Ags are not affected. As the established Ly-4$^+$ lines and clones were not tested for reactivity upon mitogenic stimulation, neither possibility could be pursued. What was unequivocally demonstrated, however, was the dose-responsiveness of Ly-4$^+$ in vivo-primed T lymphocytes to homologous pRBC lysate in vitro. An alternative explanation of these findings has been provided by Riley et al (1988 b) who found that a crude extract of P. falciparum Ags did suppress lymphoproliferative responses to mitogens. The degree of suppression appeared to correlate with the level of lymphocyte proliferation to the pRBC preparation, and was correspondingly more marked in malaria-immune donors than in non-immune donors. This study is similar to that carried out herein in the use of crude parasite preparations, and this may have been responsible for the similar patterns of T cell mitogenic activity.

The consensus of opinion now forming is that for P. falciparum in man, cellular immune responses are suppressed during acute infection and that this may contribute to the slow acquisition of protective immunity after natural exposure to infection. However, specific lymphoproliferation can be induced by crude parasite extracts and soluble purified Ag in peripheral blood lymphocytes from recently recovered individuals (Wyler & Oppenheim 1974, Troye-Blomberg et al 1983 b, Bygbjerg et al 1985). A similar response was observed using cloned lines of Ly-4$^+$ T cells derived from mice primed to P. c. chabaudi AS by multiple infection. For further dissection of this system, purified P. c. chabaudi AS Ags will have to be used for lymphocyte activation. The use of defined Ags has been used already to study the lymphocyte proliferative response to P. falciparum (Riley et al 1990) and to P. vivax (Goonewardene et al 1990) malarias, in both of which non-responsiveness was a feature.

In general, non-responsiveness to a given Ag could have several causes, including the induction of suppressor circuits (Sercatz et al 1978), the failure of Ag-MHC interaction (Allen 1987) or tolerance to MHC-Ag complexes (Vidovic & Matzinger

1988). T cell clones have also been rendered non-responsive or tolerised by exposure to high doses of IL-2 (Pawelec et al 1989). It appears that in Trypanosoma cruzi infection of mice, for which suppression of the parasite-specific proliferative response is also restricted to the acute phase of infection, the high antigenic load and the extensive polyclonal activation of lymphocytes that occur in the spleen may be responsible for the reduction in the specific spleen cell responses elicited soon after the start of infection (Curotto de Lafaille et al 1990). For falciparum malaria, there is some evidence that $CD8^+$ T cells are implicated in the Ag-specific suppression of primed lymphocyte proliferation (Riley et al 1989 a & b).

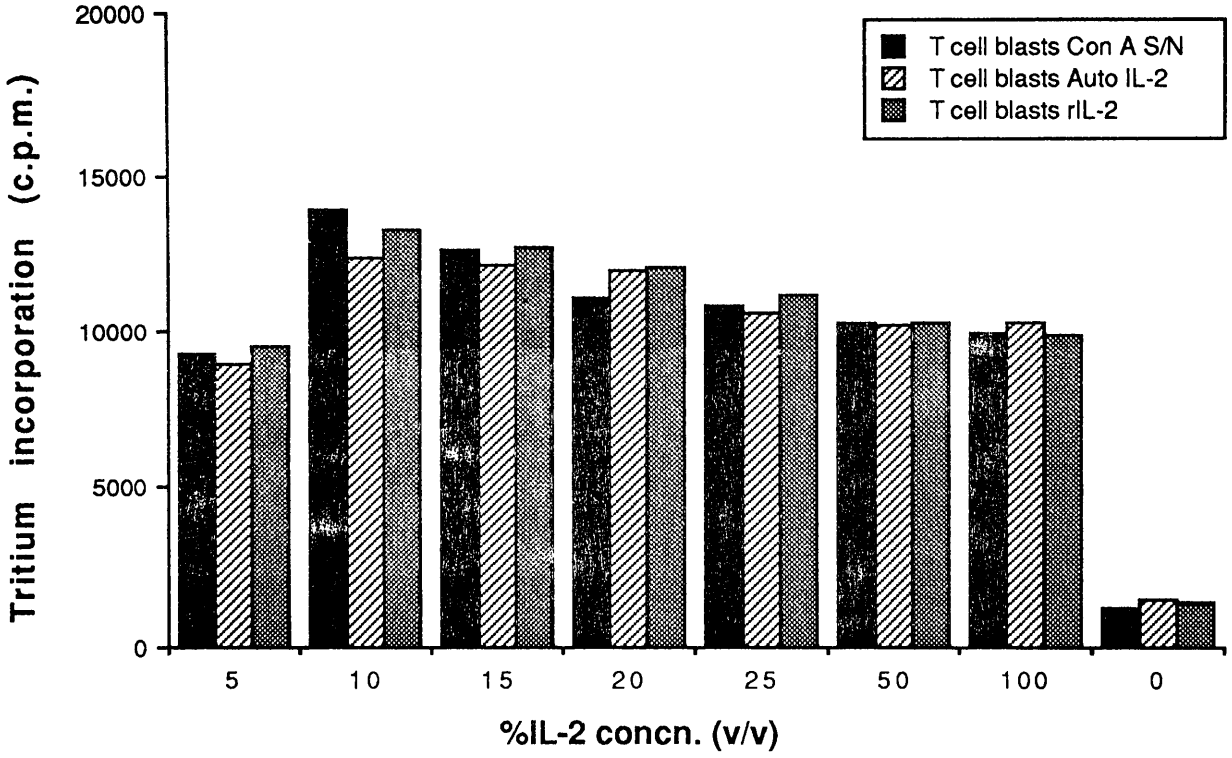


Fig. 4.2.1 Assay of IL-2 concentration on NIH T lymphocyte blasts

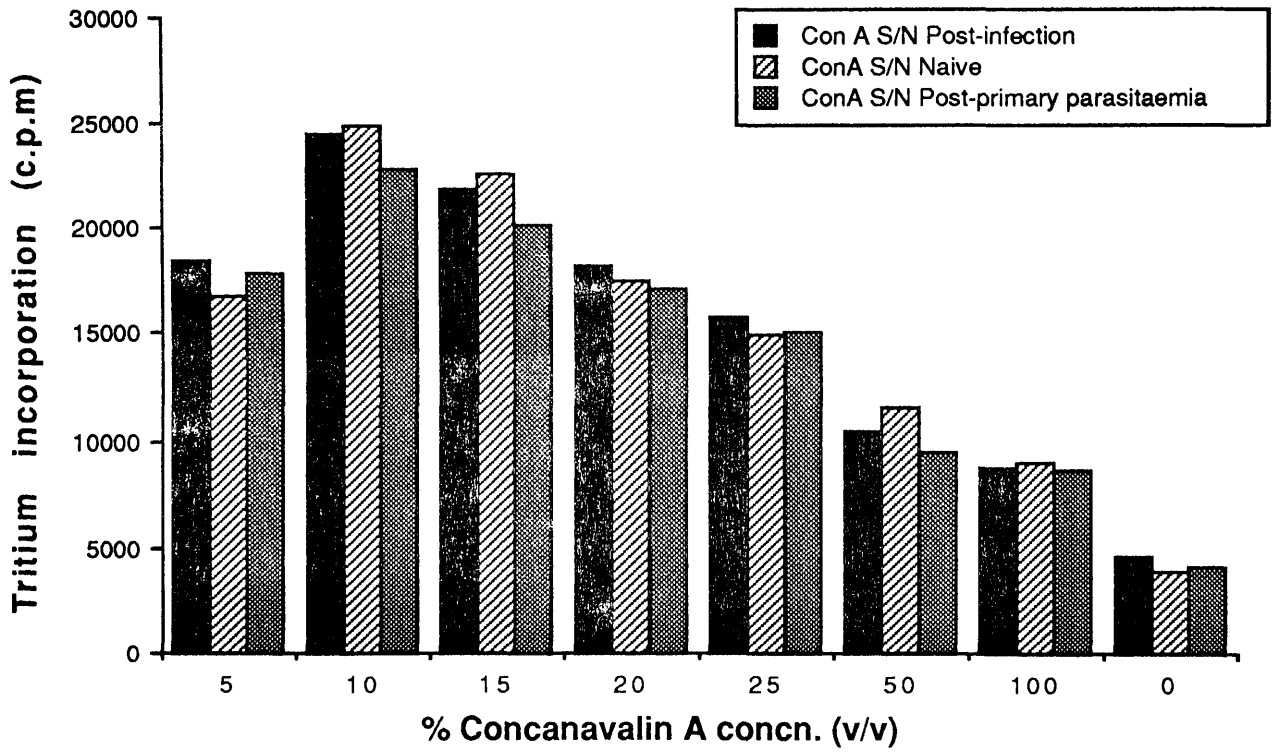


Fig.4.2.2 Assay of IL-2 concentration in Con A S/N as measured by proliferation of NIH splenic lymphocytes

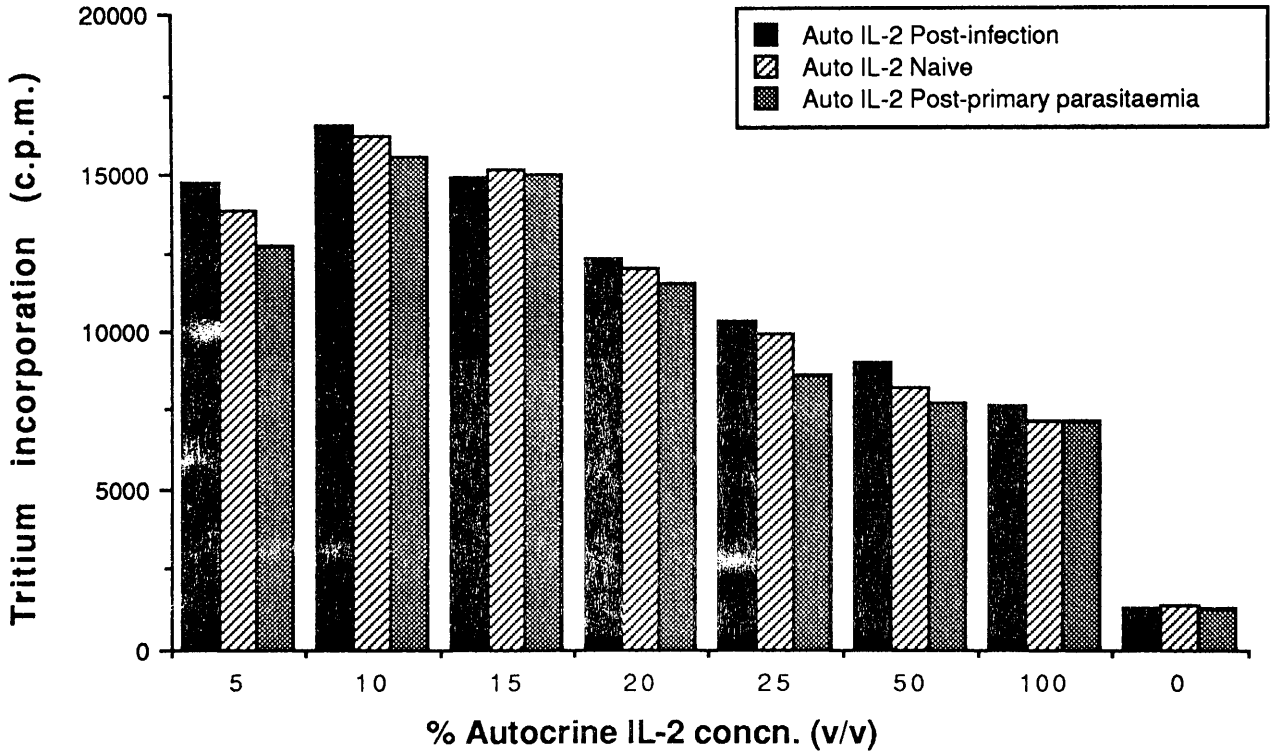


Fig. 4.2.3 Assay of IL-2 concentration in Autocrine IL-2 as measured by proliferation of NIH splenic lymphocytes

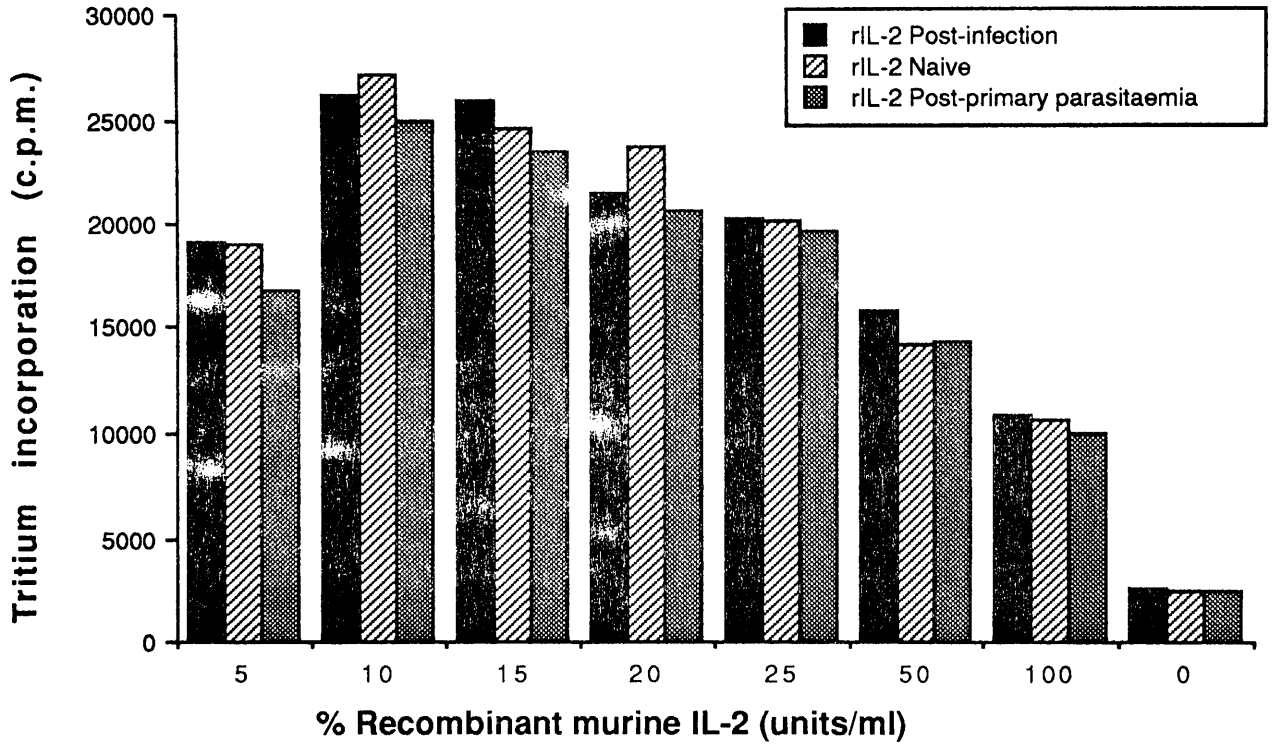


Fig. 4.2.4 Assay of IL-2 concentration in Recombinant murine IL-2 as measured by proliferation of NIH splenic lyphocytes

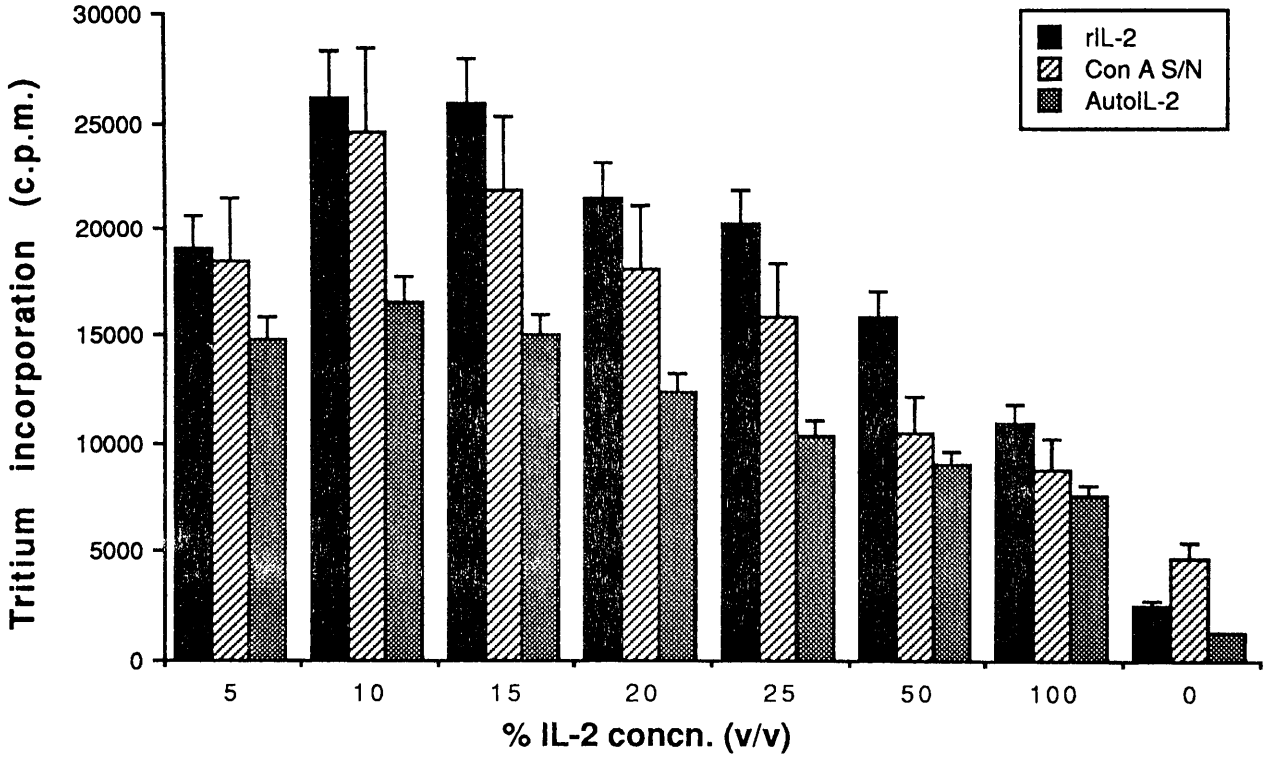


Fig. 4.2.5 Assay of IL-2 concentration as measured by proliferation of NIH post-infective splenic lymphocytes

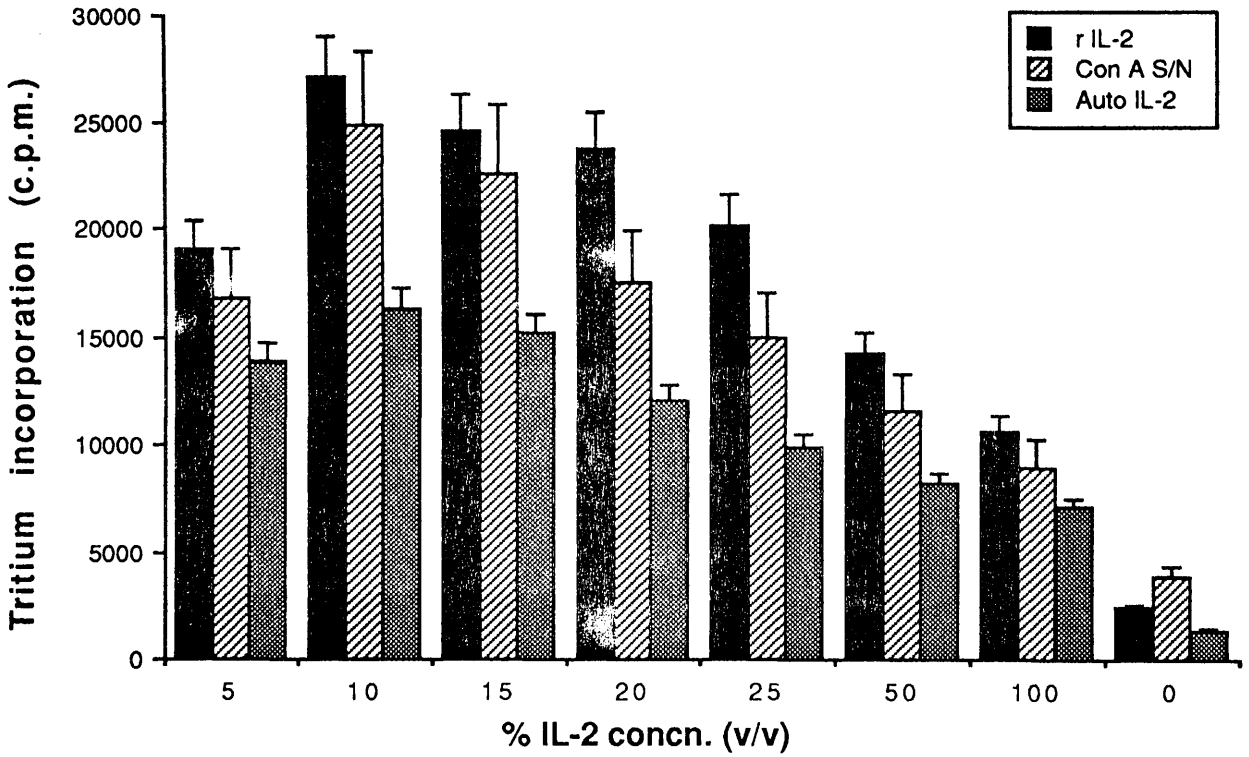


Fig. 4.2.6 Assay of IL-2 concentration as measured by proliferation of NIH naive splenic lymphocytes

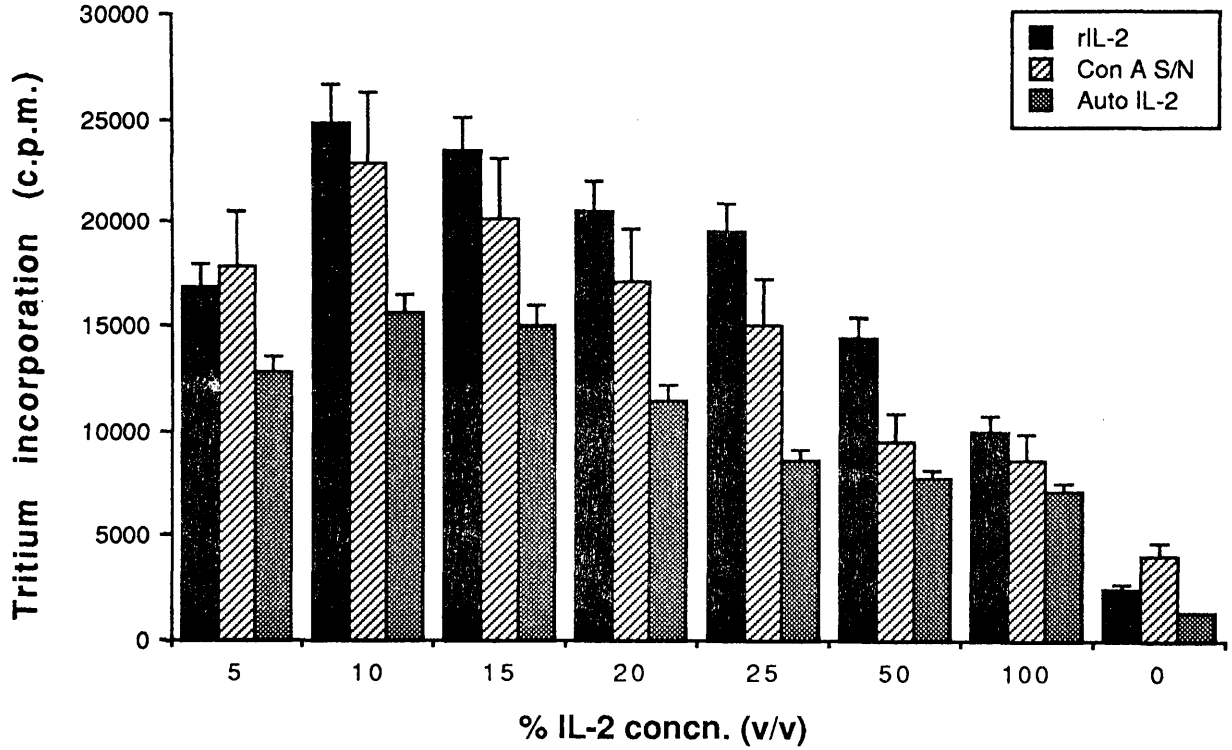


Fig. 4.2.7 Assay of IL-2 concentration as measured by proliferation of NIH post-primary parasitaemia splenic lymphocytes

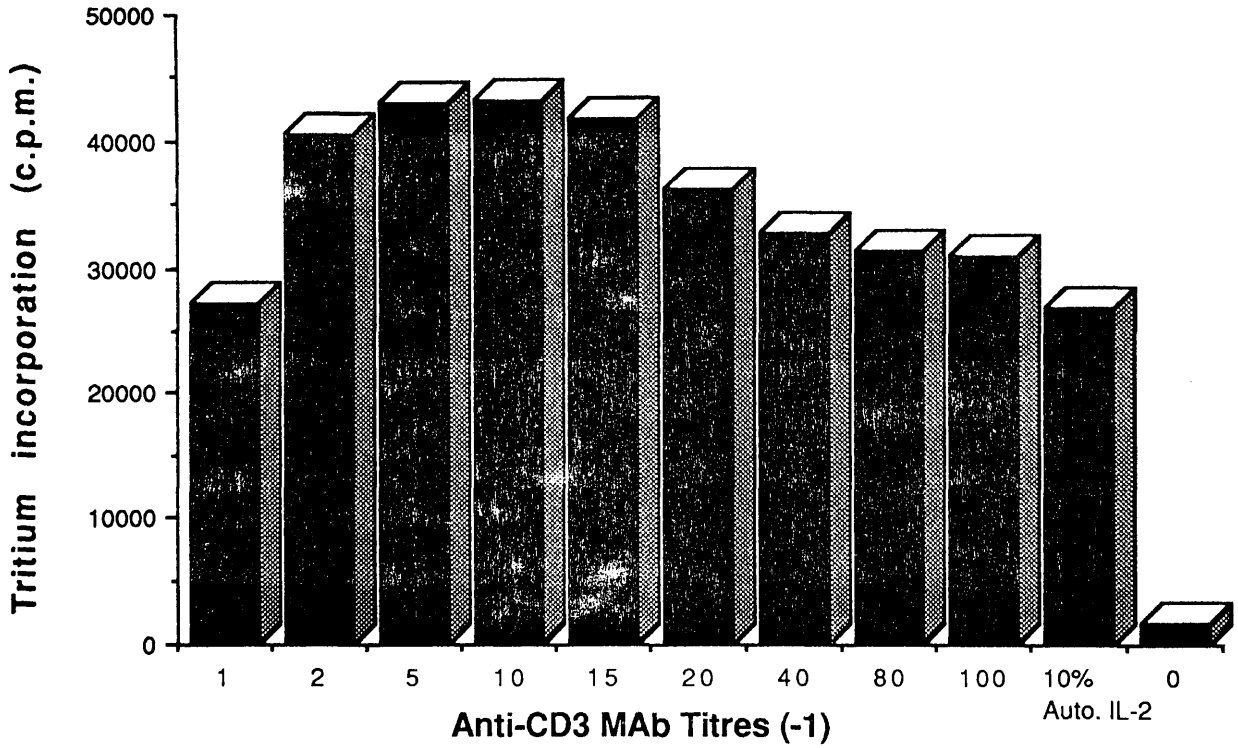


Fig. 4.3.1 Assay of anti-CD3 MAb on NIH naive splenic lymphocytes

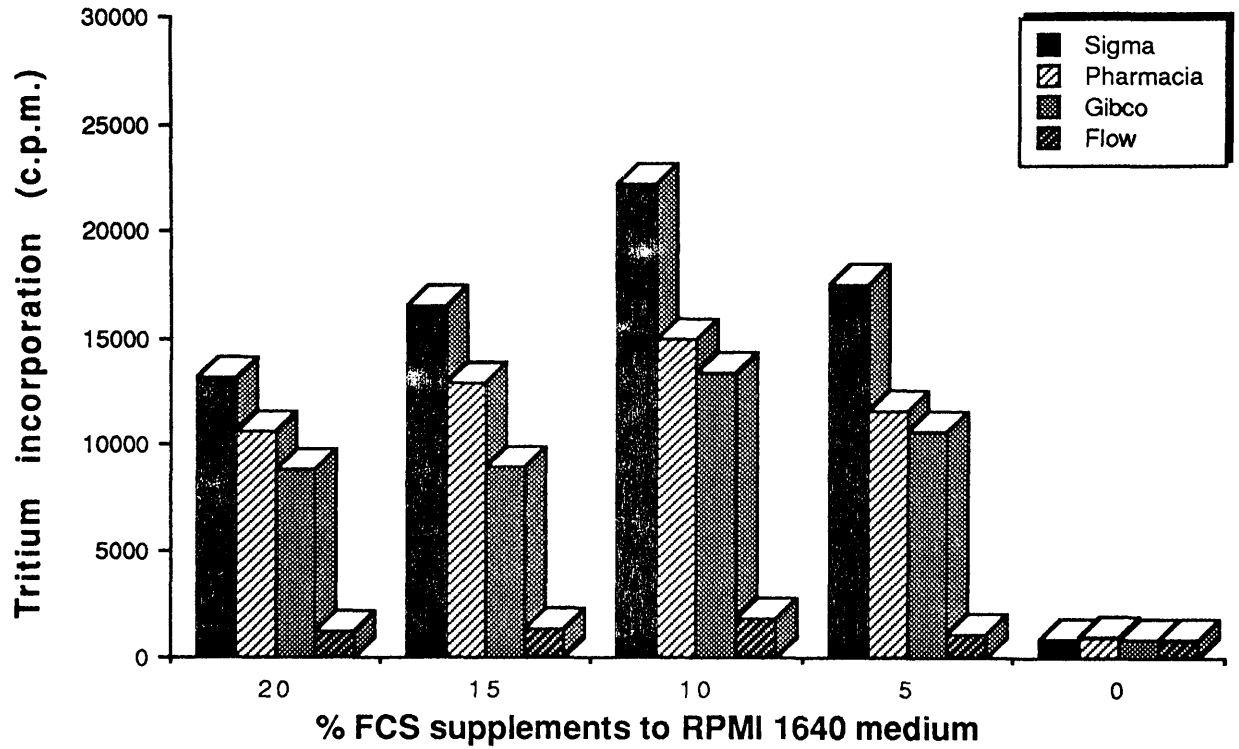


Fig. 4.4.1 Assay for the ability of FCS to support the non-specific proliferation of NIH naive splenic lymphocytes

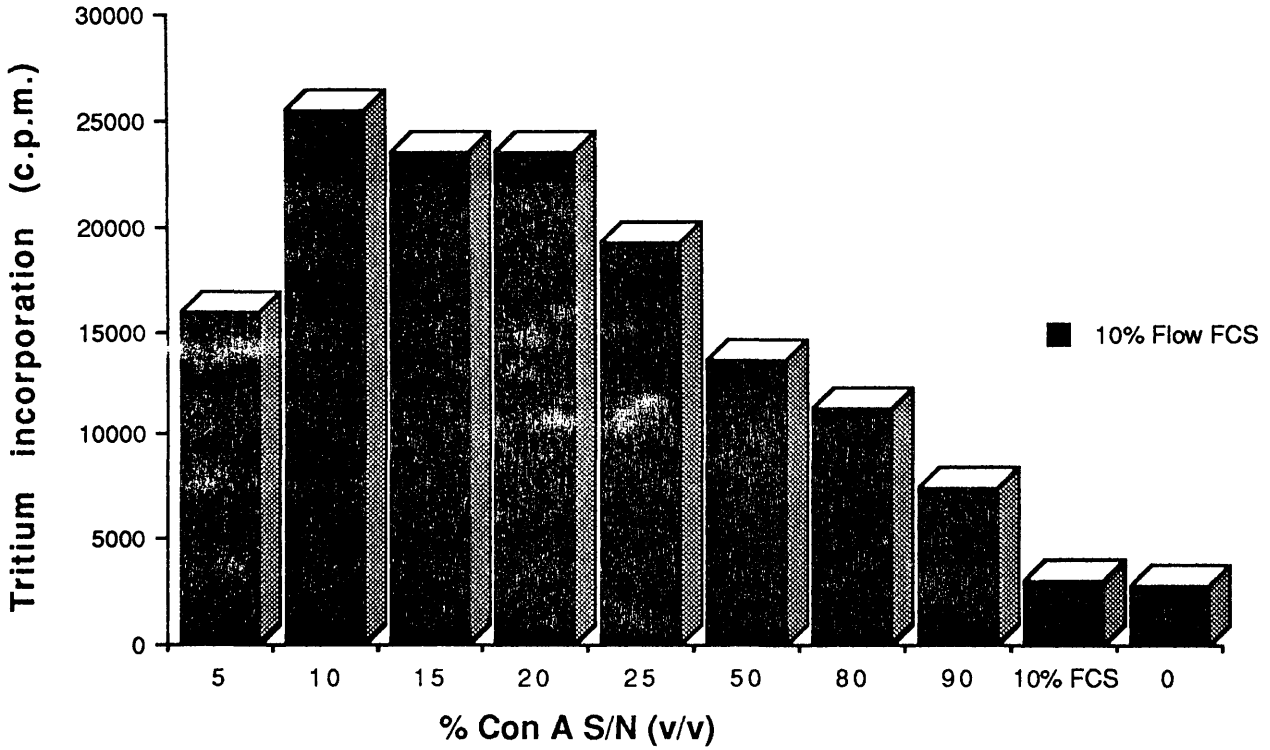


Fig. 4.4.2 Assay for the ability of a 10% Flow FCS supplement to RPMI 1640 medium to support the Con A S/N-promoted proliferation of NIH naive splenic lymphocytes

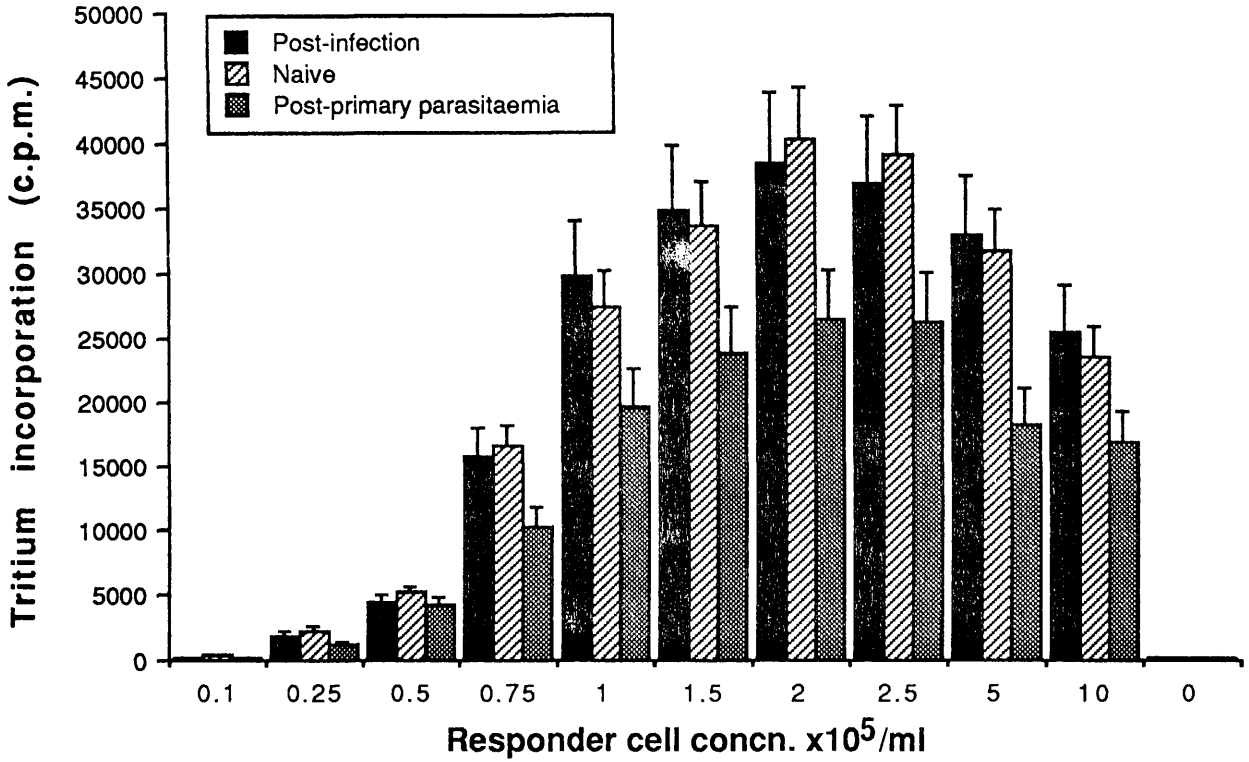


Fig. 4.5.1 Assay of responder cell concentration as measured by proliferation of NIH splenic lymphocytes in response to a 10% v/v Con A S/N supplement to RPMI 1640 medium

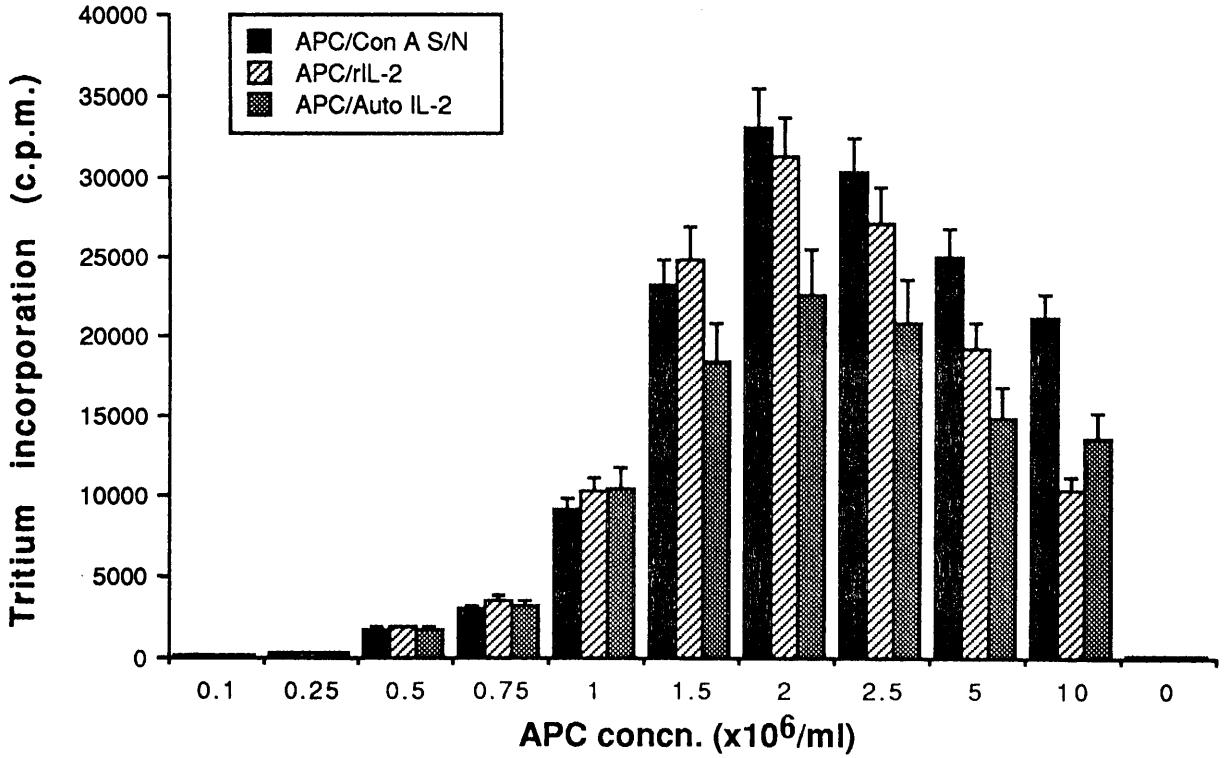


Fig. 4.6.1 Assay of APC concentration on NIH post-infection splenic lymphocytes

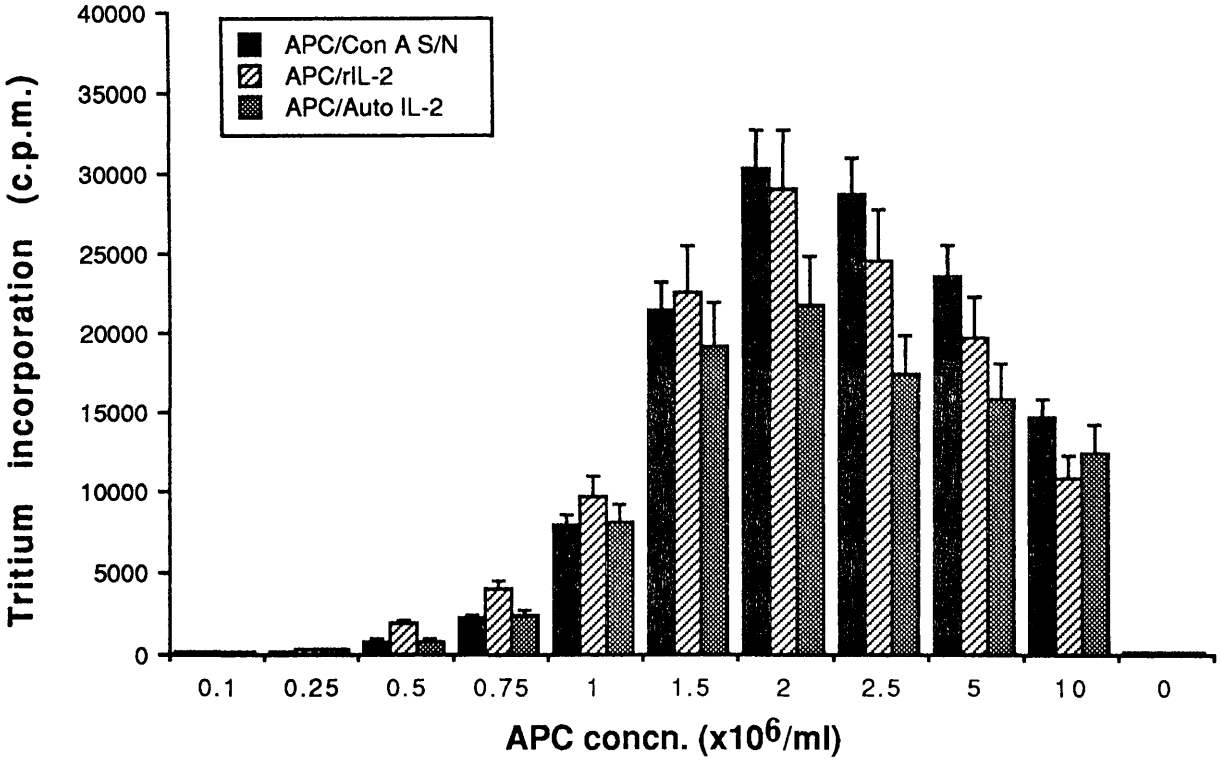


Fig. 4.6.2 Assay of APC concentration on NIH naive splenic lymphocytes

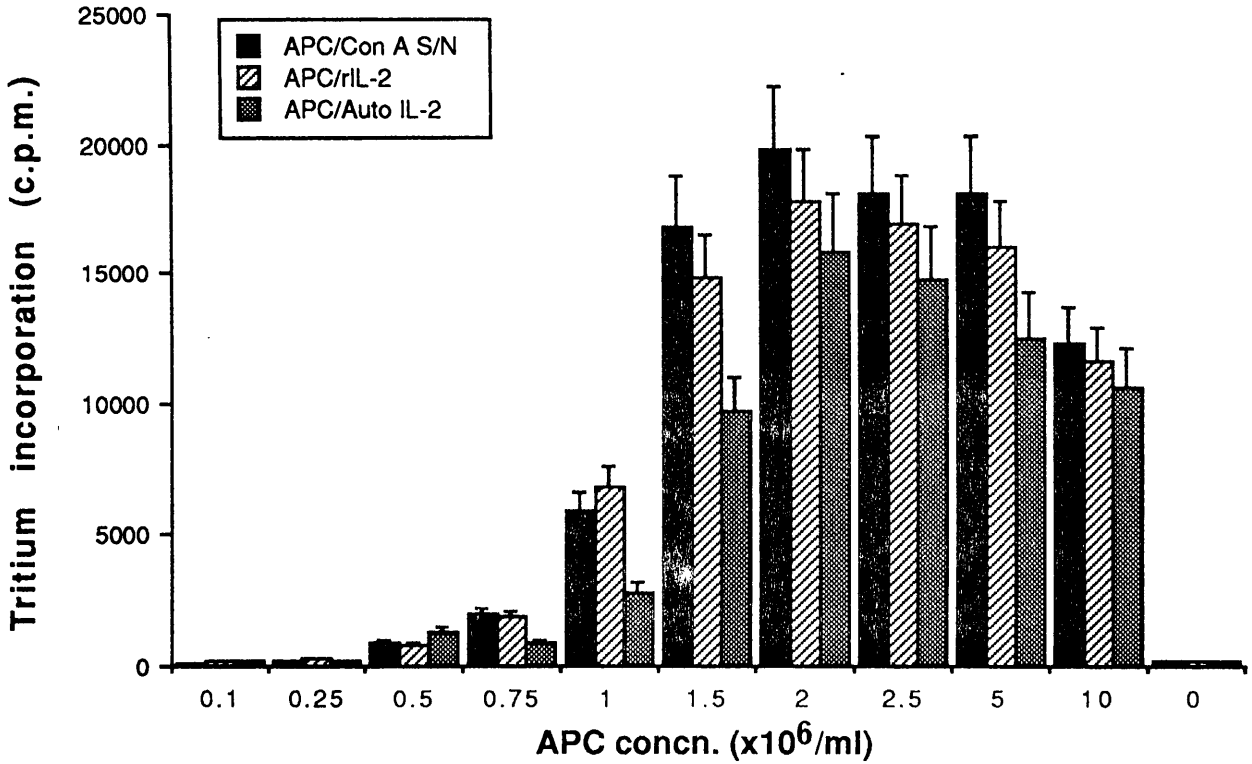


Fig. 4.6.3 Assay of APC concentration on NIH post-primary parasitaemia splenic lymphocytes

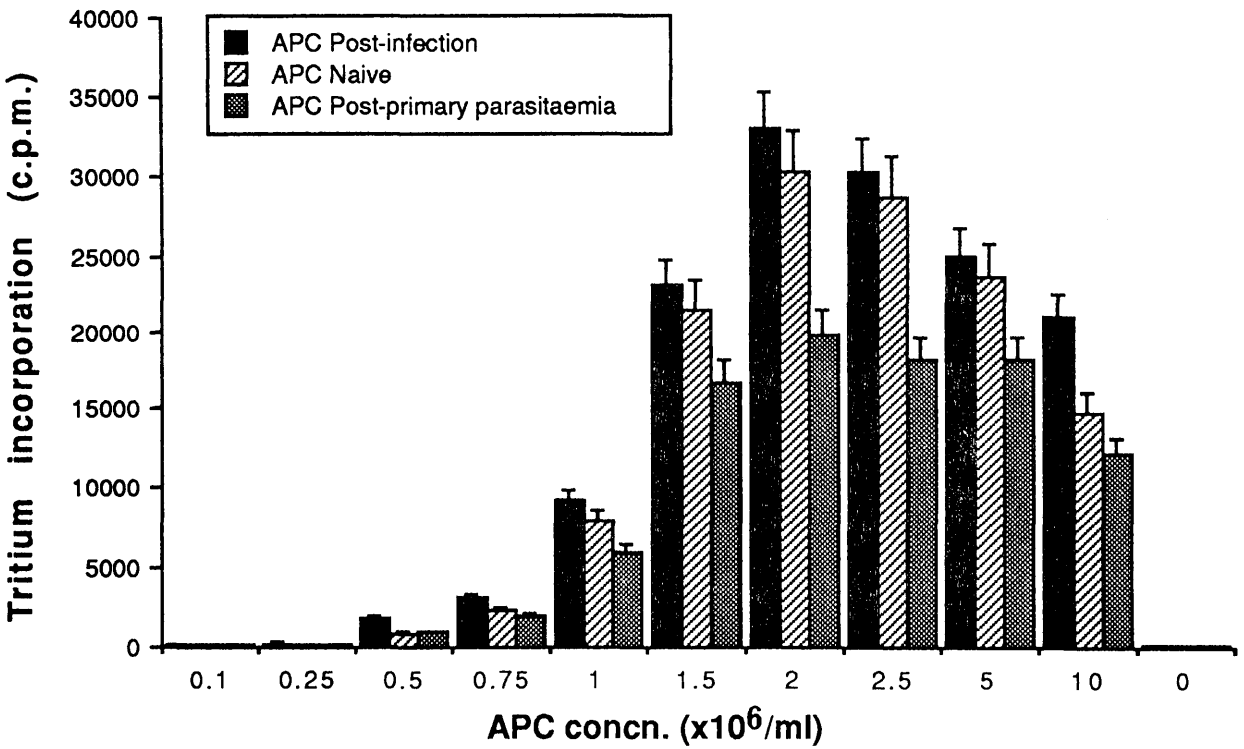


Fig. 4.6.4 Assay of APC concentration on NIH splenic lymphocytes using 10% Con A S/N

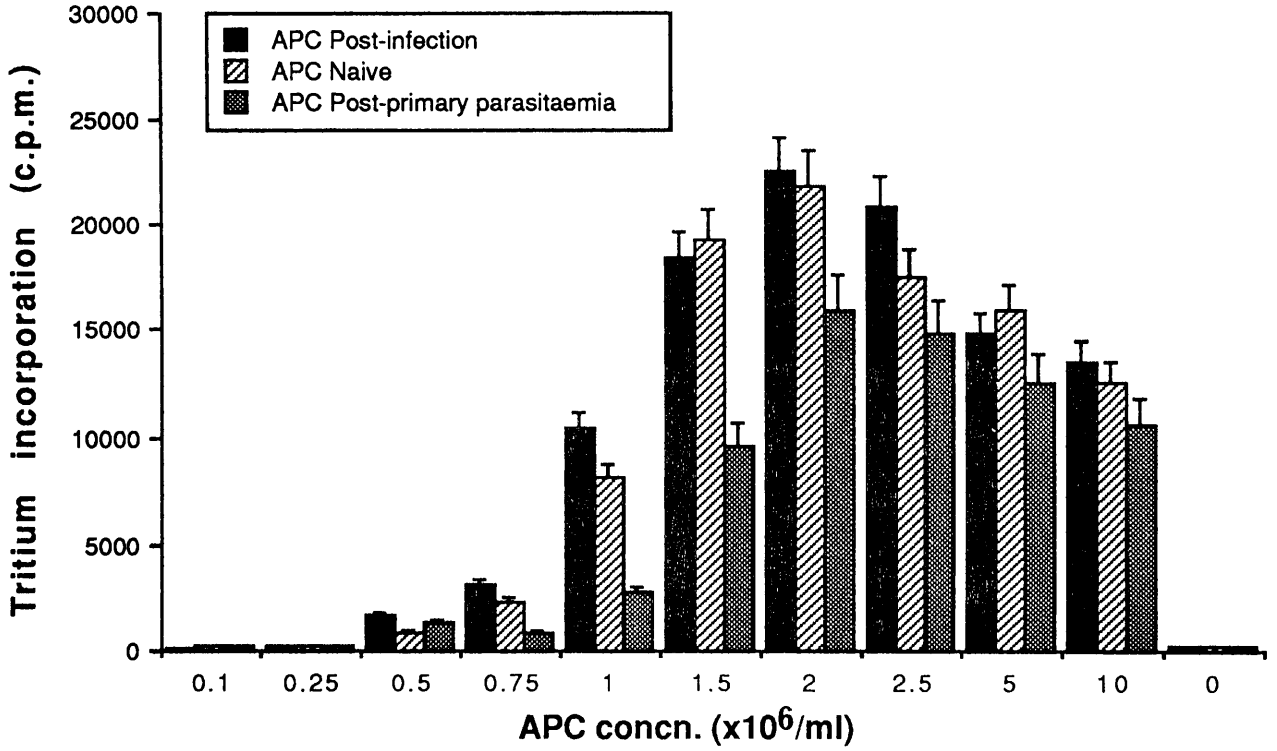


Fig. 4.6.5 Assay of APC concentration on NIH splenic lymphocytes using 10% Auto IL-2

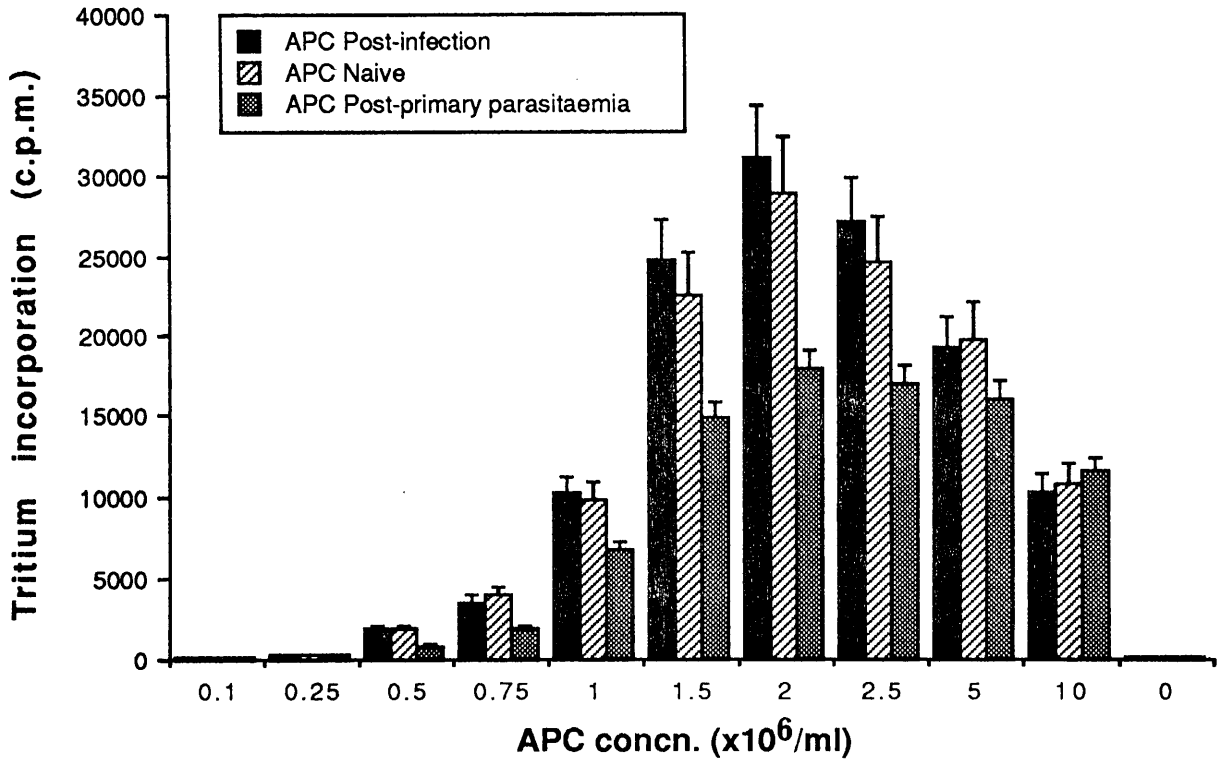


Fig. 4.6.6 Assay of APC concentration on NIH splenic lymphocytes using 10% recombinant IL-2

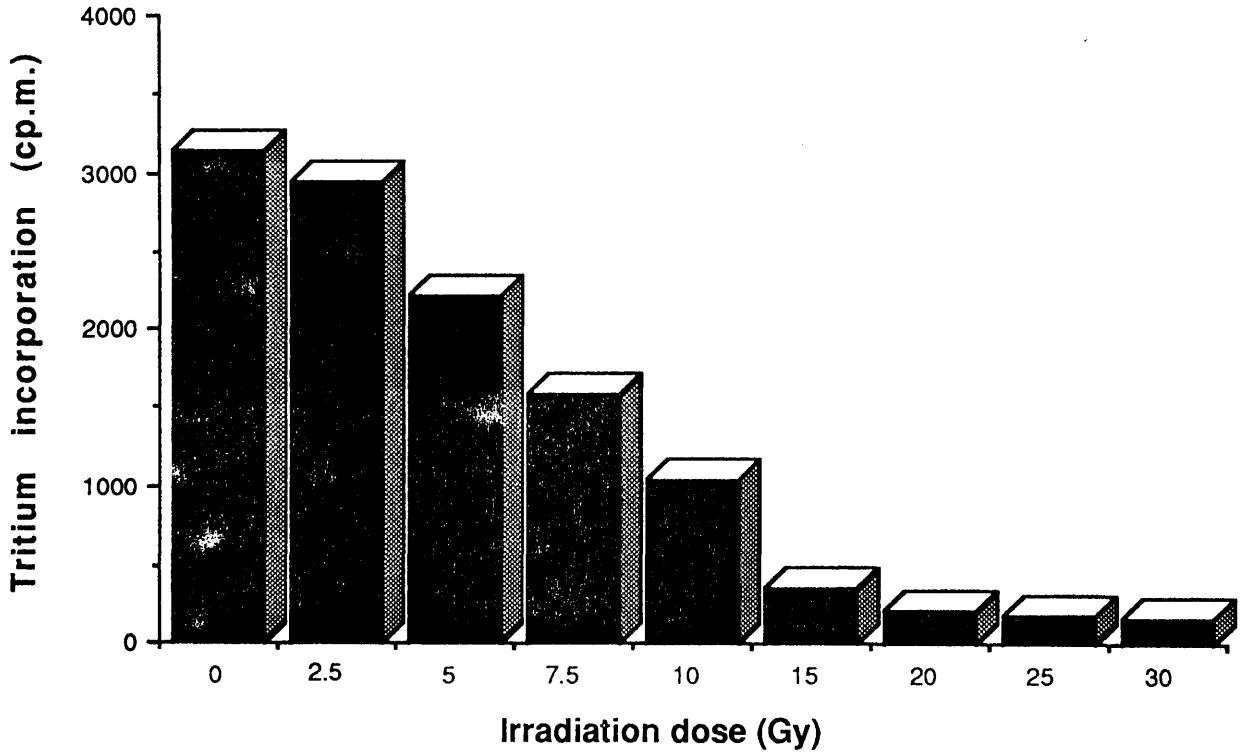


Fig. 4.7.1 Assay of inhibition of APC proliferation by measurement of NIH naive splenic lymphocyte proliferation after gamma irradiation treatment

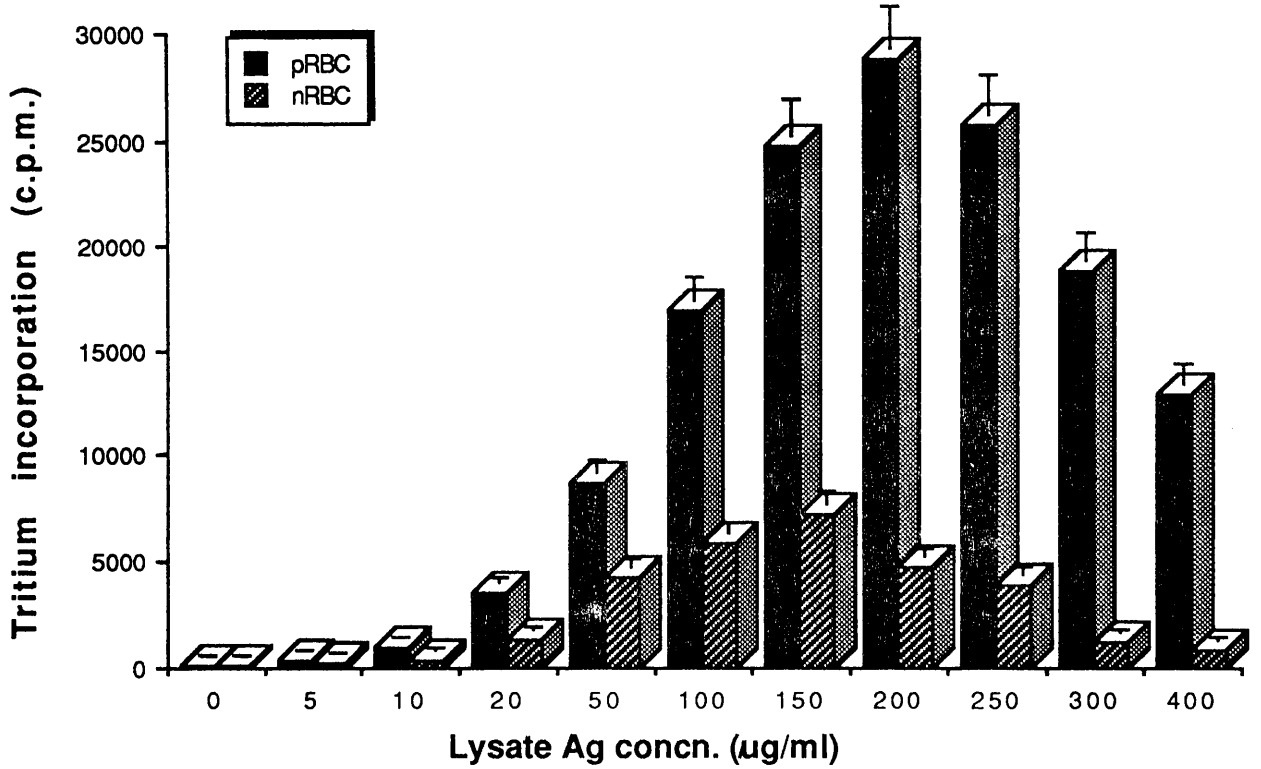


Fig. 4.8.1 Assay of proliferation of post-infection NIH splenic lymphocytes in the presence of P. c. chabaudi AS pRBC lysate

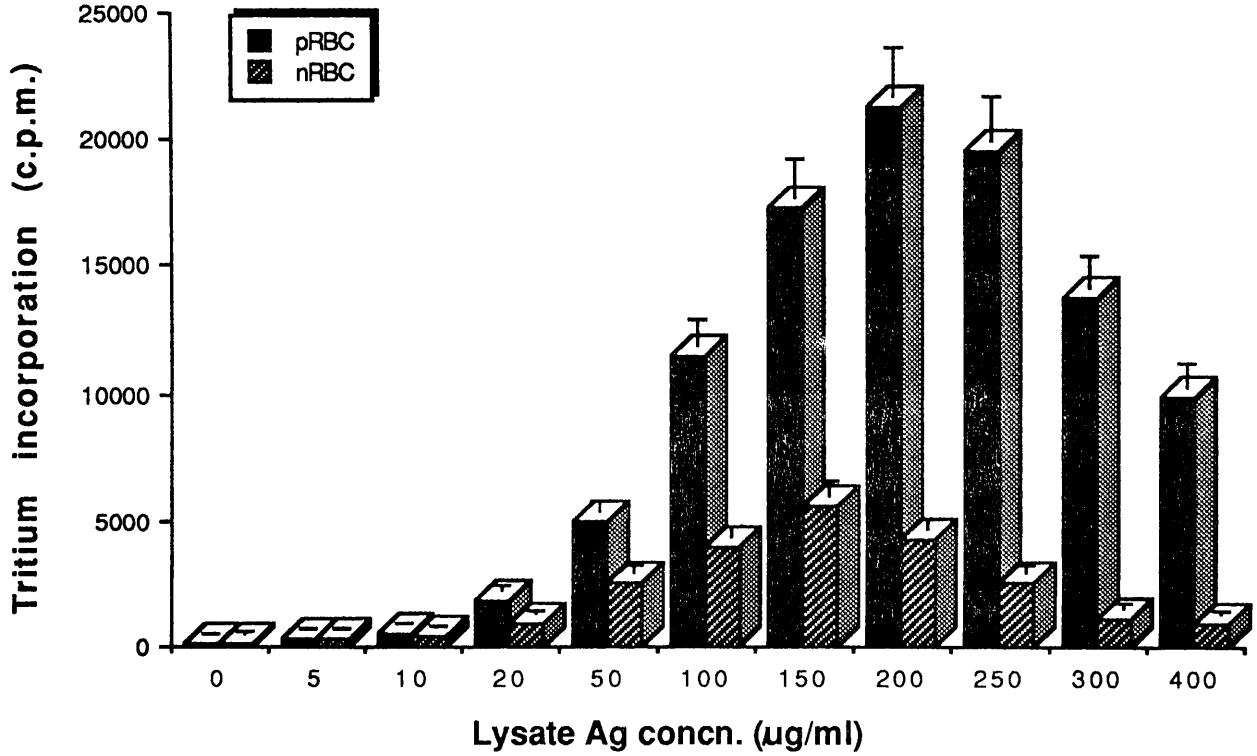


Fig. 4.8.2 Assay of proliferation of post-primary parasitaemia NIH splenic lymphocytes in the presence of P. c. chabaudi AS pRBC lysate

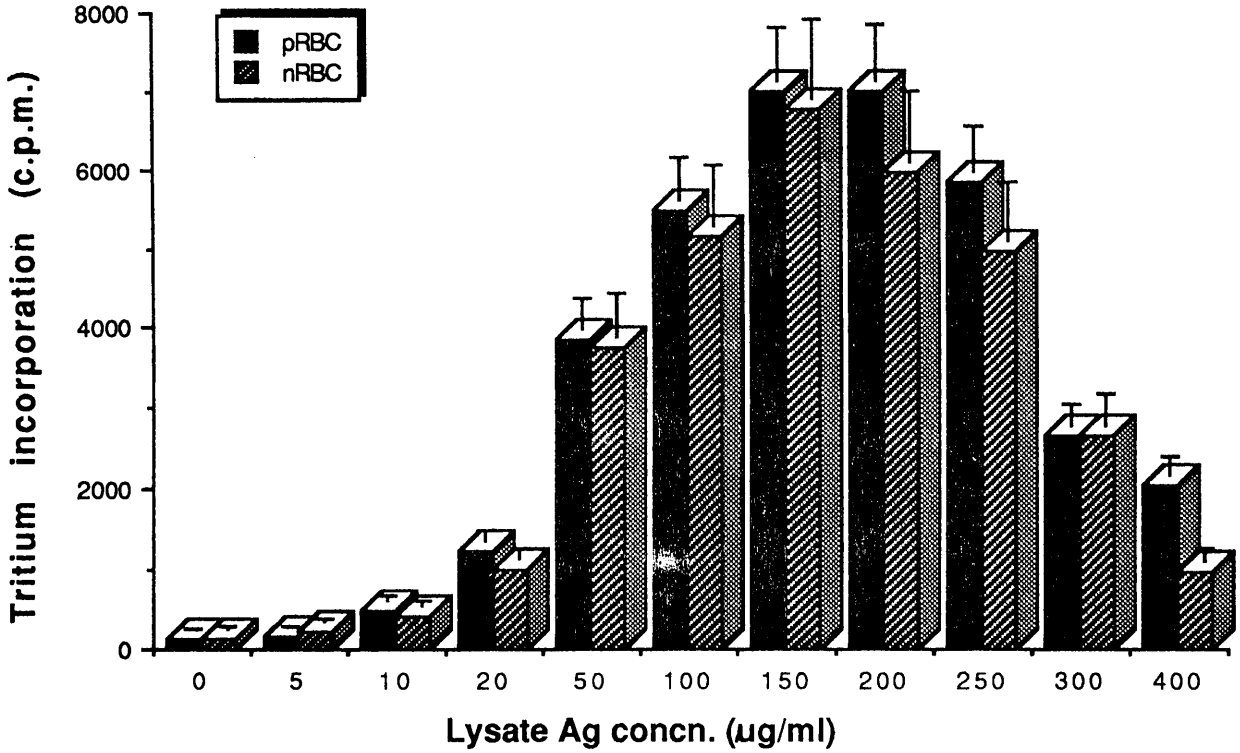


Fig. 4.8.3 Assay of proliferation of naive NIH splenic lymphocytes in the presence of P. c. chabaudi AS pRBC lysate

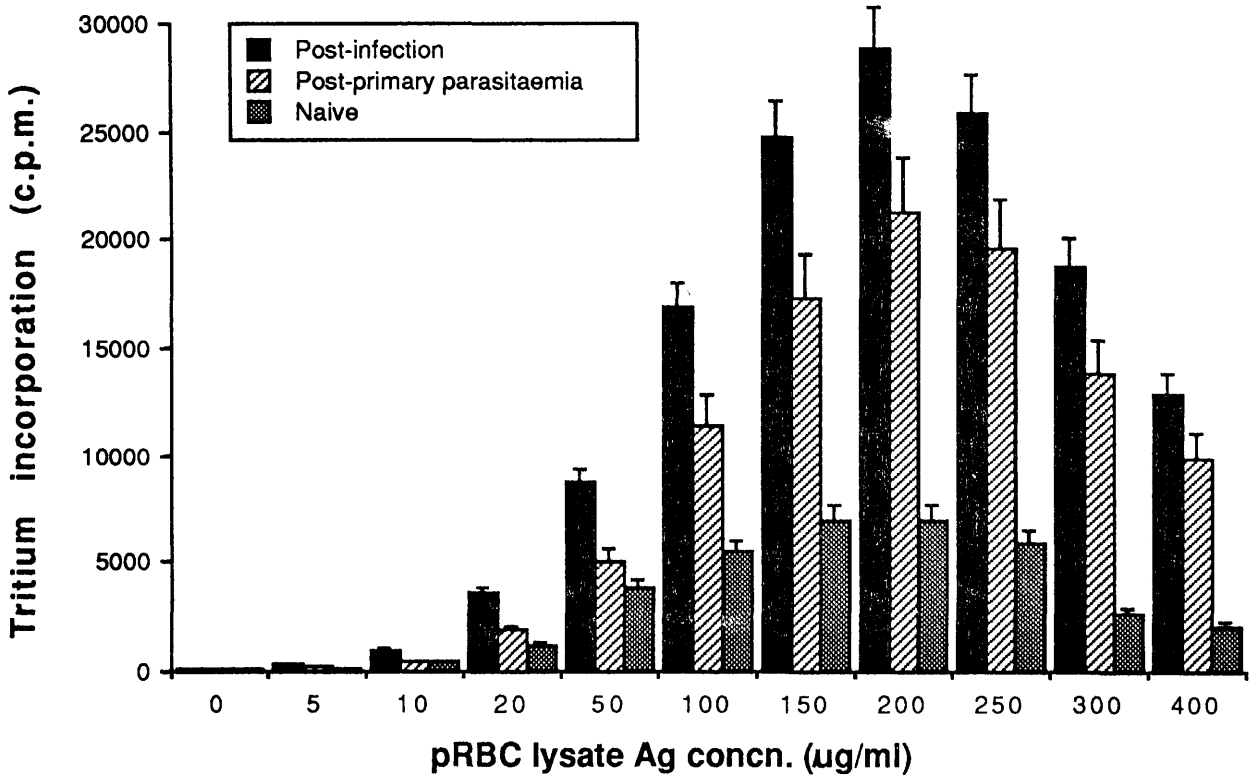


Fig. 4.8.4 Assay of proliferation of splenic lymphocytes in the presence of P. c. chabaudi AS pRBC lysate

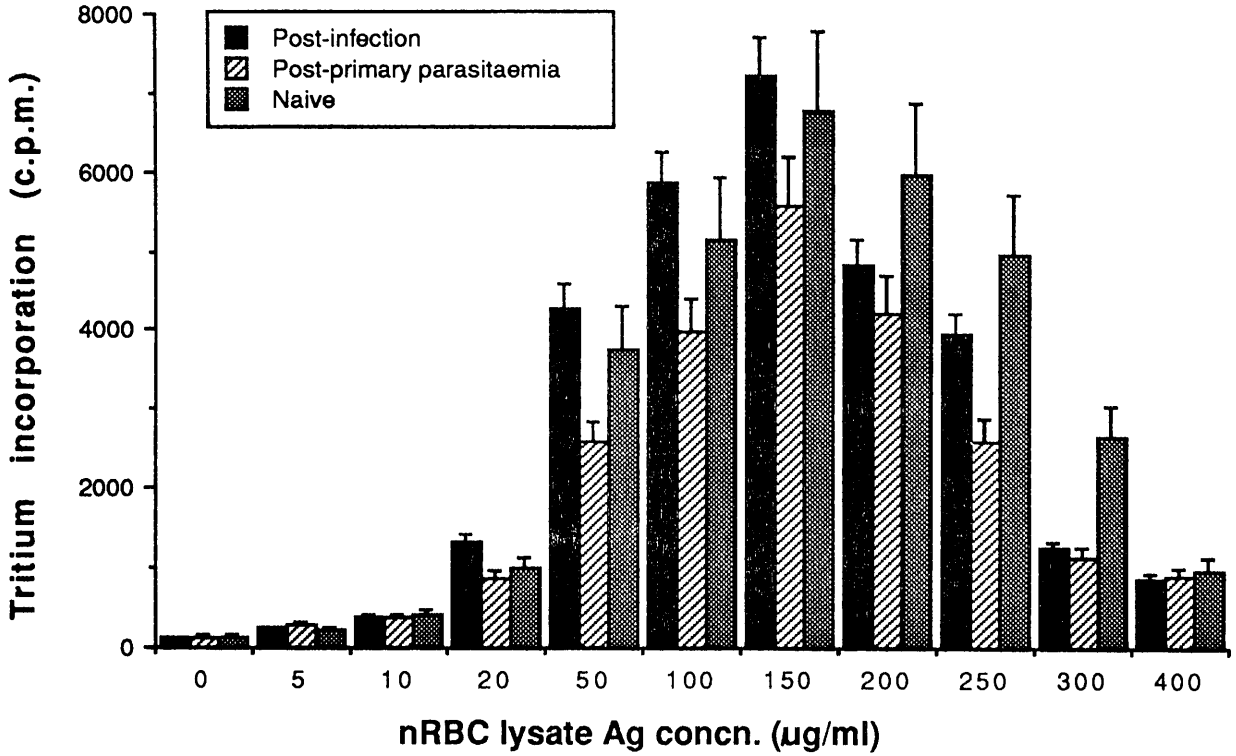


Fig. 4.8.5 Assay of proliferation of splenic lymphocytes in the presence of uninfected nRBC lysate

Table 4.9.1
Generation of Ly-4-bearing T lymphocyte lines

Identity (WEP no.)	In vivo priming period	Culture protocol	First freezing of cell stocks
723	3 infections	Original	3 d post 4th feed
737	2 infections	Original	2 d post 3rd feed
775	d 16 p.i. 1° infection (remission of acute parasitaemia)	Modified	3 d post 3rd feed
779	d 20 p.i. 1° infection (subpatent period)	Modified	3 d post 3rd feed

Table 4.10.1
Generation of Ly-4-bearing T lymphocyte clones

Identity (WEP no.)	Cloned Ly-4$^+$ line	Limiting dilution (Cells/well)
979		1.0
980		1.0
981		1.0
982		1.0
983	737	1.0
984		1.0
985		0.5
986		0.5
987		0.5
988		0.1
989		1.0
990		1.0
991		1.0
992		1.0
993		1.0
994	775	1.0
995		1.0
996		0.5
997		0.5
998		0.5
999		0.1

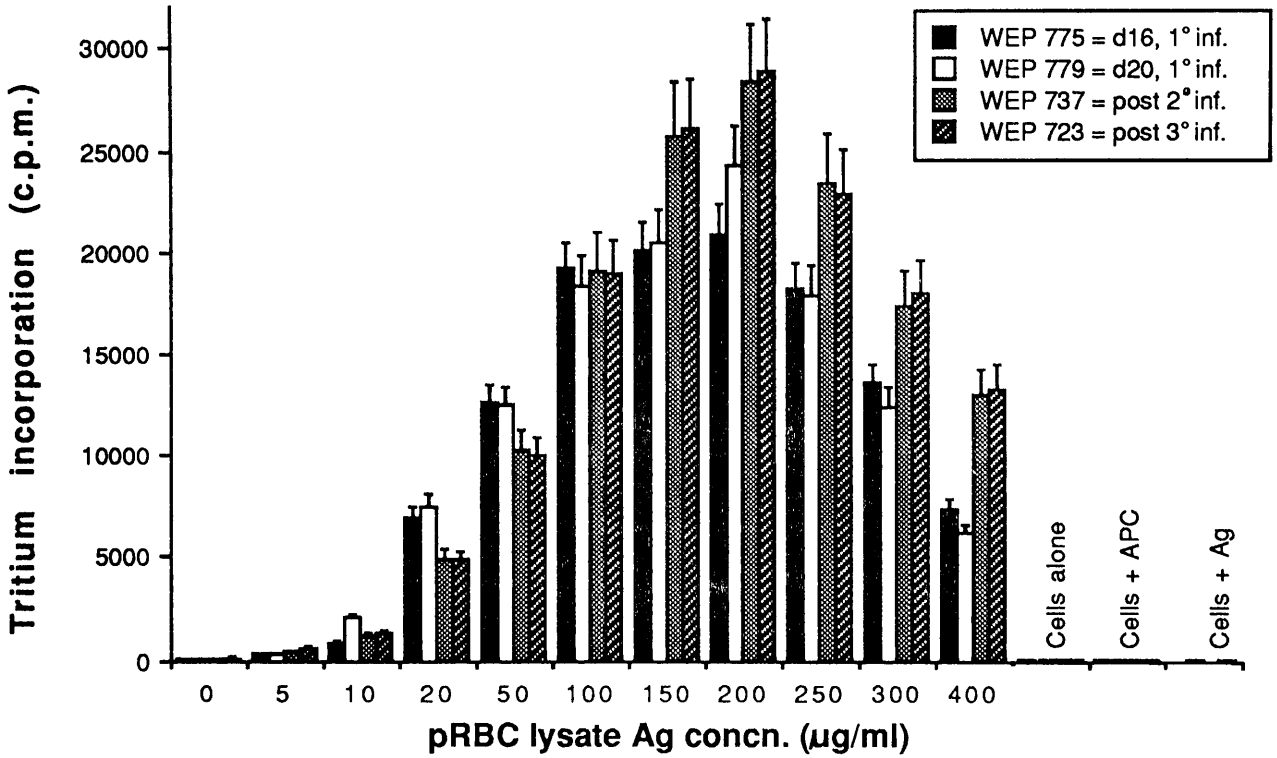


Fig. 4.11.1 Assay of proliferation of P. c. chabaudi AS-primed T lymphocyte lines in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate

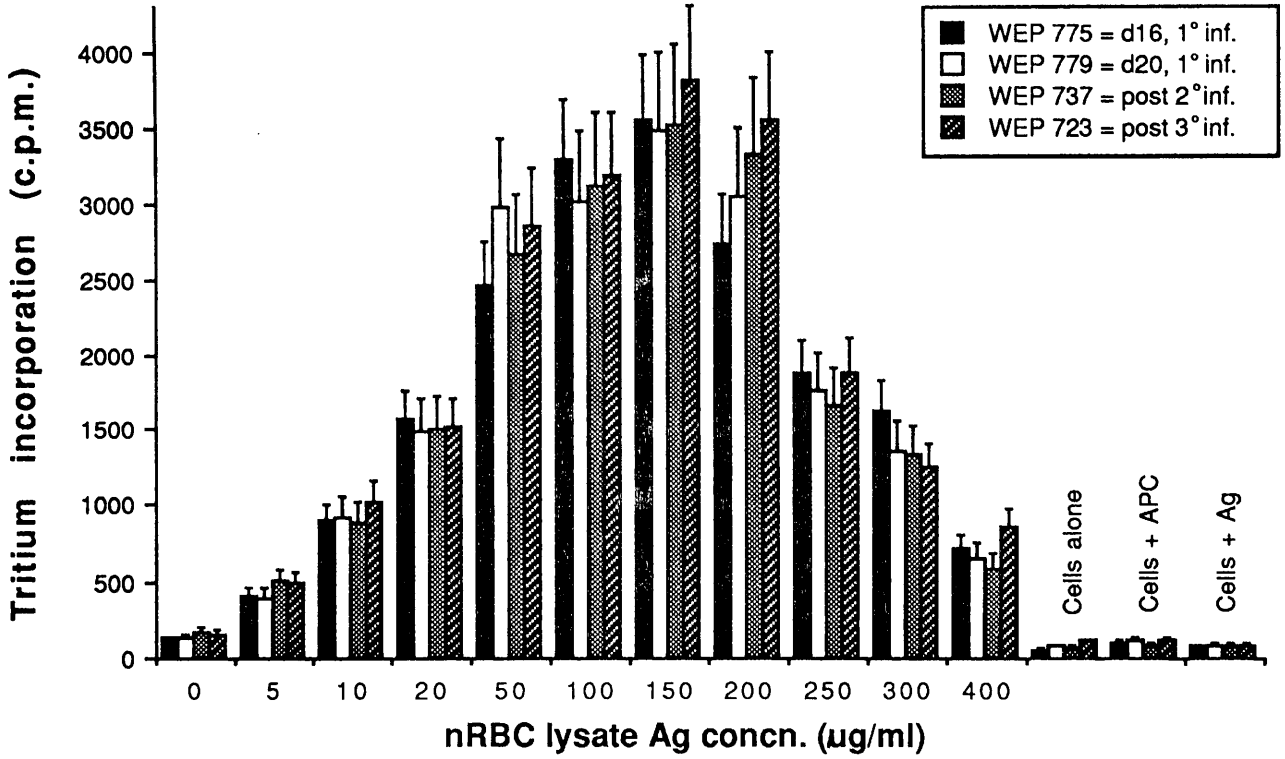


Fig. 4.11.2 Assay of proliferation of P. c. chabaudi AS-primed T lymphocyte lines in response to antigenic stimulation with uninfected nRBC lysate

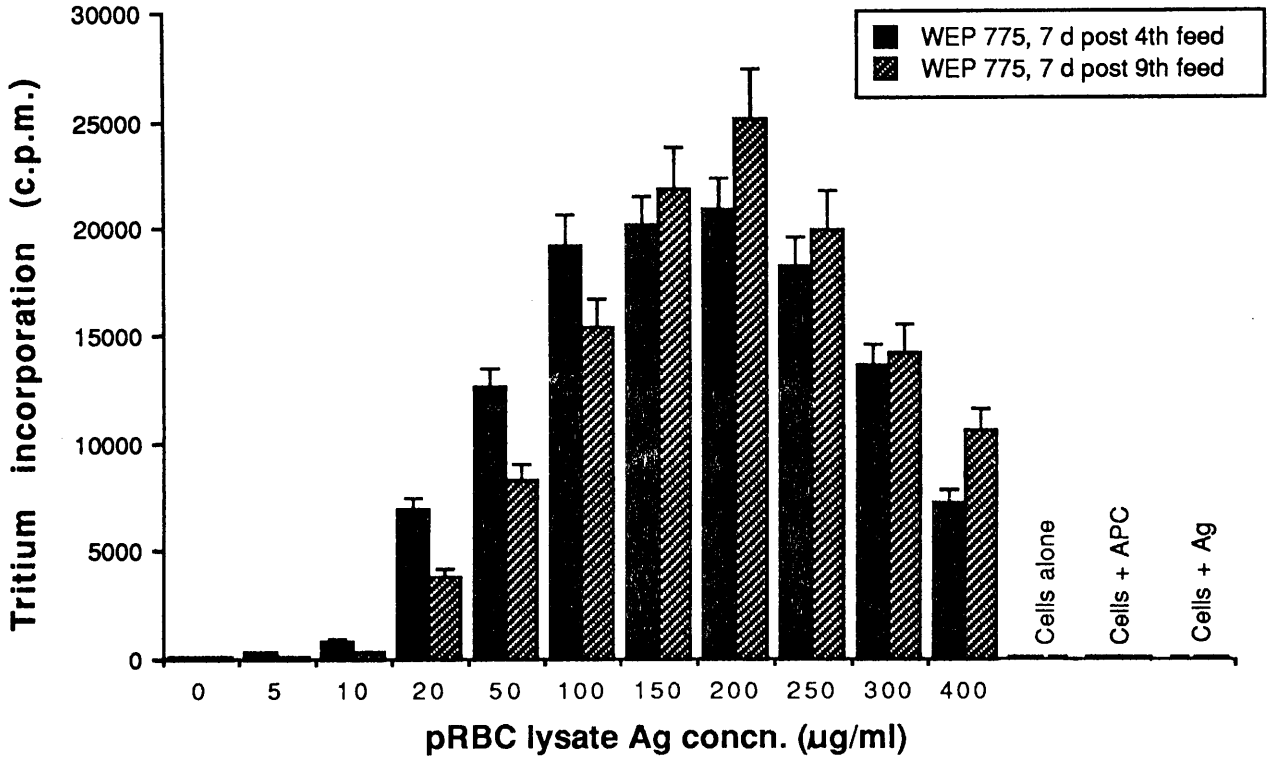


Fig. 4.11.3 Assay of proliferation of WEP 775 T lymphocyte line using P. c. chabaudi AS pRBC lysate, showing maintenance of antigenic specificity

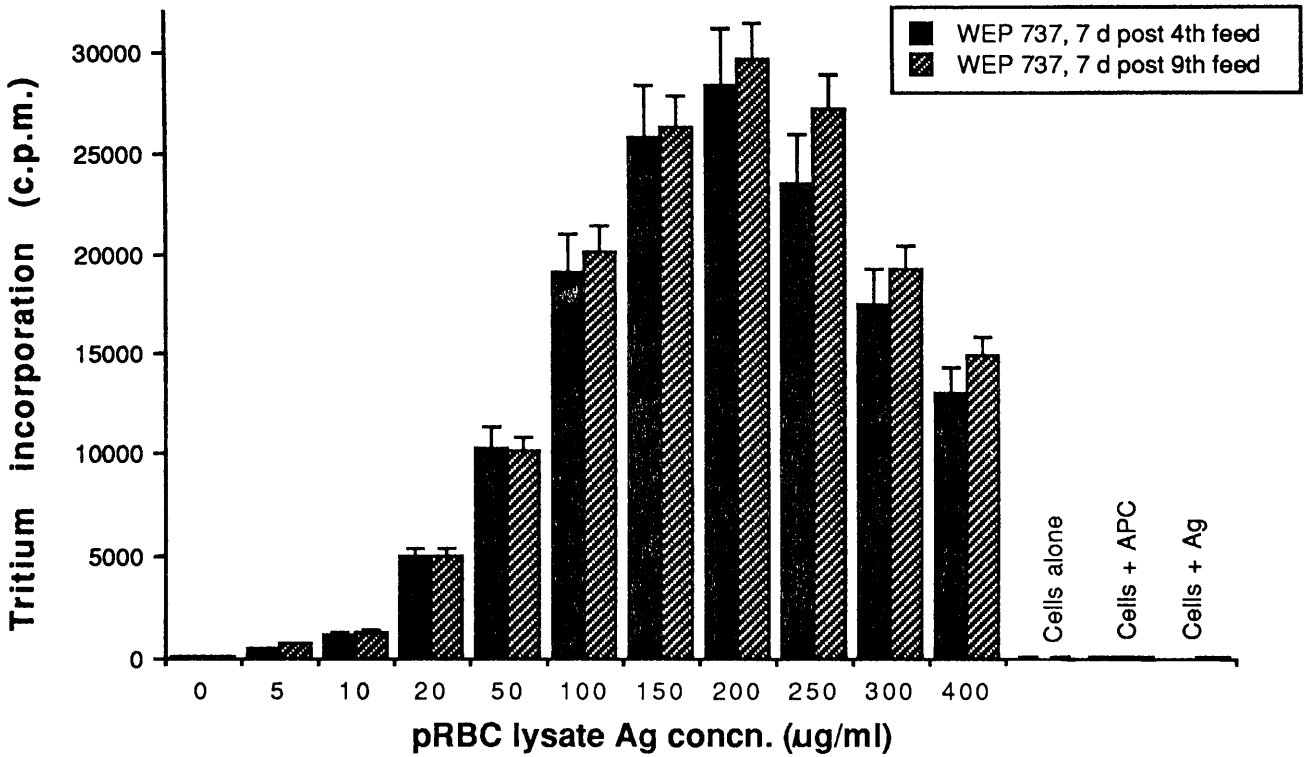


Fig. 4.11.4 Assay of proliferation of WEP 737 T lymphocyte line using P. c. chabaudi AS pRBC lysate, showing maintenance of antigenic specificity

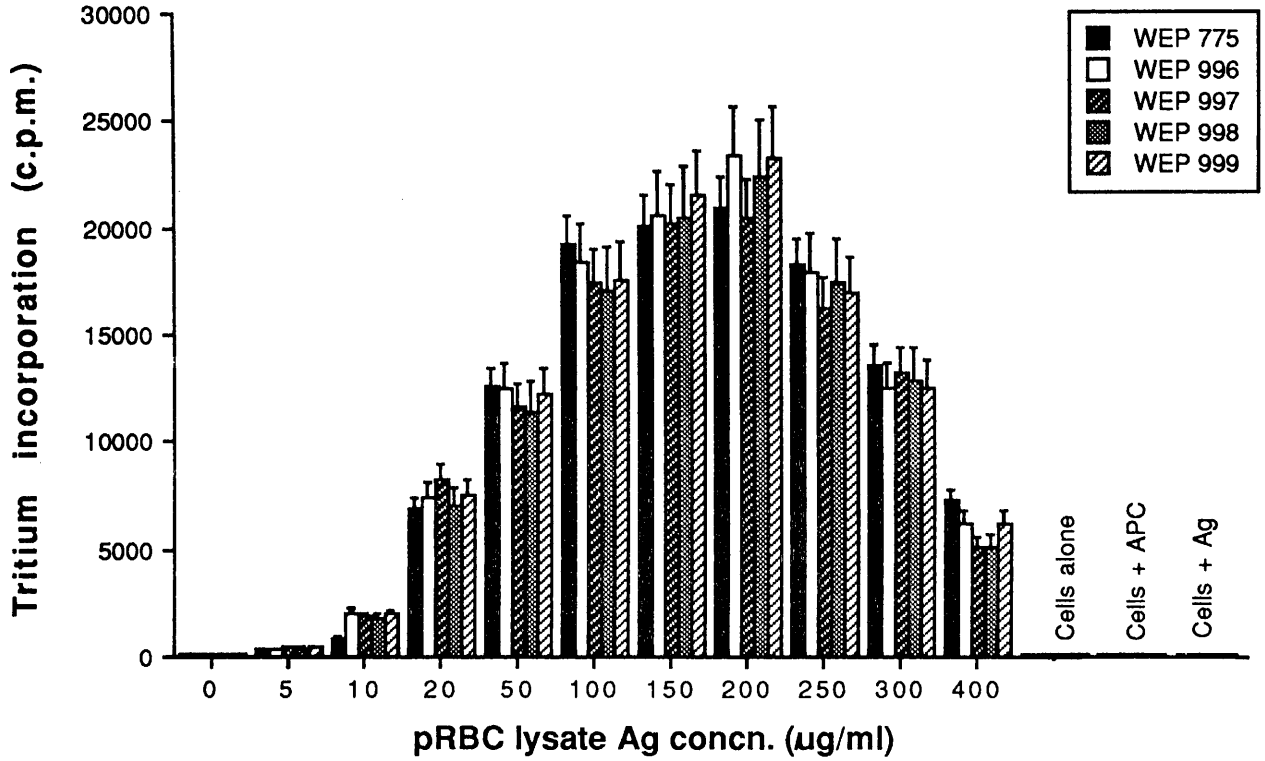


Fig. 4.11.5 Assay of proliferation of WEP 775 T lymphocyte line and of WEP 996-999 daughter T lymphocyte clones in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate

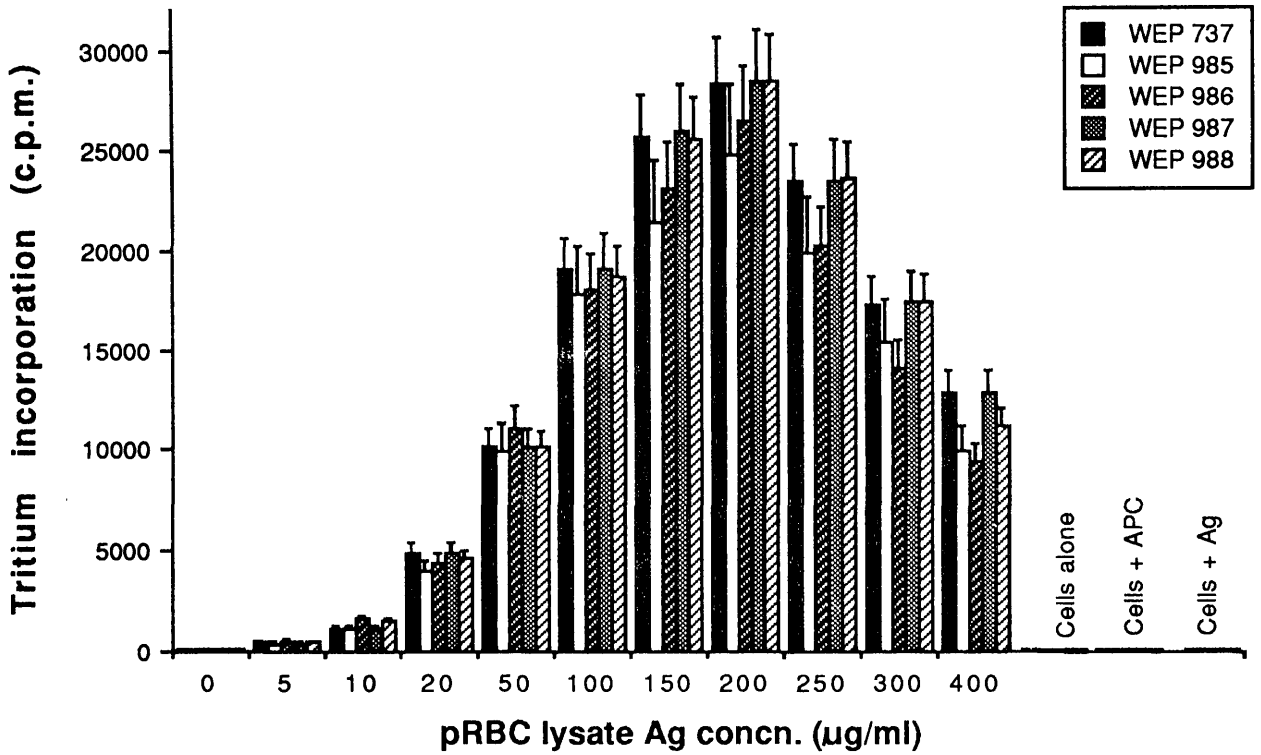


Fig. 4.11.6 Assay of proliferation of WEP 737 T lymphocyte line and of WEP 985-988 daughter T lymphocyte clones in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate

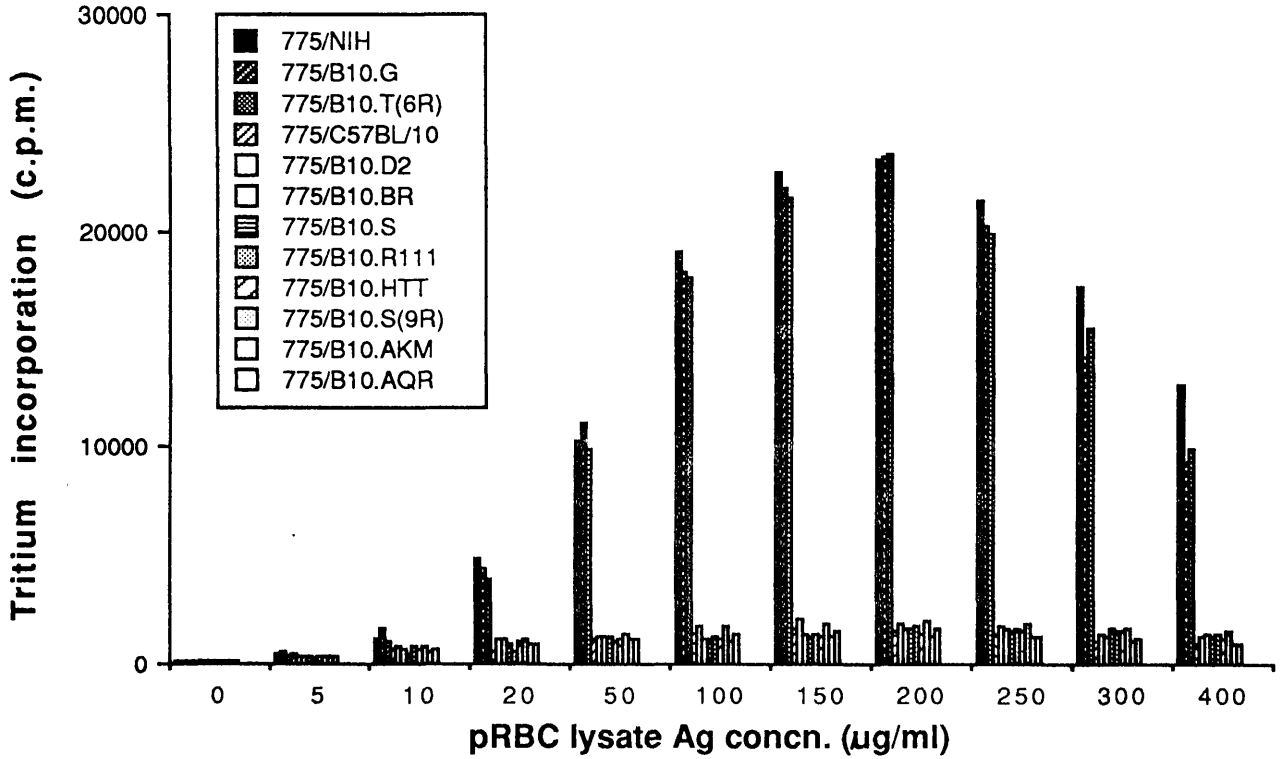


Fig. 4.12.1 Assay of proliferation of WEP 775 T lymphocyte line in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate presented by APC of varying H-2 haplotype

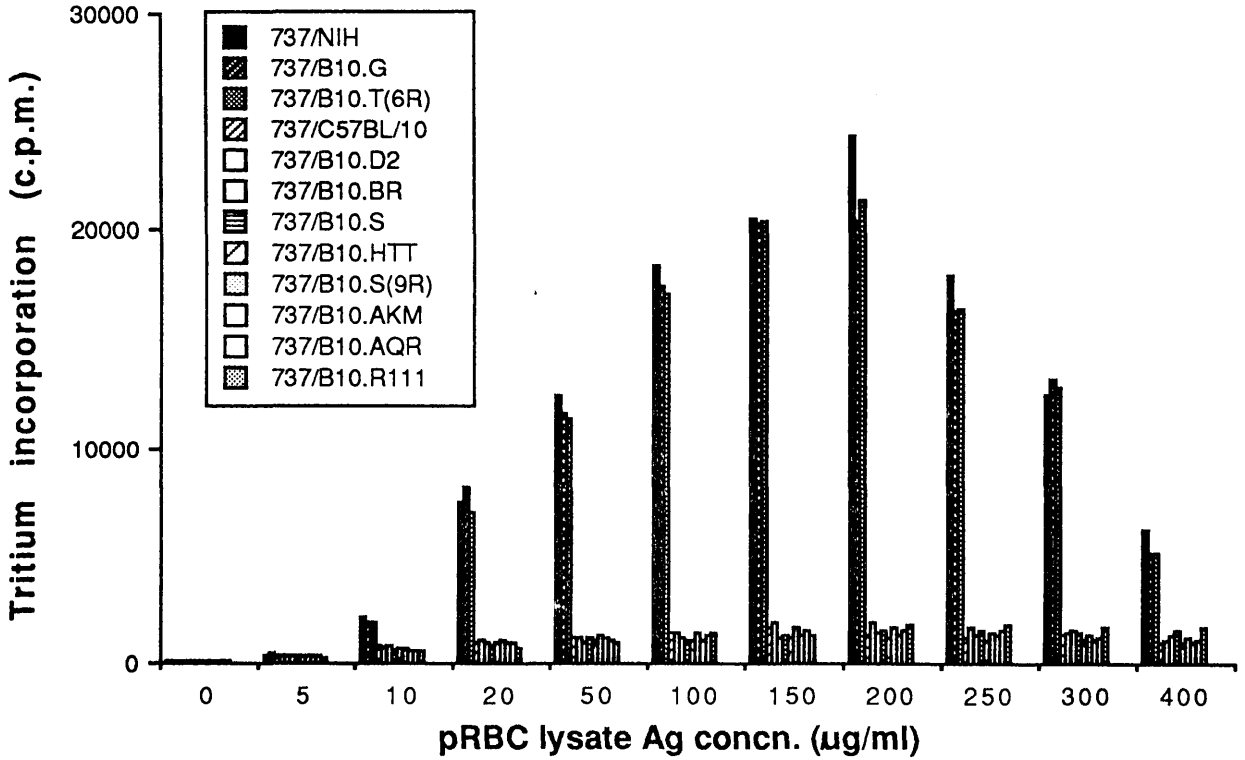


Fig. 4.12.2 Assay of proliferation of WEP 737 T lymphocyte line in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate presented by APC of varying H-2 haplotype

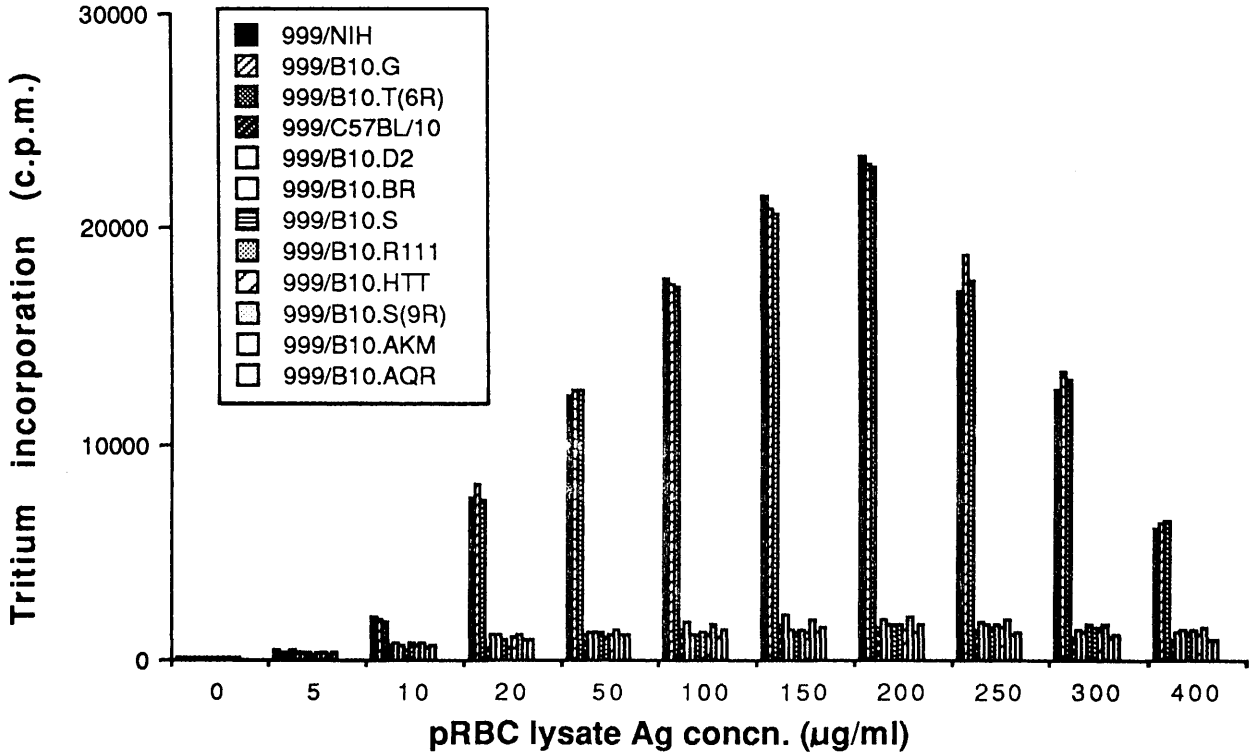


Fig. 4.12.3 Assay of proliferation of WEP 999 T lymphocyte clone in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate presented by APC of varying H-2 haplotype

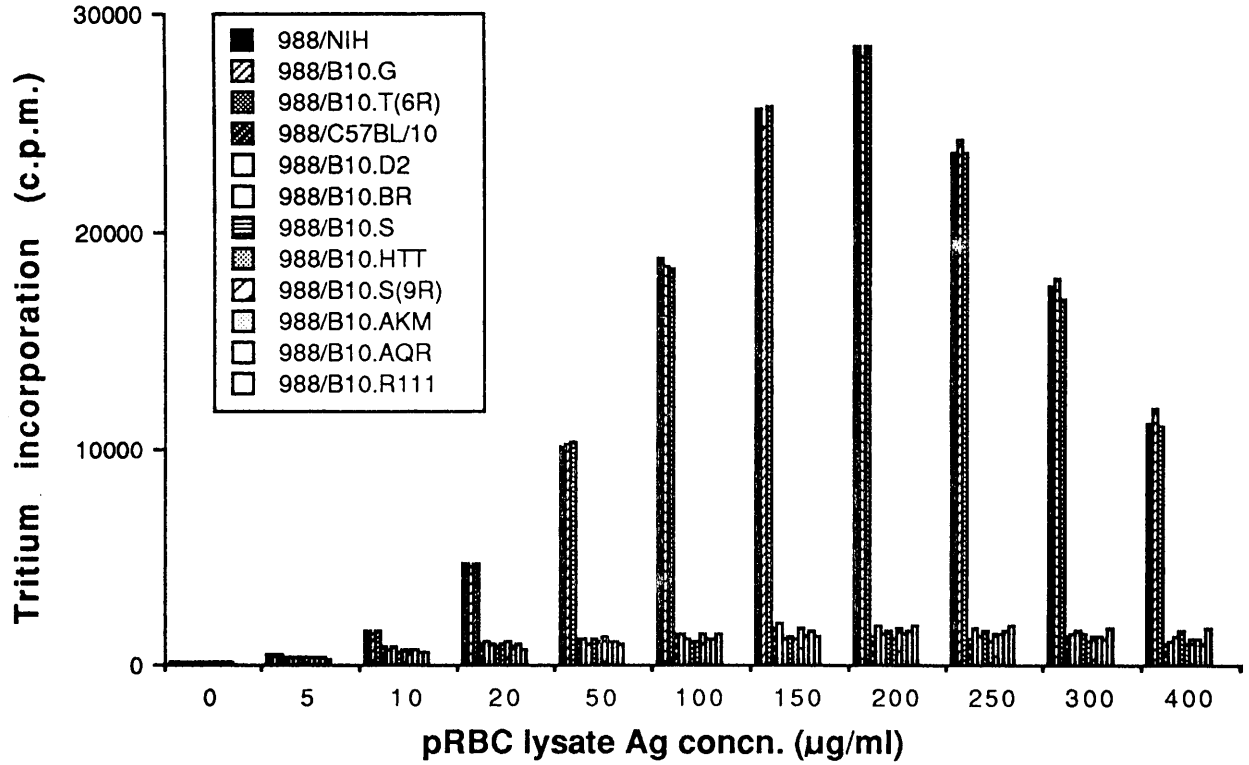


Fig. 4.12.4 Assay of proliferation of WEP 988 T lymphocyte clone in response to antigenic stimulation with P. c. chabaudi AS pRBC lysate presented by APC of varying H-2 haplotype

Table 4.12.1
MHC restriction analysis of Ag-specific proliferation:
strains of mice used and their H-2 haplotypes.

Strain	Haplotype	H-2 locus alleles					
Classical		**K**	**A-β**	**A-α**	**E-β**	**E-α**	**D**
NIH	q	q	q	q	q	q	q
B10.G	q	q	q	q	q	q	q
C57BL/10	b	b	b	b	b	b	b
B10.BR	k	k	k	k	k	k	k
B10.AKM	m	m	m	m	m	m	m
B10.R111	r	r	r	r	r	r	r
B10.S	s	s	s	s	s	s	s
Recombinant							
B10.D2	g1	d	d	d	d	d	b
B10.HTT	t3	s	s	s	s/k	k	d
B10.S(9R)	t4	s	s	s	s/k	k	d
B10.AQR	y1	q	k	k	k	k	d
B10.6(TR)	y2	q	q	q	q	q	d

CHAPTER FIVE

ADOPTIVE TRANSFER OF T CELL LINES TO NAIVE SYNGENEIC RECIPIENTS

5.1 Introduction

Adoptive transfer of immunity to malaria has been performed in the past using unfractionated or enriched lymphocytes of lymph node or splenic origin from immune rodents (Stechschulte 1969, Roberts & Tracey-Patte 1969, Kasper & Alger 1973, Gravely & Kreier 1976, McDonald & Phillips 1978, Cavacini et al 1986, Favila-Castillo et al 1990). An alternative to the use of in vivo-derived lymphocytes is to use in vitro-generated, Ag-specific T cell lines. Such cultured lines have in recent years been shown to transfer a protective immune response to Plasmodium berghei (Gross et al 1984) and to P. c. adami (Brake et al 1986). Accordingly, it was decided to adopt a similar approach for this work.

The generation of four stable Ly-4$^+$ T cell lines was described in Chapter 4. As for previous investigations, two of these lines were derived from donor mice rendered immune by two (WEP 737) or three (WEP 723) P. c. chabaudi AS infections. For the first time, however, pRBC lysate-specific, in vitro-propagated Ly-4-bearing T cells derived originally from mice undergoing a primary infection have been used in the P. c. chabaudi AS/NIH mouse system for analysing the possibility of conferring immunity using a relatively homogeneous Ly-4$^+$ cell population. Two lines were prepared from mice recovering from the primary parasitaemia, on d 16 and d 20 p.i. (WEP 775 and WEP 779, respectively).

The evidence so far points to an important role for Ly-4$^+$ T cells in the protective immune response to P. c. chabaudi (Süss et al 1988) and to the related parasite P. c. adami (Cavacini et al 1986). Furthermore, protection against an infection with P. c. adami was demonstrated with Ly-4$^+$ T cell lines specific for undetermined pRBC Ags (Brake et al 1986). Süss et al (1988) implicated Ly-4$^+$ cells in resistance to P. c. chabaudi by an alternative approach of selective immunodepletion. Thus, the parallel study to that of Brake et al (1986) had not previously been performed in this rodent model system.

Following pRBC Ag stimulation in vitro, the effects of adoptive T cell transfer of host resistance to a primary infection with P. c. chabaudi AS in immunocompetent, naive mice were assessed. Functional heterogeneity of Ly-4$^+$ T cell clones specific for a variety of antigenic determinants has been evaluated extensively (Mosmann & Coffman 1987, Abbas 1987), and it has been speculated that in vivo these T cells may play different roles in immune responses and have different activation requirements. With regard to P. c. chabaudi AS infection, the frequency of appearance of Ly-4$^+$ subsets

throughout infection suggests that the majority of Ly-4$^+$ lymphocytes responding to P. c. chabaudi AS early in infection are of the T_H1-type, whereas by the time of patent recrudescence, this pattern is reversed with a predominance of T_H2 responder cells (Langhorne et al 1989 a). Therefore, the use for adoptive transfer of Ly-4$^+$ lines taken by d 20 p.i. and of lines taken after reinfection proved pertinent in examining the nature of the in vivo T cell response.

It has been established that each of the four splenic lymphocyte lines described in this chapter respond specifically to P. c. chabaudi AS pRBC Ags in vitro, in an MHC-restricted manner (Chapter 4). The adoptive transfer studies documented herein extend this in vitro characterisation by correlating the lymphoproliferation observed under culture conditions with protection against homologous parasite challenge in vivo. This link between specific lymphoblast proliferation in vitro and immunity to malaria was originally demonstrated by Weissberger et al (1980), so it was surmised that each proliferative T cell line generated may exhibit immunological activity in vivo. Prior to the dissection of the effector mechanisms of the anti-P. c. chabaudi AS response by T cell reconstitution of immunocompromised recipient mice (Chapters 7 & 8), it was necessary to evaluate the effect of each of the Ly-4$^+$ populations on the expression of infection in normal, immunocompetent recipients. Also, further experiments were performed to determine whether or not the protection engendered by the T cell preparations inoculated into challenged mice was due to the mediation of lymphocytes of the Ly-4$^+$ phenotype, or, alternatively, to any minority of cells possibly contaminating each preparation. The value of this precaution had been highlighted by the finding of Mogil et al (1987) that the transfer of immunity to P. yoelii 17X NL by immune Ly-1$^+$ T cells (predominantly Ly-4$^+$) was absolutely dependent upon cotransfer of a non-T/non-B immune spleen cell found in splenic preparations at a low frequency. As no equivalent requirement has been reported for the elaboration of immunity to P. c. chabaudi AS, it was likely that any protective effects observed in vivo were caused by the majority Ly-4$^+$ population. However, as these lines were uncloned at the time of this study, this qualification was essential.

5.2 Adoptive transfer of in vitro generated P. c. chabaudi AS-specific T cell lines to naive, syngeneic recipients.

Each of the four Ly-4$^+$ cell lines to be used in this study was propagated in vitro in bulk cultures prior to adoptive transfer to gain sufficient viable lymphocytes for inoculation.

As a result of the intrinsic proliferative response of these Ly-4$^+$ populations to antigenic stimulation, cultures were harvested 3-4 d after the previous subculture when the cultures had grown sufficiently but when the majority of cells were still dividing logarithmically.

For each line, lymphocytes were adoptively transferred to six age-matched NIH female mice. The standard inoculum used was 3 x10^7 viable cells, injected i.v.. Control groups receiving similar sized inocula of either unfractionated splenic T & B cells or enriched T cells were also prepared. For these, naive non-immune donor mice were used. All animals were infected with 1 x10^5 pRBC P. c. chabaudi AS immediately after adoptive transfer, and the consequent infection followed by daily blood smears. Two normal mice were also challenged as a control of infection in immunocompetent individuals.

Each of the Ly-4$^+$ T cell lines gave detectable protection against homologous P. c. chabaudi AS challenge after adoptive transfer into naive syngeneic recipient mice. This was manifested as a significantly lowered primary peak parasitaemia, a more rapid remission phase and a shift in the recrudescence parasitaemia which was also usually depressed, compared with control mice given a similar number of naive splenic T or T & B cells (Figs. 5.2.1 & 2). This was a general trend of enhanced immunity to infection. There were, however, exceptions; although all mice inoculated with WEP 775, 779 or 737 resolved the acute infection by d 16 p.i., mice given WEP 723 showed a subpatent parasitaemia only at the same time, d 19 p.i., as did those mice given naive spleen cells. Also, for WEP 723, the reduction of peak primary parasitaemia (mean = 23%) compared to control groups (e.g. splenic T & B recipients, mean = 35%) was not as significant ($p < 0.05$) as those seen upon transfer of the other cell lines (e.g. WEP 779, mean = 11%, $p < 0.01$). However, WEP 723-transferred mice did show a lower recrudescence than did mice receiving either WEP 737 or splenic T & B cells.

For recipients of all lymphocyte lines, the subpatent period was of longer duration than for control groups, in which subpatency lasted either 7 or 8 d (Figs. 5.2.1 & 2). The length of the subpatent period ranged from 10 d (WEP 723) to 21 d (WEP 779). Despite the fact that the subpatency was so extended for recipients of WEP 779, the mean parasite clearance for this group was at the same time as that of mice inoculated with naive spleen cells (Fig. 5.2.1). For WEP 737 and WEP 723, recipients cleared the infection by d 43 and 44 p.i., respectively, well in advance of the d 54 p.i. clearance time for mice injected with naive T & B lymphocytes (Fig. 5.2.2).

It was apparent from this experiment that each Ly-4$^+$ cell line was capable of

conferring considerable protection to naive mice against P. c. chabaudi AS challenge. This was observable as each of the criteria used to evaluate the effects of protective immune responses on challenge parasitaemias (2.8). However, it was also evident from the parasitaemia determinations that the courses of infection in the recipients of these T cell lines fell into two distinct groups, WEP 775 and WEP 779 (Fig. 5.2.3), and WEP 737 and WEP 723 (Fig. 5.2.4). Within each category, the patterns of parasitaemia were broadly similar, but between categories, there were clear differences (Figs. 5.2.3 & 4). In the case of WEP 775 and WEP 779, those lines derived early during primary P. c. chabaudi AS infection, the subpatent period was considerably longer than those in recipients of the multiple infection-derived cell lines, WEP 737 and WEP 723. Consequently, the recrudescent parasitaemias occurred earlier and were cleared earlier for mice receiving either lymphocyte population from the latter group. It should be noted, however, that the divergency in the patterns of parasitaemia observed upon adoptive transfer of different P. c. chabaudi AS-specific T cell lines to naive recipients was noticeable principally only after remission of the acute infection to subpatent levels. During the first wave of infection, the pattern of the primary parasitaemia and the timing of its peak, if not its level, were essentially similar. This dichotomy in the manifestation of the protection conferred by the various in vitro-propagated cell lines is exemplified by Fig. 5.2.5. The courses of infection of mice receiving WEP 775 and WEP 737 began to differ significantly only on d 26 p.i., before which the pattern and degree of parasitaemia were indistiguishable. In each case, the level of immunity conferred by transfer of these parasite-specific populations was greater than either naive spleen cell preparation used. It was encouraging to see P. c. chabaudi AS-specific immune responses exhibited by both WEP 775 and WEP 737 in the immunocompetent transfer system (Fig. 5.2.5), as these were the two Ly-4$^+$ lines which were subsequently cloned by limiting dilution. This enhanced protection beyond that engendered by either naive transferred lymphocytes or naive background lymphocytes already present in the immunocompetent host demonstrated that there was a direct correlation between the plasmodial specificity for lymphoproliferation in vitro and the protective immune response observed in vivo. In turn, this finding showed that it was not necessary to prime donor animals by a full course of infection to achieve effective protection in vivo, for WEP 775 and WEP 779, derived from infected mice on d 16 and d 20 of primary infection, adoptively transferred specific anti-P. c. chabaudi AS immunity. Clearly, the priming of these two Ly-4$^+$ lines to only early appearing, and not recrudescent,

antigenic determinants did not prevent or reduce their ability to confer protection in vivo. Also, for all cell lines, the fact that a heightened immune response was observed upon adoptive transfer indicated that each Ly-4$^+$ population had maintained its specific recognition of P. c. chabaudi AS Ags throughout in vivo priming, in vitro propagation and then adoptive transfer. This vindicated the selection of a pRBC lysate enriched for late trophozoite and schizont stages of the malaria parasite for antigenic stimulation in vitro, and showed that the same pRBC Ags were recognisable in vivo as in vitro.

5.3 In vitro depletion of Ly-4$^+$ T cell lines

The adoptive transfer experiments described in 5.2 would suggest that each of the T cell lines established in vitro was capable of conferring immune protection in vivo. However, from surface immunofluorescence and complement-mediated cytotoxicity studies (Chapter 9), it was known that there was a residual population of cells present in each T lymphocyte population that was not phenotyped to either the Ly-4$^+$ or Ly-2$^+$ T cell subset. To examine the proliferation of these Ly-4$^-$ Ly-2$^-$ cells in vitro, a population of a given T cell line could be depleted of lymphocytes bearing either or both Ly-4 or Ly-2 cell surface markers (2.41). This was achieved by incubation of the cell line with either one or both MAbs to Ly-4 or Ly-2 Ags to give Ly-4$^-$, Ly-2$^-$ or Ly-4$^-$ Ly-2$^-$ populations. The cellular response of any cells surviving this treatment was measured by a proliferation assay in the presence of P. c. chabaudi AS pRBC lysate as the source of stimulation (2.41 a).

Figs. 5.3.1 & 2 show the proliferative response, as determined by [^{3}H] thymidine incorporation, of the WEP 775 and WEP 737 T cell lines after various in vitro depletion treatments. The levels of tritium uptake measured were of those cells not lysed by incubation with specific MAb and complement. As the lymphocyte lines were known to be predominantly of the Ly-4$^+$ phenotype (Chapter 9), use of the anti-Ly-2 MAb had negligible effect on the proliferation detected (Figs. 5.3.1 & 2). In contrast, use of both MAbs abrogated completely the proliferative response of the test cells. Incubation of the cultured populations with the anti-Ly-4 MAb had a similar effect in reducing the subsequent cell growth to a minimum level. Thus, the effective loss of responder cell growth by treatment with the anti-Ly-4 MAb alone would suggest strongly that it was the majority Ly-4-bearing lymphocytes that were responsible for proliferation in the presence of P. c. chabaudi AS Ags under optimal in vitro culture conditions. Upon depletion of Ly-4$^+$ cells, the small Ly-2-bearing population did not grow, showing that

these lymphocytes were incapable of proliferating in response to P. c. chabaudi AS pRBC Ags. This was because the Ly-2^+ T cell subset has an absolute requirement for class I H-2-restricted Ag presentation. Moreover, the fact that there was little detectable growth in the absence of both Ly-4^+ and Ly-2^+ T lymphocytes showed that the minority fraction of Ly-4^- Ly-2^- cells contaminating each preparation was unable to overgrow the T cells under physiological conditions.

That the non-proliferation of the WEP 775 and WEP 737 cell lines upon incubation with the anti-Ly-4 MAb was due to specific depletion of Ly-4-bearing lymphocytes and not to some non-specific cytotoxic effect was demonstrated by assaying control cultures of naive splenic T cells with each of the MAbs (Figs. 5.3.3). For these preparations, the depletion by a combination of anti-Ly-4 and anti-Ly-2 MAbs was nearly complete. However, the proliferation attained after incubation with either MAb by itself was just over half that attained by the negative control to which no MAbs was added. Allowing for contamination, especially by B cells, of this nylon wool-enriched preparation, the degree of tritium incorporation after incubation with either anti-Ly-4 or anti-Ly-2 MAbs was commensurate with the fact that each subset comprises approximately half of the splenic T cell complement.

As each MAb was cultured with both Ly-4^+ lines at a range of dilutions, this assay also served as a quantitative determination of the minimum titre of both anti-Ly-4 and anti-Ly-2 MAbs that could be used to deplete effectively these T cell subsets in vitro. As is evident from Fig. 5.3.4, the highest titre of the anti-Ly-4 MAb used in this study, 1:1000, was sufficient to eliminate most Ly-4^+ lymphocytes present in the culture wells. All activity was lost at lower dilutions. There was a similar situation in the case of anti-Ly-2 depletion, exemplified by Fig. 5.3.5. This is not as straightforward to interpret, as the depletion occurred only for splenic T cells, and then it was only partial. However, examination reveals that the level of depletion was similar at all MAb titres, and was not dependent upon the dilution used. Figure 5.3.6 shows similarly that for all preparations, including the splenic T cell control, total lymphocyte depletion was achieved by incubation of cultures with both MAbs at all dilutions tested. This was manifested as a lack of proliferation of any remaining viable cells present in the test wells. Thus, it appeared that for both MAbs, against Ly-4 and Ly-2 T cell surface determinants, depletion could be effected at quite low titres and therefore the dilutions used to remove subsets from culture for adoptive transfer of those cells surviving such treatment would not be critical (5.4). In the event, the relatively high titre of 1:50

was used, as recommended (2.41 b) (Cobbold et al 1984, Harte et al 1985 a).

5.4 Adoptive transfer of in vitro Ly-4$^+$- and/or Ly-2$^+$-depleted T cell lines to naive syngeneic recipients

The adoptive transfer of Ly-4$^+$ T cell lines to immunocompetent recipients showed that in this system these relatively homogeneous populations of lymphocytes were able to give detectable protection against primary P. c. chabaudi AS infection (5.2). Furthermore, depletion of the majority Ly-4$^+$ cellular component of either WEP 775 or WEP 737 using a specific MAb abrogated the P. c. chabaudi AS Ag-specific proliferation that was a characterisic of these cell lines (4.11). There was, therefore, indirect evidence that it was the Ly-4-bearing proportion of lymphocytes in each population that was responsible for the protection conferred by these cell lines upon adoptive transfer. To demonstrate unequivocally in which T cell subset or other cellular fraction the protective activity resided, a further adoptive transfer was performed. The courses of infection of challenged mice were followed after inoculation of either WEP 775 or WEP 737 cell lines which had been previously treated in vitro to deplete differentially either Ly-4$^+$ or Ly-2$^+$ T lymphocyte subset or deplete totally both (2.41 b). Most transfers involved the administration of complete cell culture preparations, which usually contained large numbers of dead cells. To control for the possibility of exacerbation of parasitaemia due to immunological consequences of the inoculation of a large dose of dead material and non-cellular debris that may conceivably have occurred, control groups were prepared. Mice in these groups received in vitro-depleted lymphocyte preparations that had been cleansed of the non-viable fraction so that only extant cells were transferred. This was desirable to show whether or not Ly-4$^-$ Ly-2$^-$ cells, in the absence of cellular interaction with these T cell subsets, could transfer any degree of resistance against P. c. chabaudi AS challenge.

Groups of five NIH mice were set up, including undepleted WEP 775 and WEP 737 T cell lines. For recipients of these lymphocytes, each mouse was given 3.0 x10^7 cells. In the case of Ly-2$^+$-depleted cultures, the total number of cells to be transferred was adjusted so that 3.0 x10^7 viable cells were administered to recipient animals. However, in the instances where MAb treatment resulted in a very low yield of surviving cells, such as Ly-4$^+$-depletion or combined MAb depletion, it was impracticable to inoculate a similar number of viable cells; thus, a total of 3.0 x10^7 cells was used for adoptive transfer, and the actual number of viable cells inoculated was proportional to the

percentage of cells in the original T cell line preparation which the cells surviving treatment comprised. No effort was made to inoculate equal numbers of all phenotypically distinct subpopulations within each T cell line, as the numbers administered reflected their proportions in these populations, and thus, the effects of each under experimental conditions. It is probable that if 3.0 $x10^7$ Ly-4^- Ly-2^- cells had been transferred to naive recipients, a non-specific protective effect may have been observed. This would be a dose-dependent phenomenon and would not reflect the specific anti-P. c. chabaudi AS immune activity conferred by the in vitro-propagated lines upon inoculation of the equivalent of a splenic population, i.e. 3.0 $x10^7$ undepleted lymphocytes of either WEP 775 or WEP 737 Ly-4^+ cell line.

The courses of infection upon adoptive transfer of in vitro-depleted WEP 775 cells to challenged naive mice is shown in Fig. 5.4.1. For recipients of either Ly-4^+-depleted or Ly-4^+-/Ly-2^+-depleted preparations, the resultant course of infection was similar to that seen in negative controls of mice challenged without adoptive transfer. This was observed as a similar pattern of parasitaemia with the same degree of acute parasitaemia, the same length of subpatency and the presence of a secondary recrudescence. Indeed, the remission of the acute infection occurred later, though not significantly ($p > 0.05$), than for the normal P. c. chabaudi AS infection in naive mice. Thus, it appeared from these results that the Ly-4^- or Ly-4^- Ly-2^- populations of the original WEP 775 line, upon adoptive transfer, gave negligible protection against homologous parasite infection. That the patterns of infection in these two groups of mice were very similar also inferred that the lack of protective activity was not due to the loss of the Ly-2^+ component of the WEP 775 cell line. This view was confirmed by examination of the courses of infection in mice receiving either the anti-Ly-2 MAb-treated cell line or the undepleted population (Fig. 5.4.1). In these cases, there was a considerable protection conferred, and the level of immunity transferred by the Ly-2^- WEP 775 preparation was very much the same as that transferred by the complete population. This was manifested as a significantly depressed peak primary parasitaemia, a quickened remission to subpatency, which was double the time of that observed in other groups, a lower recrudescence and an overall quicker clearance time. Thus, by all the criteria used to evaluate immune protection, it was clear that the Ly-2^- population was able to confer as effective an activity as did the Ly-4^+ Ly-2^+population. It would seem, therefore, that the Ly-4^+ majority population within the WEP 775 cell line was responsible for the anti-P. c. chabaudi AS activity in vivo as well as in vitro.

That the non-staining Ly-4$^-$ Ly-2$^-$ residual fraction of cells failed to confer any protection was established by adoptive transfer of the double depleted WEP 775 preparation, which showed a total lack of ability to alter the normal course of infection in immunocompetent recipient mice.

Very similar results were attained for the adoptive transfer of in vitro-depleted WEP 737 populations (Fig. 5.4.2). Here too, an effective enhancement of protection was observed only in mice receiving either the original lymphocyte line or a Ly-2$^-$ derivative of it. Preparations lacking a Ly-4$^+$ population failed to give any protection upon adoptive transfer, compared to the negative control group.

An interesting feature of this experiment was that the patterns of recrudescent parasitaemia varied between differentially depleted populations of cells. For preparations lacking cells of the Ly-4$^+$ phenotype, after adoptive transfer, the course of recrudescent infection was identical to that normally observed in naive mice, irrespective of the cell line depleted (Fig. 5.4.3). For these Ly-4$^-$ populations, the dichotomy of the appearance of the secondary parasitaemia that was a feature of the adoptive transfer of WEP 775 and WEP 737 lines to immunocompetent recipients (both here and in 5.2) was not apparent. However, for the corresponding depletion of Ly-2$^+$ cells from each lymphocyte line, the Ly-4$^+$ Ly-2$^-$ populations did retain the divergent recrudescent parasitaemias that were characteristic of the original cell lines (Fig. 5.4.4). This analysis supported the view that the protective activity resided with the Ly-4-bearing lymphocyte population, but extended it further to suggest that the different effector mechanisms underlying the observable manifestation of a shift in the recrudescent parasitaemia were dependent on the presence of P. c. chabaudi AS-primed Ly-4$^+$ lymphocytes.

All the results reported thus far are of adoptive transfers of total cell populations. For these inoculations, recipients were given culture preparations containing a mixture of dead and live cells, the ratio of which was dependent upon the particular depletion carried out. An additional set of transfers was performed for both WEP 775 and WEP 737 cell lines, for which only viable cells retained after complete lymphocyte depletion, were injected into challenged animals. Figures 5.4.5 & 6 indicate that for the adoptive transfer of Ly-4$^-$ Ly-2$^-$ populations to naive recipients, there was no significant difference between the parasitaemias of mice given total or cleansed preparations. There was a slight exacerbation of the primary parasitaemia in recipients of complete populations, seen as a remission to subpatency 2 d or 1 d later than for the other two

groups, for WEP 775 (Fig. 5.4.5) and WEP 737 (Fig. 5.4.6), respectively. This phenomenon was consistent, suggesting a possible inhibition of clearance of parasites to subpatent levels in mice given a large inoculum of non-viable material, but this effect notwithstanding, the presence of dead cells appeared to have no gross non-specific deleterious effect on the course of infection attained upon adoptive transfer to competent mice. Moreover, this study demonstrated clearly that splenic Ly-4$^-$ Ly-2$^-$ cells were incapable of transferring protection in this system, and thus the minimal level of contamination of all four T cell lines typed to the Ly-4$^+$ subset that were used for adoptive transfer experiments was irrelevant to the immune reactivity engendered by these lines.

5.5 Discussion

The results of the two adoptive transfer studies described in this chapter showed that each of the four Ly-4$^+$ cell lines used was capable of providing adoptive protection against P. c. chabaudi AS in vivo. This protection appeared to be aimed specifically at the malaria parasite against which the lines had been raised. This was surmised because for the two representative lines assayed, WEP 775 and WEP 737, the Ly-4-bearing cells which comprised the overwhelming majority of cells in each lymphocyte population showed a marked proliferative response upon stimulation with pRBC lysate in vitro. Furthermore, it was this same T cell subset which was shown to confer the immune protection in vivo in the absence of the cotransfer of other cell types. These findings vindicated the use of employing long term in vitro-propagated populations of uncloned Ly-4$^+$ T cell lines of a defined Ag specificity for the study of host immunity to P. c. chabaudi AS. It was the intention at the outset of this project to use only completely homogeneous populations, but as cloning of T cells of the Ly-4$^+$ phenotype proved to be initially problematic (4.10), stable Ly-4-bearing T cell lines were used successfully in place of clones.

That the anti-P. c. chabaudi AS lines did confer protection against primary parasite challenge in an immunocompetent host is in accord with the findings of Gross et al (1984) and Brake et al (1986), who had both previously raised T cell lines to the blood stages of P. berghei or P. c. adami, respectively, and shown these lines to be protective upon adoptive transfer. Brake et al (1986), however, did not use naive mice as recipients but instead congenitally T cell-deficient nude mice. This is an immunologically incompetent host in which any differences between the presence and

absence of protection tend to be amplified. Thus, that the manifestations of immune reactivity upon adoptive transfer of the Ly-4$^+$ lines used were so clearly evident in the more immunologically complex normal mouse recipient showed the considerable degree of this protection. For the P. berghei model, it was also possible to demonstrate protection without artificially inducing an immunocompromised state in the host animal. In this study, recipient mice all became infected and always developed a significant parasitaemia before suppression of the acute infection. It would appear, therefore, that the transferred cells did not function immediately as effector cells, but rather that they activated other mechanisms within the competent host or possibly elsewhere within the donor population to effect resolution of infection. Such activation is presumably the consequence of lymphokine secretion by the grafted lymphocytes. This finding concurred with that of Brake et al (1986). These workers subsequently developed a T cell clone from a cell line possessing protective activity; this homogeneous population was shown to secrete IFN-γ and IL-2 in response to homologous malarial Ag in vitro (Brake et al 1988). A similar characterisation of lymphokine production by the Ly-4$^+$ lines and clones described in this thesis is detailed in Chapter 9. Both these findings differ from those of Gross et al (1984), who utilised T cell lines derived from rats that had recovered from a P. berghei infection to transfer protection against homologous parasite challenge of naive rats. In this instance, the transferred lines appeared to induce protection by secreting mediators which activated macrophages and/or lymphocytes non-specifically.

Although the cellular events occurring during the prepatent period are not known, it appeared that the trigger for their activation was specific P. c. chabaudi AS Ag. This is because of the well-defined pRBC lysate-induced proliferative response of these lymphocytes (4.11 & 5.3). It is likely that this function observed in vitro would be maintained in vivo, and thus parasite-specific Ly-4$^+$ lymphocytes could be similarly induced to grow to large numbers in vivo and to survive long term with retention of specific anti-malarial activity upon introduction into the host by adoptive transfer. Indeed, in an analogous model using tumour-specific T cells, Chen et al (1990) reported an 11-fold increase in total donor T cell numbers recoverable from host ascites and spleen a week after adoptive transfer. They showed that the intermittent restimulation with specific Ag that presumably occurs in vivo induced spasmodic regrowth of donor T cells, maintained the number of lymphocytes of donor origin at greater than the number transferred for longer than a month, and allowed detection of substantially augmented

donor T cell-mediated specific anti-tumour function over that period of time. There is no reason why such a proliferative response in vivo should not occur upon stimulation through a natural P. c. chabaudi AS infection. Indeed, the optimal conditions for Ly-4$^+$ cell growth in vitro, including restimulation with pRBC lysate followed by periods of rest, are only an attempt to recreate the in vivo environment under artificial culture conditions. T cell proliferation in vivo can be studied by transferring T cells i.v. into irradiated H-2-different mice, pulsing the recipients with radiolabelled DNA precursors such as tritiated thymidine (Sprent & Miller 1972) or ^{125}I-iododeoxyuridine (Bennett 1971) and then removing the spleen or lymph nodes to measure radioisotope uptake. Although lymphocyte migration patterns have been examined for the host lymphocyte reservoir during malaria infection (e.g. Kumararatne et al 1987), it is believed that no similar studies have been performed on the distribution of lymphoid cells introduced by artificial means into a malaria-challenged animal. Such a study would reveal the homing patterns of inoculated cells after their introduction into the host blood stream, and show whether or not they have the capacity to migrate to appropriate sites. Many cloned T cells display aberrant trafficking patterns, a phenomenon which may be due, in part, to the loss of the MEL-14 homing receptor (Gallatin et al 1986). In the case of malaria, it is thought that a small subpopulation of the cultured anti-P. c. adami T cell lines (Brake et al 1986) may still express the MEL-14 cell surface Ag (Weidanz & Long 1988) and thus may distribute to immunologically critical sites. Much evidence exists pointing to the role of the spleen in resolution of malaria infections. Therefore, it may be important for the grafted lymphocytes to reach the spleen, and perhaps even microenvironments within it, in order to fulfill their protective capacity. Indeed, Kumar et al (1989) have recently stressed the interdependence of Ly-4$^+$ cells and the host spleen in immunity to P. vinckei vinckei.

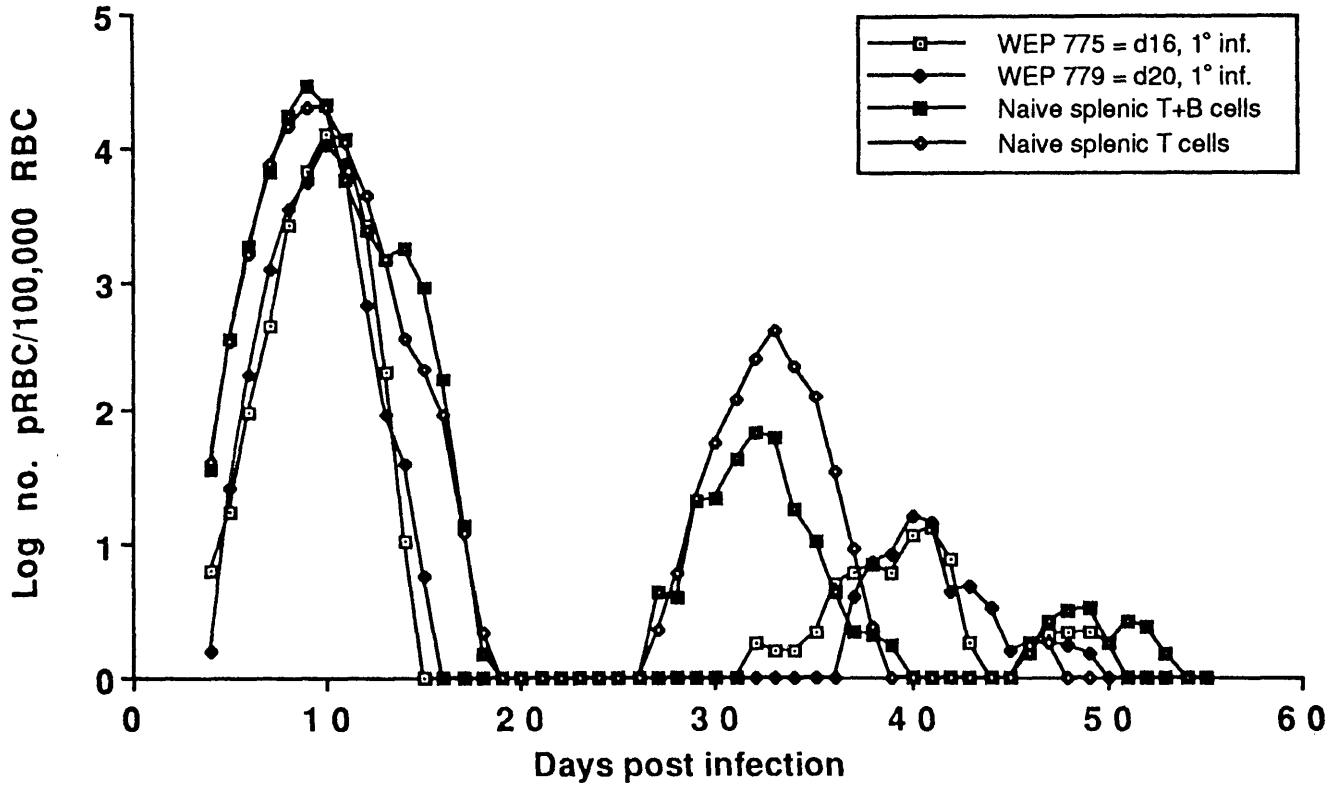


Fig. 5.2.1 Course of infection in NIH naive recipients of WEP 775 & WEP 779 T lymphocyte lines challenged with 1 x10^5 pRBC i.v..

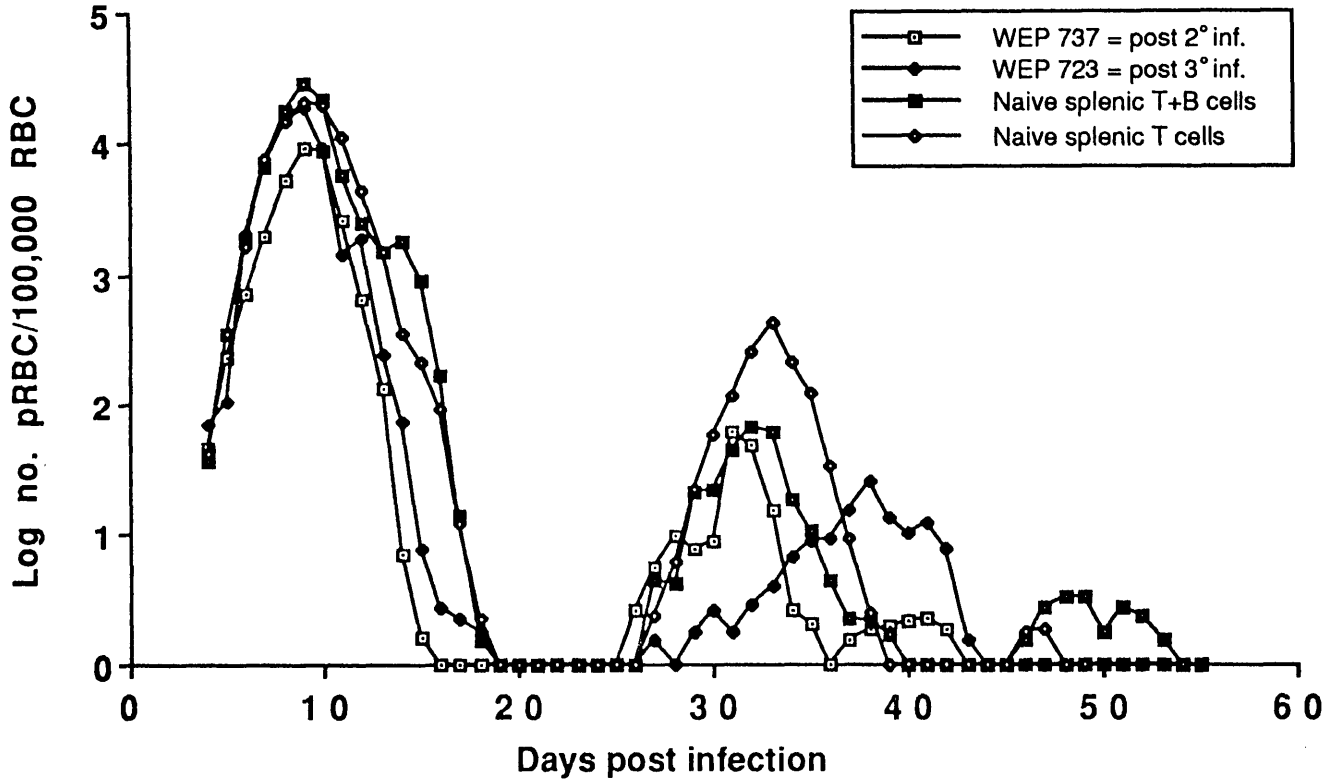


Fig. 5.2.2 Course of infection in NIH naive recipients of WEP 737 & WEP 723 T lymphocyte lines challenged with 1 x10^5 pRBC i.v..

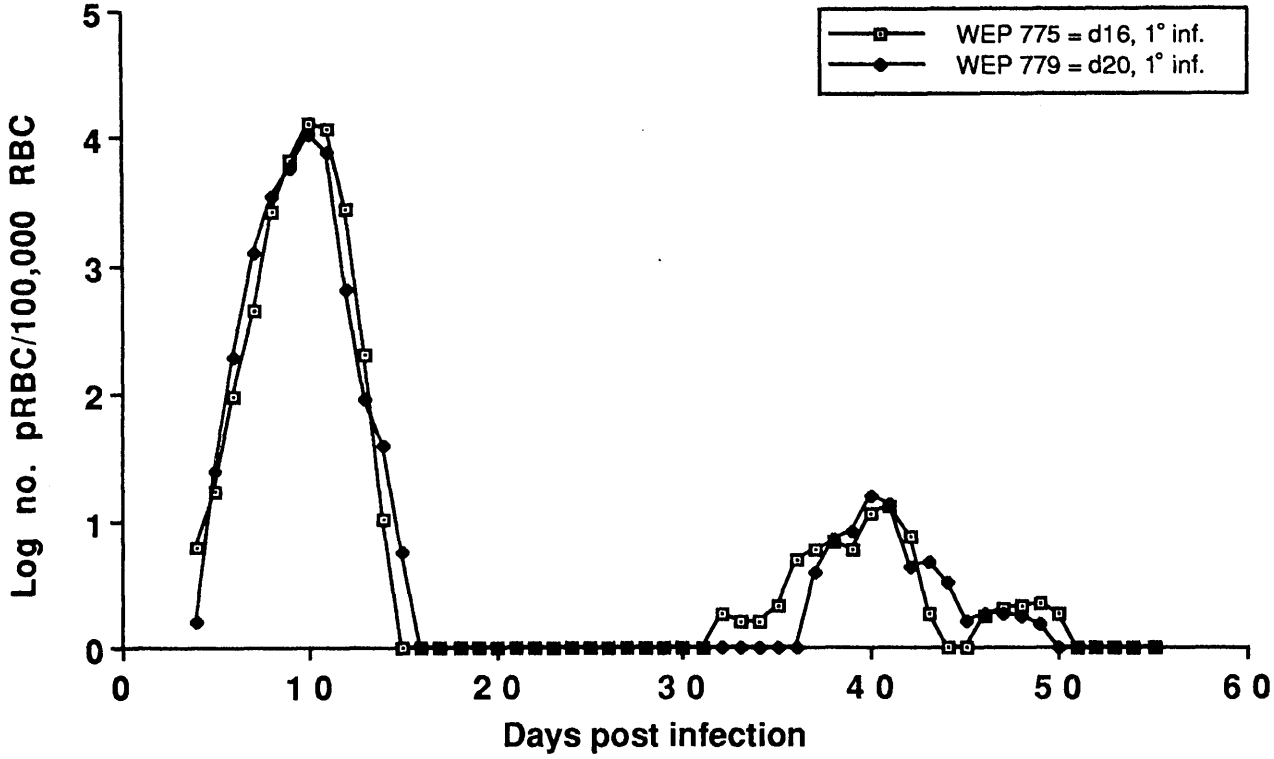


Fig. 5.2.3 Course of infection of NIH naive recipients of WEP 775 and WEP 779 challenged with 1 x10^5 pRBC i.v..

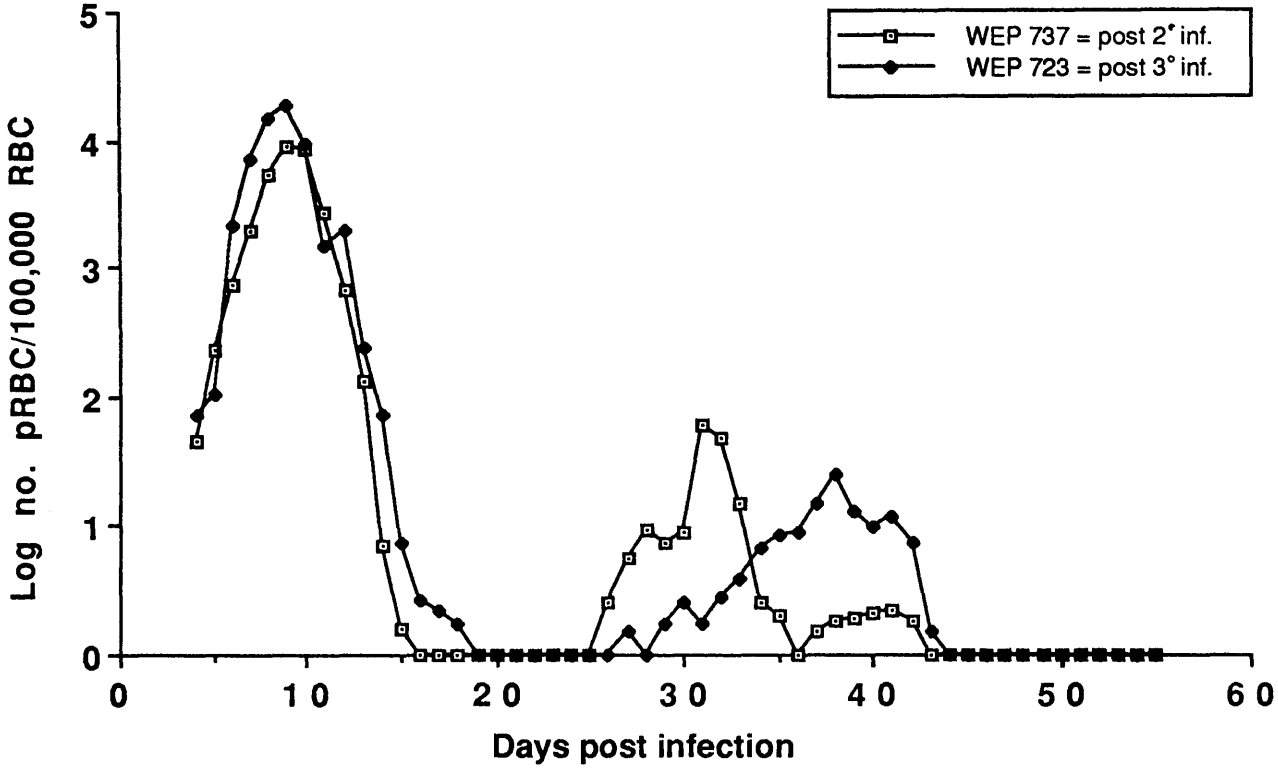


Fig. 5.2.4 Course of infection of NIH naive recipients of WEP 737 and WEP 723 challenged with 1 x10^5 pRBC i.v..

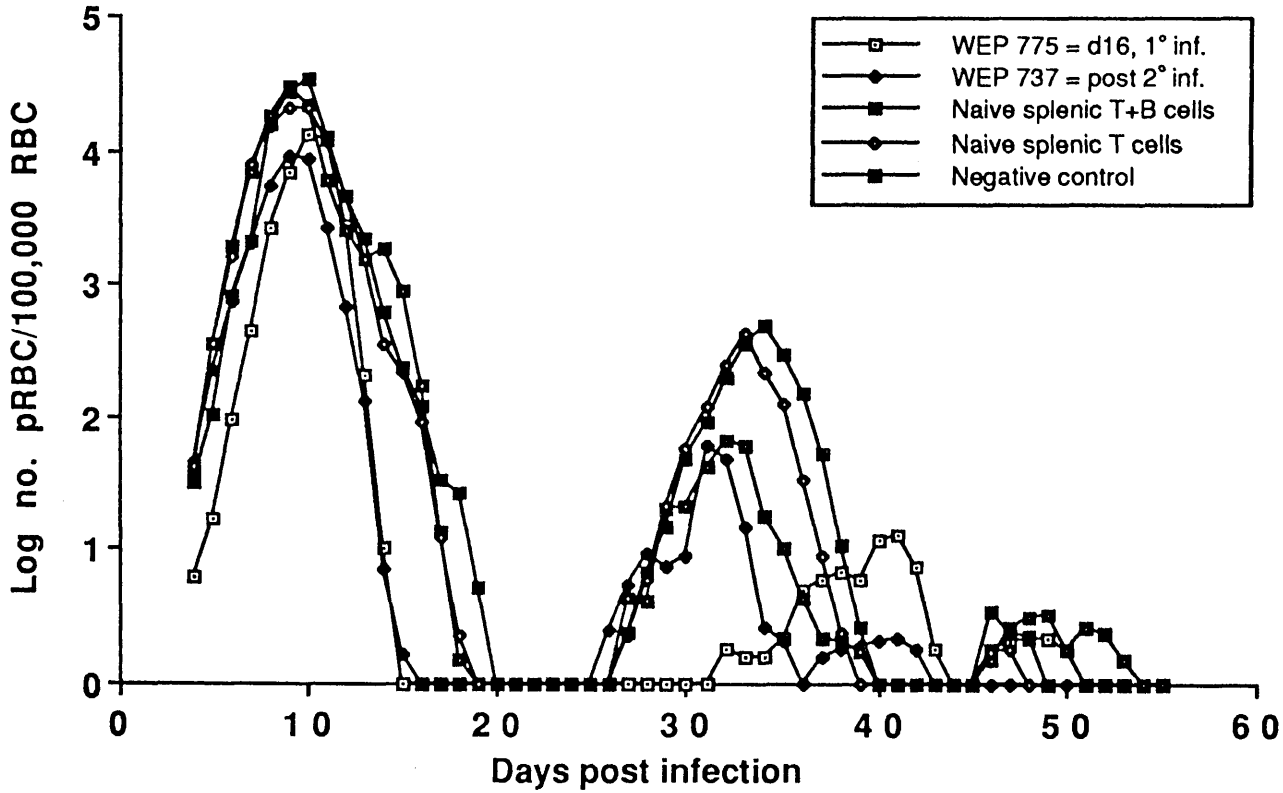


Fig. 5.2.5 Comparison of the courses of infection in NIH naive recipients of WEP 775 and of WEP 737 challenged with 1 x10^5 pRBC i.v..

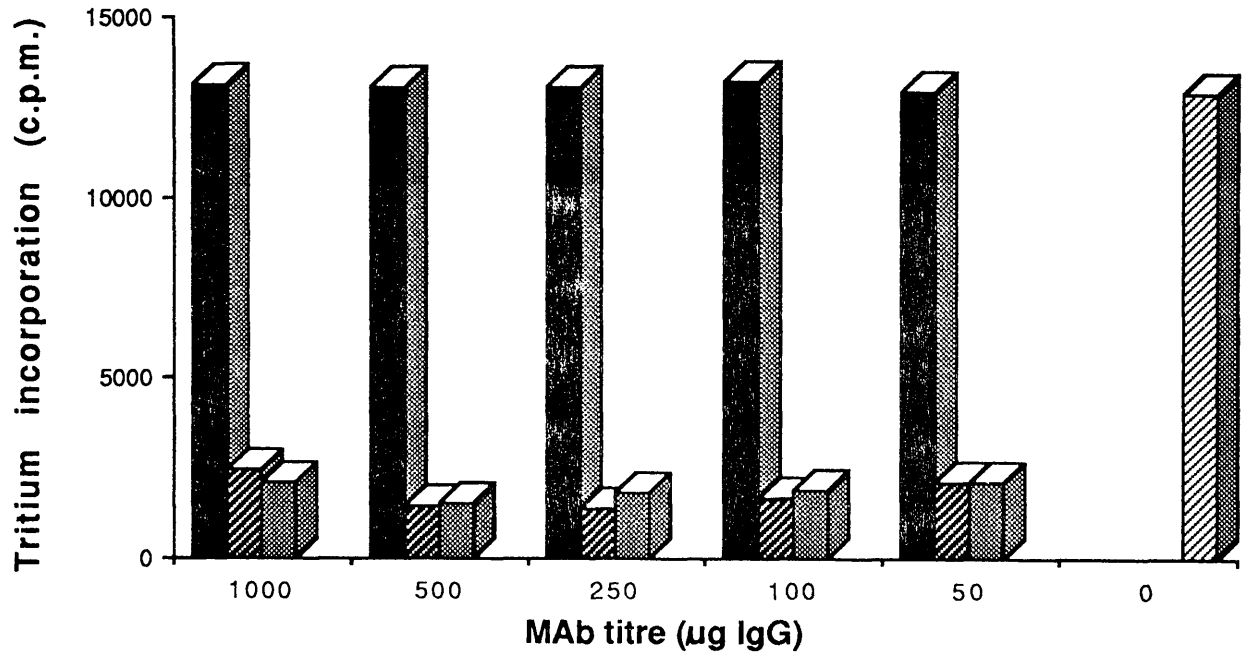


Fig. 5.3.1 Assay of Ag-specific proliferation of WEP 775 T lymphocyte line following in vitro MAb depletion treatment

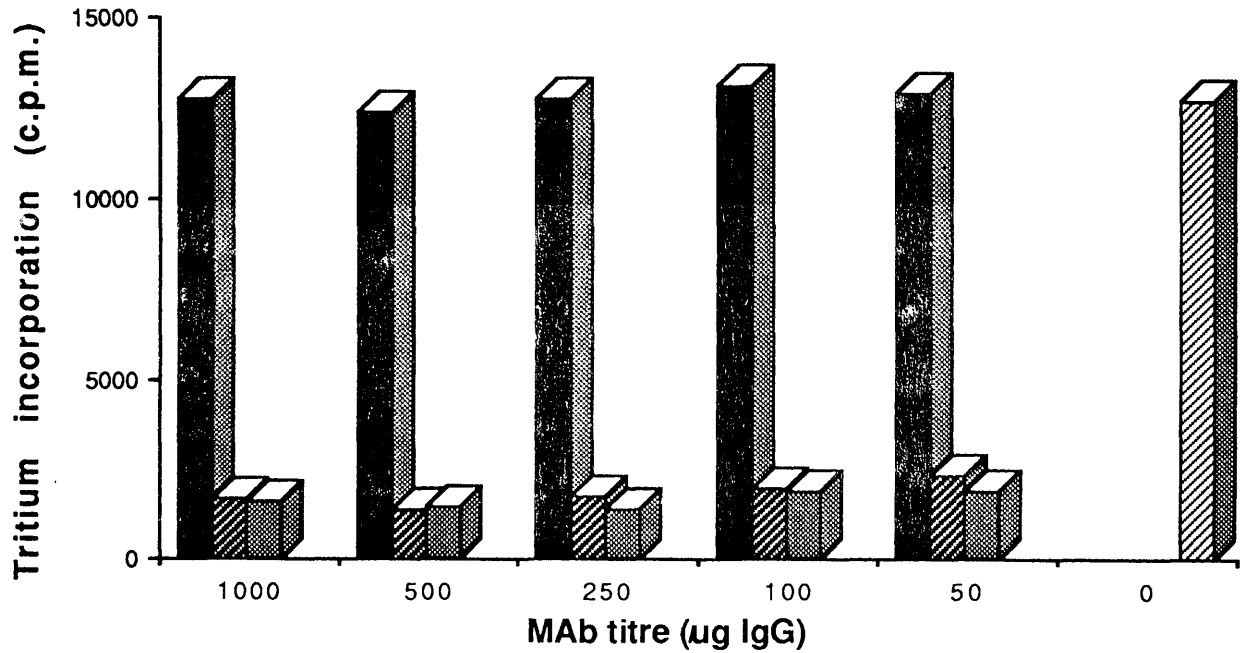


Fig. 5.3.2 Assay of Ag-specific proliferation of WEP 737 T lymphocyte line following <u>in vitro</u> MAb depletion treatment

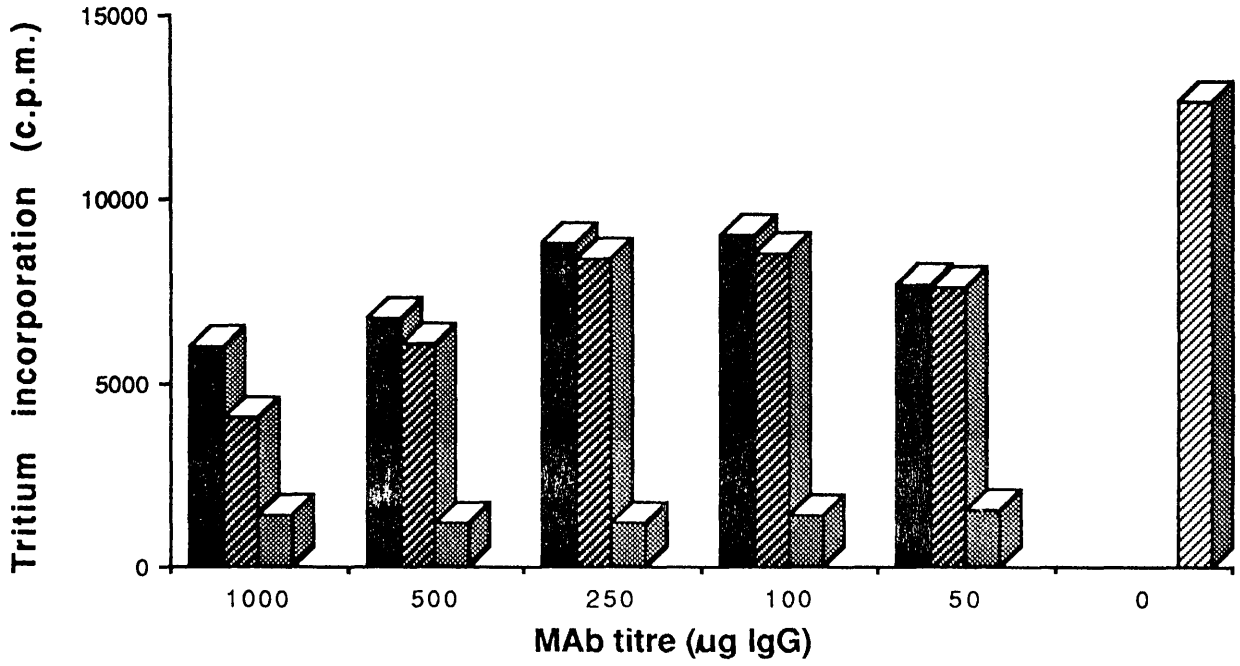


Fig. 5.3.3 Assay of proliferation of NIH naive splenic T lymphocytes following in vitro MAb depletion treatment

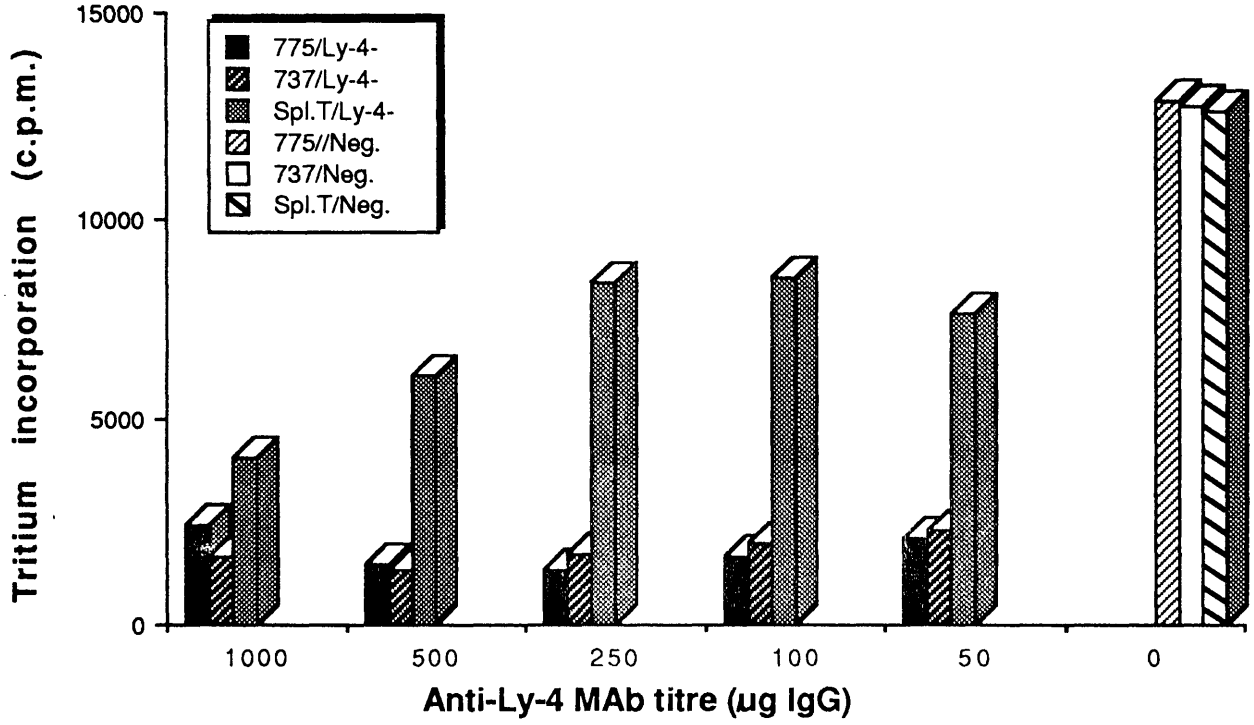


Fig. 5.3.4 Assay of Ag-specific proliferation of T lymphocyte lines following in vitro anti-Ly-4 MAb depletion treatment

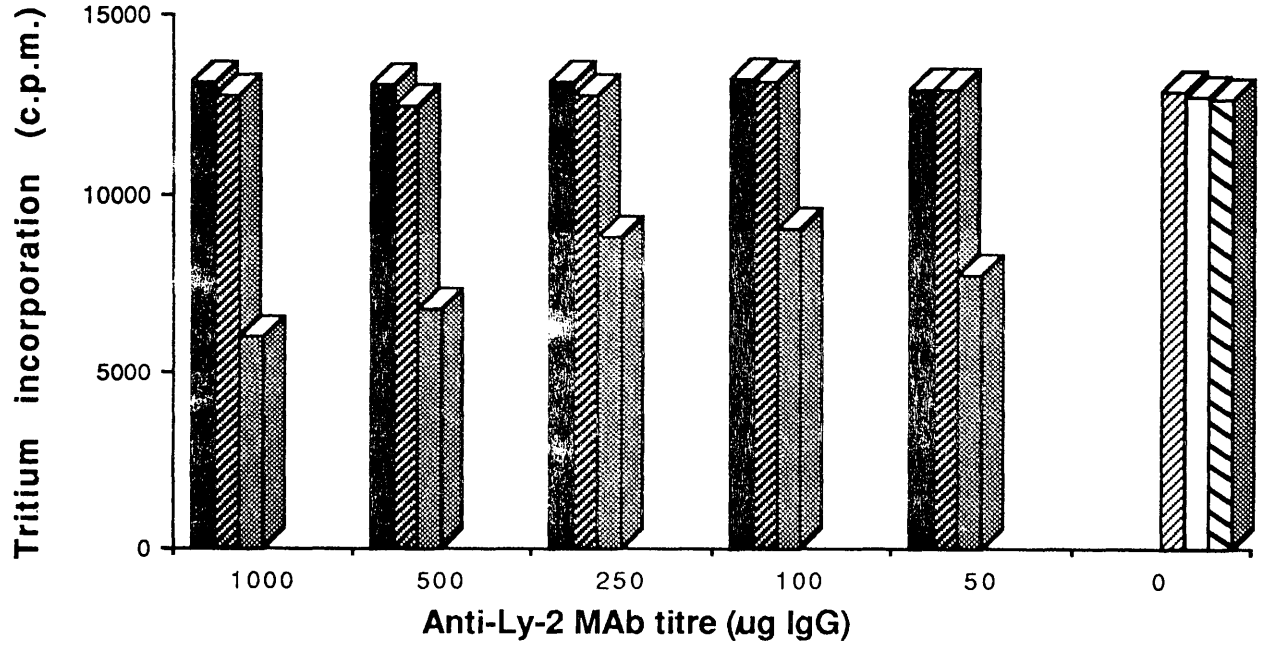


Fig. 5.3.5 Assay of Ag-specific proliferation of T lymphocyte lines following in vitro anti-Ly-2 MAb depletion treatment

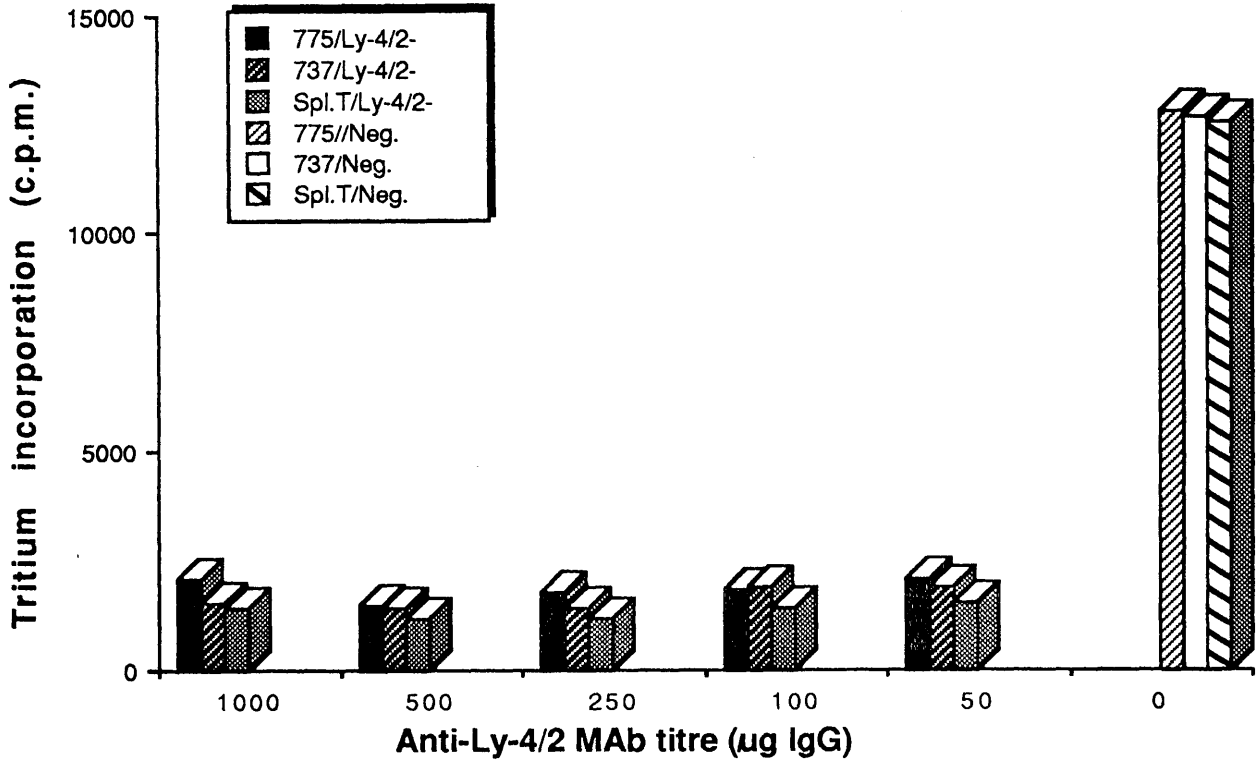


Fig. 5.3.6 Assay of Ag-specific proliferation of T lymphocyte lines following in vitro anti-Ly-4 & -Ly-2 MAb double depletion

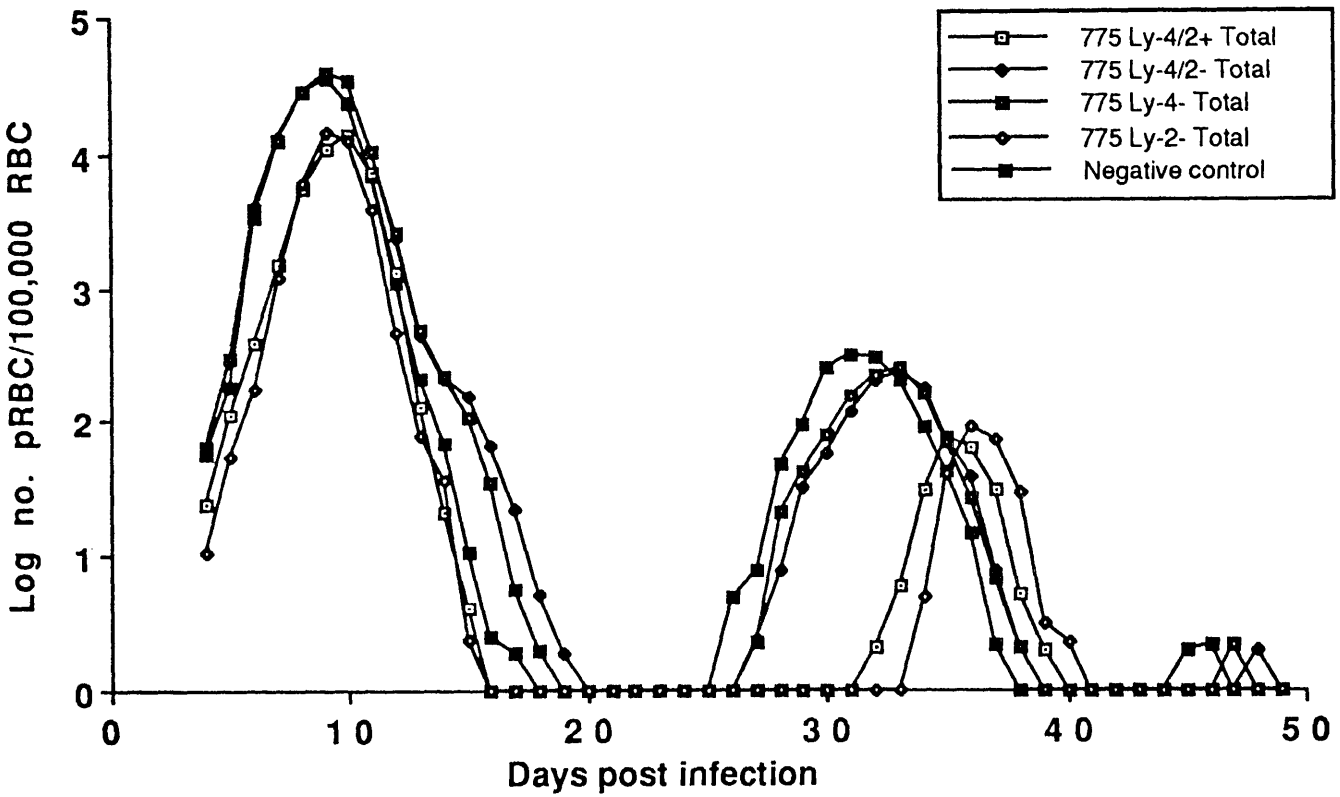


Fig. 5.4.1 Courses of infection in NIH naive recipients of WEP 775 following <u>in vitro</u> depletion of Ly-4$^+$ and/or Ly-2$^+$ T cell subsets upon challenge with 1 x10^5 pRBC i.v..

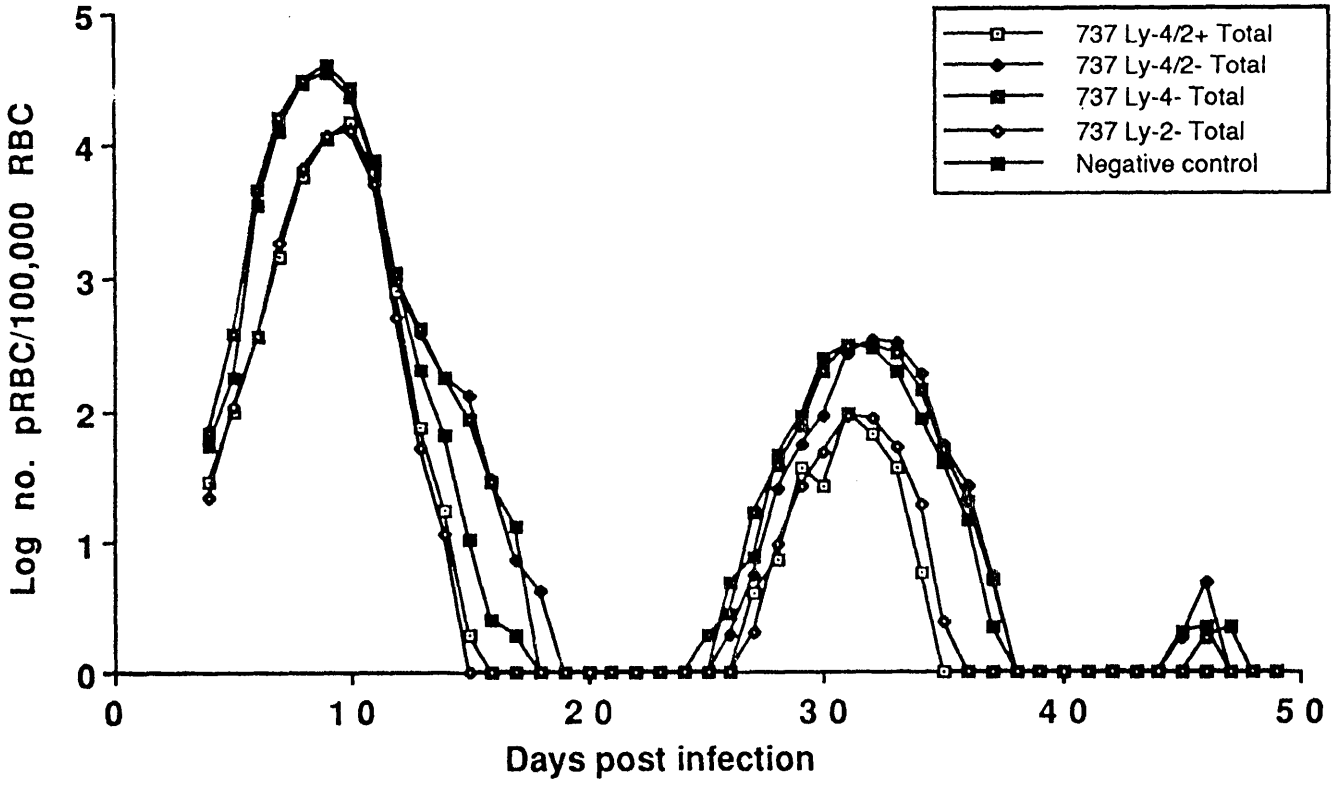


Fig. 5.4.2 Courses of infection in NIH naive recipients of WEP 737 following <u>in vitro</u> depletion of Ly-4$^+$ and/or Ly-2$^+$ T cell subsets upon challenge with 1 x10^5 pRBC i.v..

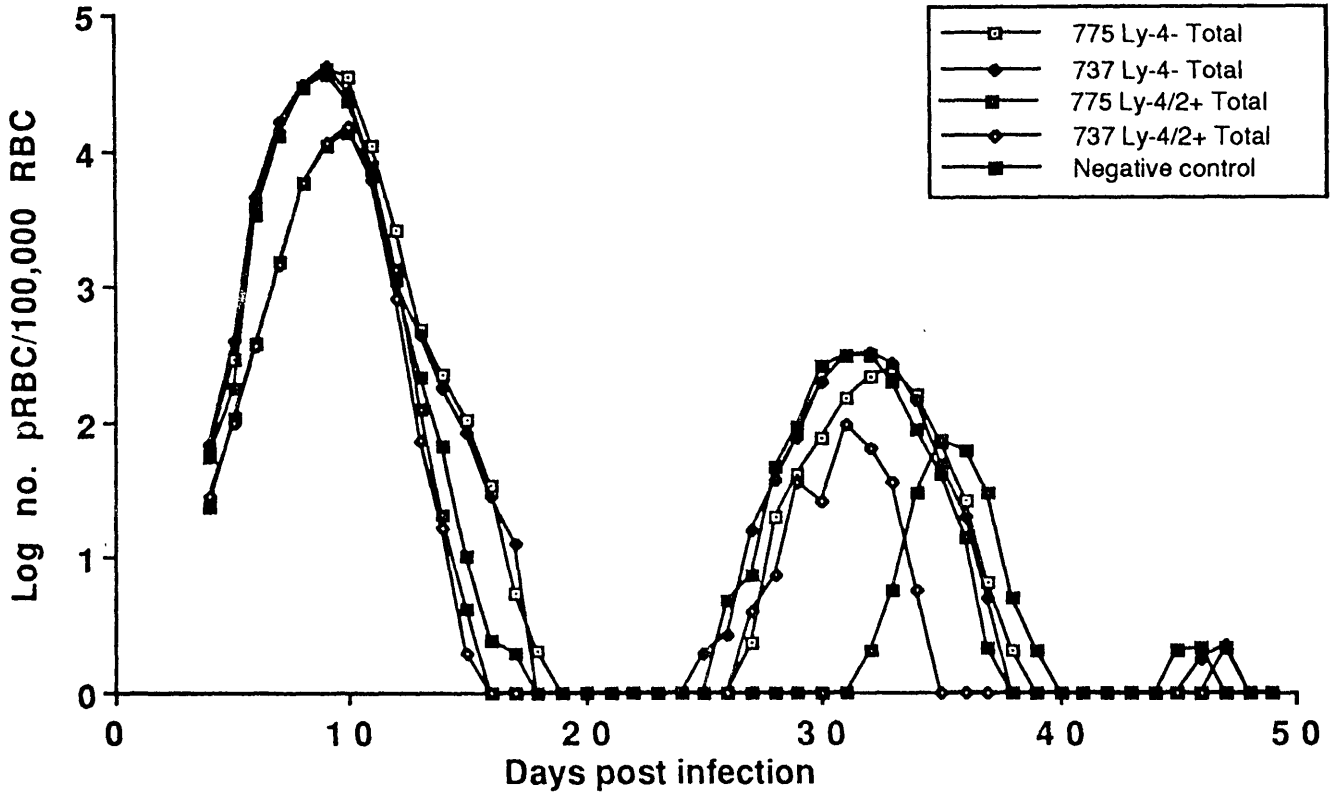


Fig. 5.4.3 Comparison of the courses of infection in NIH recipients of WEP 775 and of WEP 737 following <u>in vitro</u> depletion of the Ly-4$^+$ T cell subset upon challenge with 1 x10^5 pRBC i.v..

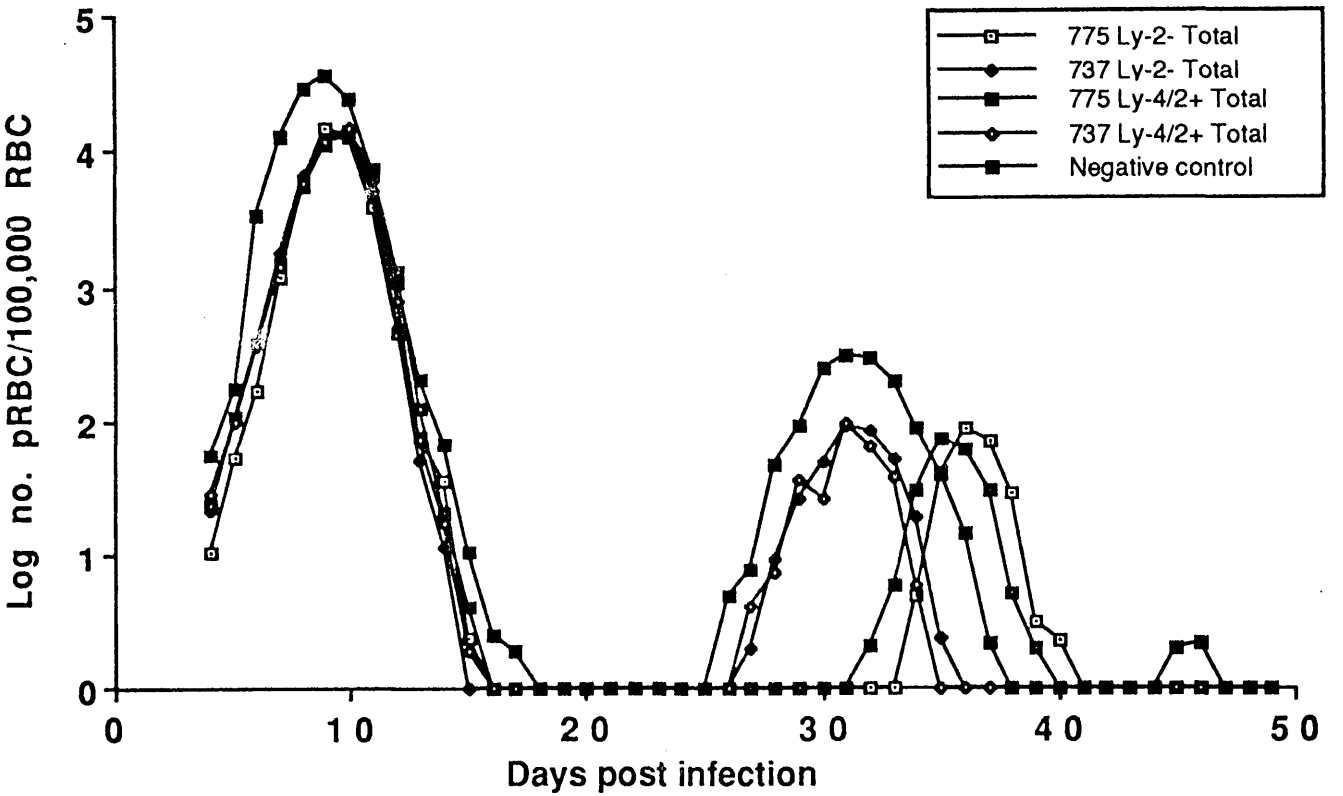


Fig. 5.4.4 Comparison of the courses of infection in NIH recipients of WEP 775 and of WEP 737 following <u>in vitro</u> depletion of the Ly-2$^+$ T cell subset upon challenge with 1 x10^5 pRBC i.v..

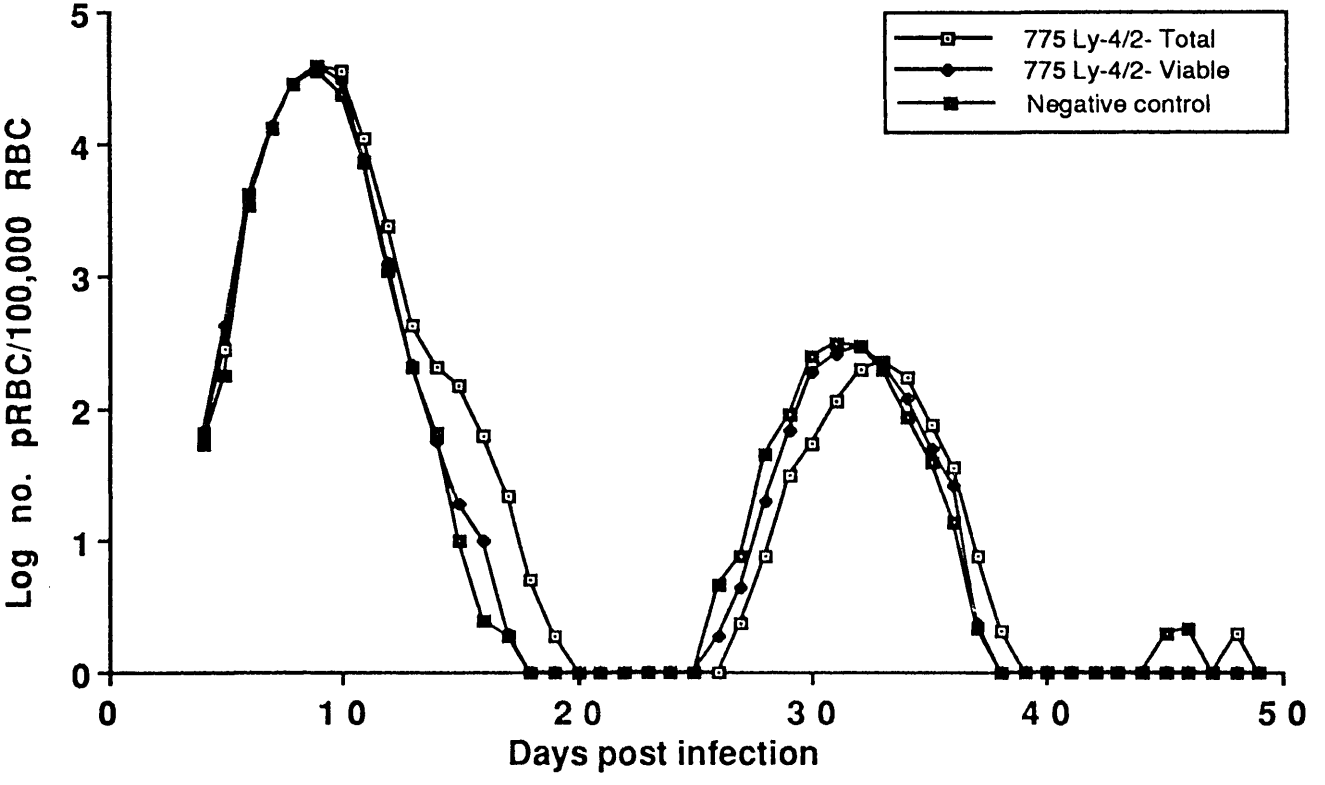


Fig. 5.4.5 Comparison of the courses of infection in NIH naive recipients of WEP 775 following different <u>in vitro</u> anti-Ly-4 & -Ly-2 MAb double depletion treatments upon challenge with 1 $x10^5$ pRBC i.v..

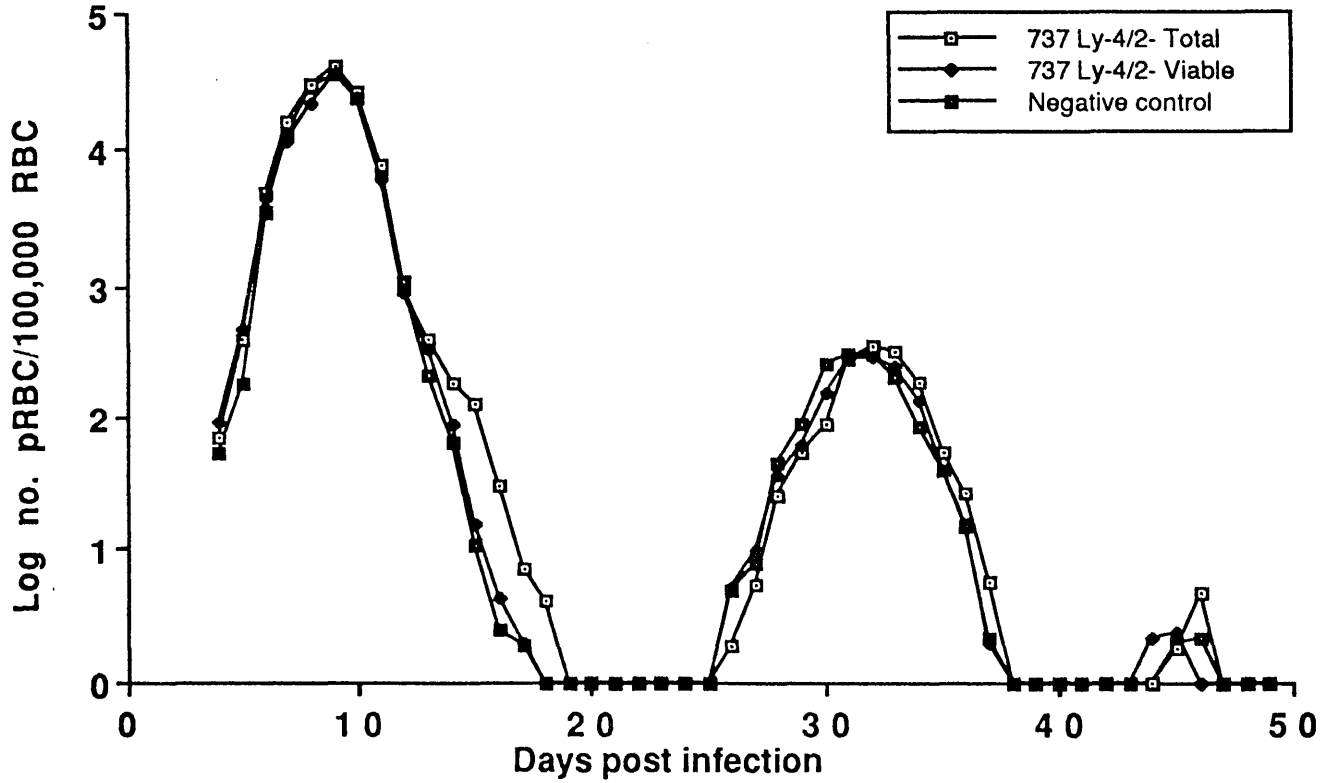


Fig. 5.4.6 Comparison of the courses of infection in NIH naive recipients of WEP 737 following different <u>in vitro</u> anti-Ly-4 and -Ly-2 MAb double depletion treatments upon challenge with 1 $x10^5$ pRBC i.v..

The Ly-4^+ T lymphocyte subset in the host immune response to the asexual stages of *Plasmodium chabaudi chabaudi*

Volume II

by

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Doctor of Philosophy in the University of Glasgow

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CHAPTER SIX

ADOPTIVE TRANSFER OF PRIMED LYMPHOCYTE SUBPOPULATIONS AND OF T CELL LINES AT HOMOLOGOUS AND HETEROLOGOUS PARASITE CHALLENGE

6.1 Introduction

The specificity of the host immune response to falciparum malaria has been a subject of much controversy for many years. Species- and strain-specific immunity (James et al 1932, Covell & Nicoll 1951), heterologous immunity (Boyd & Kitchen 1945, McCarthy et al 1978, Pazzaglia & Woodward 1982) or the non-existence of any effective immunity (Jeffrey 1966) have all been proposed. Early studies on rodent malarias showed that prior immunisation or infection induced solid immunity against homologous reinfection (Cox & Voller 1966, Nussenzweig et al 1966, Cox 1978). These studies also indicated the existence of interspecies cross-immunity detectable in the IFAT, which reflected the appreciable homology in Ags of plasmodia of different species.

Recent studies using more homogeneous parasite preparations have confirmed that homology, as well as extensive genotypic and phenotypic variabililty, exists in antigenic determinants of cloned lines of asexual stage parasites of the same species derived from different isolates or even from a single isolate (e.g. Schofield et al 1982, Newbold et al 1984). Due to the diversity of the parasite population, analysis of the induction of protective immunity can be carried out only in extremely well defined host-parasite systems. For this reason, most advances in this area have come by studying cloned isolates of rodent malarias in syngeneic hosts. For instance, Jarra & Brown (1985) demonstrated more intense parasitaemias upon heterologous strain (genotype) infection in the cloned P. c. chabaudi/ CBA/Ca mouse model, but the absence of significant recrudescence even after heterologous challenge. These observations and others (Brown et al 1970 a & b, Phillips 1970, Barnwell et al 1983 a, Howard & Barnwell 1985, Marsh & Howard 1986) are indicative of a considerable degree of immunity transcending intrastrain phenotypic diversity, and to a lesser extent, interstrain genotypic differences. Both effects may be incomplete, reducing parasitaemia and clinical disease without necessarily clearing infection. At least part of this immunity is associated with a serum factor which retains species, strain and phenotypic variant specificity (McLean et al 1982 a, Jarra et al 1986). However, Abs of a given specificity are unlikely to be a sufficient prerequisite for immunity. Rather, T cell recognition leading to protective helper T cell responses is considered to be of central importance to anti-malarial protective immunity (Jayawardena 1981, Brown et al 1986). This is because it has been shown that the spleen, highly primed at the helper T cell level, is required to mount an effective immune response (Brown 1971, Brown et al 1976 a, Jayawardena 1981). The sensitised spleen has the capacity for rapid

anamnestic parasiticidal Ab responses to the extensive range of parasite-derived surface exposed epitopes which the Plasmodium gene pool is capable of encoding, and also for the T_H2 cell-mediated mechanisms of immunity that may be necessary for reduction of the clinical manifestations of disease.

A feature of all in vivo models of malaria is that immunisation with lysed parasites or whole non-irradiated pRBC, or with purified Ags, requires potent adjuvants for even modest effect. Immunisation without adjuvant requires a previous priming infection or the use of pRBC rendered non-replicating, usually by irradiation or chemotherapy. Brown et al (1986) showed that after a primary P. c. chabaudi AS infection, there is a clear difference between mice immunised by purified Ag and mice immunised by infection or gamma-irradiated pRBC. Infections in the former approximate to those suffered by naive mice undergoing a primary, recrudescing infection, except that after homologous challenge, the initial parasitaemia is delayed and the level of recrudescence is increased. In contrast, mice challenged after a primary infection or immunisation with gamma-irradiated pRBC have no recrudescence, even when challenged with a different isolate.

These findings prompted an examination of the specificity of immunity generated by infection of NIH mice with P. c. chabaudi. In all, three different adoptive transfer experiments were performed to dissect the genotypic and phenotypic specificity of immunological reconstitution. To do this, cloned parent populations of the AS and CB strains of P. c. chabaudi were used, as was an antigenically variant recrudescent population collected from a P. c. chabaudi AS primary infection. Previously, it had been demonstrated that lymphocytes primed to P. c. chabaudi AS for a relatively brief period were capable of conferring protection against homologous parasite challenge upon adoptive transfer to both competent and compromised recipients (Chapter 3). As an extension of this study, the differences in host susceptibility to homologous and heterologous strain challenge were examined upon reconstitution of immunosuppressed recipients with either splenic or peripheral blood lymphocytes primed to either AS or CB strain P. c. chabaudi. The study of interstrain variation in immunity to homologous or heterologous infection on either an AS- or CB-primed background was thought worthwhile as all previous studies bar one had adoptively transferred hyperimmune cells from superinfected mice (Cox 1970, Grun & Weidanz 1981, 1983, Cavacini et al 1986, Falanga & Pereira da Silva 1989, Favila-Castillo et al 1990) Using P. yoelii, however, Fahey & Spitalny (1986) had shown that adoptive immunisation of A/Tru mice

with spleen cells from syngeneic donors with a primary, non-virulent 17X strain infection conferred on recipients the capacity to resist a challenge infection with the virulent YM strain. Of importance with regard to the methodology used herein, unfractionated splenic lymphocytes as well as spleen cells enriched for T or B cells capable of transferring protective immunity were detected as early as d 7 of the primary avirulent infection. This showed that it was possible to generate an adequate level of immunity against parasites of a different genotype to those used to initiate the primary infection, and, furthermore, that this strain-transcending immune response could be adoptively transferred to syngeneic recipients. Rather than using P. yoelii parasites, immunity to which is known to be Ab-dependent (Weidanz & Long 1988), P. c. chabaudi AS and CB strains were used in this study, but lymphocytes were taken from donor mice similarly during infection, at the time of peak peripheral blood lymphocytosis.

A further experiment was performed in which the WEP 775 and WEP 737 in vitro-propagated Ly-4^+ T cell lines were adoptively transferred into naive, syngeneic mice. These recipients were challenged either with the homologous AS strain of P. c. chabaudi to which they had been primed, or with heterologous strain (CB) or phenotypic variant (RC10) parasites. Already, it had been shown that these lines could transfer successfully immune protection to the AS parent population in vivo (Chapter 5), but the specificity of this reactivity had not been determined. As the proliferation of these lymphocyte populations in vitro was P. c. chabaudi AS pRBC Ag-specific, it was thought likely that these cells would exhibit a limited specificity in vivo. However, as the plasmodial epitope(s) to which these lines had been exposed were not identified, either or both lines tested may have shown a specificity of protection indicative of priming to a determinant common to all phenotypes of the AS strain, or even possibly all strains of the P. c. chabaudi species. To ascertain the specificity of protection of these AS-primed lines, an adoptive transfer was necessary.

6.2 Adoptive transfer of P. c. chabaudi AS-primed lymphocyte populations at homologous and heterologous parasite strain challenge in immunologically compromised recipients

40 NIH female mice were exposed to 4 Gy gamma irradiation and 24 hr later infected with either 1 $x10^5$ P. c. chabaudi AS or CB strain pRBC. At the time of challenge, each mouse was also inoculated with an aliquot of 1.0 $x10^7$ lymphocytes. Groups of 10 mice

each received populations of primed splenic T & B cells, normal splenic T & B cells or normal peripheral blood T & B cells. Within each group, batches of five mice were infected with either AS or CB strains of P. c. chabaudi.

To enable the adoptive transfer of AS semi-immune lymphocytes, a group of 20 syngeneic female mice was infected with 1 x10^5 P. c. chabaudi AS pRBC and the course of infection and absolute lymphocyte counts determined. When the mean peak peripheral lymphocytosis was reached, d 12 p.i., each donor mouse was bled and splenectomised. 14 uninfected mice were used as a source of naive lymphocytes for adoptive transfer.

As it had been shown previously that a mixed population of T & B lymphocytes gave better protection to homologous challenge than either separated cell fraction (Chapter 3), whole preparations of either splenic or peripheral blood lymphocytes were used for reconstitution.

A further group of controls was set up to determine the normal courses of infection of AS and CB parasites in irradiated recipients, and to check for the non-transfer of pRBC in donor lymphoid cell preparations.

There was an element of strain-specificity in the protection conferred to immunocompromised recipients upon challenge infection. This is observed most clearly in Fig. 6.2.1 for P. c. chabaudi AS-primed spleen cell transfers. The greatest protection was given by AS semi-immune splenic lymphocytes, all of the recipients of which were able to resolve the primary infection, subpatency being achieved by d 22 p.i., then lasting 15 d. However, a marked recrudescence did take place in all five mice studied. In contrast, for the heterologous CB strain challenge of mice inoculated with AS-primed cells, the primary parasitaemia, though of a similar peak level to that shown for homologous challenge, was of considerably longer duration, remission occurring on only d 37 p.i.. Although three of five mice died at crisis, no recrudescent parasitaemia was observed for the two surviving animals (Fig. 6.2.1). It appeared, therefore, that by one of the parameters of protection used in this study, the presence and/or level of recrudescence, that homologous challenge gave an exacerbated infection. It should be stressed, however, that this effect occurred only in mice surviving acute infection, which led to fatalities amongst the heterologous challenge group.

Naive spleen cells were quite capable of transferring protection to sublethally irradiated mice infected with P. c. chabaudi AS, three of five mice clearing blood-borne parasites (Fig. 6.2.2). For naive splenic lymphocyte transfer to CB-challenged mice, all recipients died by peak primary parasitaemia. This difference in the protection given

by spleen cells not previously exposed to P. c. chabaudi Ags reflects the increased virulence of the CB strain over the AS strain of P. c. chabaudi in the NIH mouse.

That some recipients of splenic lymphocytes primed to a different strain of P. c. chabaudi were able to clear CB strain challenge, yet no mice adoptively transferred normal spleen cells were able to do so (Fig. 6.2.3), shows that there was a protective activity present in the sensitised lymphocytes not present in the naive lymphocytes, and, furthermore, that this protection was able to transcend interstrain differences. Thus, with the CB strain of P. c. chabaudi, adoptive transfer of cells primed to the AS strain were capable, to a limited degree, of conferring an immune reactivity sufficient to clear challenge infection.

Unlike P. c. chabaudi CB challenge, immunosuppressed mice receiving naive spleen cells were able to clear AS strain parasites, at least in some cases. This protection was not as effective as that given by the AS semi-immune splenic lymphocytes (Fig. 6.2.4). Although onset of acute infection was similar in the two groups of mice, recipients of homologously-primed lymphocytes showed a significantly lower peak parasitaemia ($p < 0.05$), with remission 3 d ahead of the naive spleen cell recipients. However, for the mice adoptively transferred AS-primed cells, the secondary parasitaemia reached a significantly greater level ($p < 0.05$), and was of longer duration, than that shown by the normal spleen cell controls (Fig. 6.2.4). Thus, prior sensitisation to the challenge parasites led to a quickened remission to subpatency but appeared to cause an immunosuppressive effect during the recrudescent phase of infection.

It had been shown previously that AS semi-immune peripheral blood lymphocytes were capable of engendering protection to homologously challenged 4 Gy-irradiated mice (Chapter 3). However, in this particular study, mice receiving peripheral blood cells died at crisis whereas those receiving spleen cells all cleared P. c. chabaudi AS infection (Fig. 6.2.5). Likewise, adoptive transfer of AS-primed splenic lymphocytes conferred sufficient cross-strain immune activity for two of five mice to control CB infection, whereas recipients of a similar number of donor AS lymphocytosis-derived peripheral blood lymphocytes all succumbed at crisis (Fig. 6.2.6). These findings do not negate those in 3.4 and 3.5, wherein it proved possible to transfer protection against homologous challenge in the P. c. chabaudi AS/NIH mouse system by the inoculation of peripheral blood lymphocytes. Instead, this study highlights the difficulty encountered attempting to transfer protection with populations of blood lymphocytes that are present only transiently in the peripheral circulation. Furthermore, it confirms the spleen as

the lymphoid organ of choice for such adoptive transfers, for not only is the spleen a concentrated source of lymphocytes and as a secondary lymphoid organ, a site of immunological memory, it has a well documented role in malarial parasite clearance. This role of the spleen in anti-malaria immunity is reflected by the protection conferred by AS-primed spleen cells at both homologous and heterologous challenge (3.6 and this experiment).

All recipients of peripheral blood cells, either semi-immune or naive, died by d 14 p.i. of homologous AS infection, as did negative controls showing a normal course of infection in sublethally irradiated mice (Fig. 6.2.7). There was no significant difference in the timing or level of parasitaemia between the three groups studied. For the corresponding heterologous CB challenge, peripheral blood lymphocyte-transferred recipients were equally unable to control infection and all succumbed at crisis, if a little earlier, d 11 p.i., than did AS-infected mice (Fig. 6.2.8).

6.3 Adoptive transfer of P. c. chabaudi CB-primed lymphocyte populations at homologous and heterologous parasite strain challenge in immunologically compromised recipients.

As this was the reciprocal challenge experiment to that described in 6.2, identical conditions were followed but using P. c. chabaudi CB-infected syngeneic donor mice as the source of semi-immune lymphocytes. As before, all recipients were exposed to 4 Gy sublethal irradiation 24 hr prior to adoptive transfer of 1×10^7 lymphocytes and challenge with 1×10^5 pRBC.

There was a clearly defined strain specificity of protection conferred by adoptive transfer of P. c. chabaudi CB-primed splenic lymphocytes to challenged immunocompromised mice. Homologously infected recipients were able to control infection effectively, with the acute parasitaemia reaching subpatent levels by d 21 p.i., at which time only one of five mice had died (Fig. 6.3.1). Thereafter, there were two brief patencies before parasitaemia was cleared on d 46 p.i.. For mice receiving an identical inoculum of CB-semi-immune spleen cells but challenged with the AS strain parasite, all five recipients suffered an elevated peak primary parasitaemia and died shortly afterwards (Fig. 6.3.1). This marked specificity of resistance was in contrast to the analogous challenge on an AS-primed background (Fig. 6.2.1), where two of five CB-challenged mice recovered completely. As the CB strain of P. c. chabaudi is more virulent in the NIH murine host than is the AS strain, this finding was unexpected, but

may reflect a better priming to P. c. chabaudi antigenic determinants common to all strains upon AS rather than CB strain infection.

The substantial degree of immunity conferred by transfer of CB-primed splenic lymphocytes to homologously infected mice can be appreciated by comparing the mean course of infection for this group with that for mice receiving naive spleen cells at P. c. chabaudi CB challenge (Fig. 6.3.2). Whereas only one mouse succumbed to infection after receiving parasite-primed spleen cells, all five mice receiving normal spleen cells did so. Although the effects of adoptive immunity were apparent during primary parasitaemia, at which time the normal lymphocyte recipients all died, the actual levels of ascending parasitaemia for the two groups were identical. Thus the differences in the protective activity of reconstituted cells was observed only beginning at the time of peak acute infection.

The protection given by transferring spleen cell preparations taken at peak lymphocytosis from P. c. chabuadi CB-infected donor animals was not matched by that conferred by peripheral blood lymphocytes collected from the same donors (Fig. 6.3.3). As for normal spleen cell recipients, mice inoculated with CB-primed peripheral blood lymphocytes all died during the remission of the primary parasitaemia. This finding was similar to that for the reciprocal AS challenge (Fig. 6.2.5) and showed that, irrespective of the infecting strain of P. c. chabaudi, a greater protection is conferred by homologously primed lymphocytes of splenic origin rather than those taken from the peripheral circulation. It is of no surprise that since recipients of CB-primed splenic lymphocytes failed to clear challenge P. c. chabaudi AS infection, mice injected with the same numbers of similarly primed peripheral blood cells did not do so either (Fig. 6.3.4). All mice died between 11-14 d p.i., at or just after peak primary parasitaemia, a feature common to all recipient animals succumbing to infection.

Irradiated mice died at crisis irrespective of whether they had received primed or normal peripheral blood lymphocytes and whether or not they were challenged by homologous CB or heterologous AS strain P. c. chabaudi parasites (Figs. 6.3.5 & 6). It would be reasonable to consider, therefore, that any enhanced immune effects observed upon adoptive transfer were not of ultimate survival value to the host animals. Indeed, in the case of AS challenge (Fig. 6.3.6), there was no significant difference in the patterns of ascending parasitaemia for recipients of either CB-primed or naive peripheral blood cells compared to negative control mice. This was similar to the previous results for both AS and CB challenges on an AS-primed background (Figs. 6.2.7

& 8). However, in the case of the reciprocal P. c. chabaudi CB infection (Fig. 6.3.5), mice receiving circulatory lymphocytes of either CB-sensitised or naive status showed a delayed onset of patent parasitaemia of between 1-2 d, compared to negative controls of CB-infected mice. For both groups, this observable lag in the appearance of blood stream parasites was statistically not significant ($p > 0.05$), nor was the later deaths of recipients of primed lymphocytes. However, these observations are suggestive of a protective effect upon transfer of peripheral blood cells, as has been demonstrated previously (Chapter 3). In this instance, however, the virulence of the CB strain parasite ensured that the biological significance of this minimal immune reactivity observed during the ascending acute infection was not fully realised.

The cumulative findings of both AS- and CB-primed lymphocyte adoptive transfers to homologous and heterologous P. c. chabaudi infection (6.2 & 3) showed a pronounced strain specificity. Of mice infected with AS strain pRBC, only those inoculated with AS-primed spleen cells could control parasitaemia. Priming to the heterologous CB parasite was insufficient to prevent recipient animals succumbing to infection (Fig. 6.3.7). For the corresponding P. c. chabaudi CB challenges, a strain-specific protective effect was also evident, though less marked (Fig. 6.3.8). Two of five AS-primed splenic lymphocyte recipients did survive challenge infection with the heterologous CB strain, indicating the presence of a strain-transcending immunity. In such cases where mice were able to control the acute phase of a heterologous challenge, no subsequent patencies occurred once the primary infection was resolved (Figs. 6.2.1 & 6.3.8). However, for both sets of homologous challenges, where recipient fatality was minimal, recrudescences were always observed (Figs. 6.2.1, 6.3.1, 7 & 8). This was indicative of an immunosuppressive effect of homologously-primed lymphocytes later in the course of infection of recipient animals. Such a phenomenon did not detract from the greatly enhanced overall protection engendered by spleen cells previously sensitised to that strain of P. c. chabaudi used to infect the mice to which these cells were transferred. The ability to confer a protective activity sufficient to control acute infection was achieved by semi-immune lymphocytes taken from the spleens but not from the peripheral blood of donor animals.

6.4 Adoptive transfer of *in vitro* generated *P. c. chabaudi* AS-specific T cell lines at homologous and heterologous parasite challenge in naive syngeneic recipients.

Although enriched T cell populations were obtained from the peripheral blood and spleen of infected mice and assessed for their protective activity by adoptive transfer at homologous (Chapters 3 & 6) and heterologous (Chapter 6) challenges, the heterogeneous nature of these populations made it difficult to determine accurately the phenotype and subset of T cells mediating protection. For this reason, T cell lines were prepared from the spleens of *P. c. chabaudi* AS-infected donor mice and cultivated *in vitro* to give relatively homogeneous Ly-4$^+$ T cell populations (Chapters 4 & 9). These preparations showed a *P. c. chabaudi* AS-specific responsiveness *in vitro*. Furthermore, each of the four different lines was then shown to be protective against homologous AS challenge in naive NIH mice (Chapter 5). The experiment below details the adoptive transfer of these Ly-4$^+$ lines to fully competent syngeneic recipient animals at both homologous AS strain and heterologous CB strain challenges. In addition, the intrastrain specificity of adoptive protection was examined using a phenotypically variant AS population, collected from the recrudescence of an AS parent infection, to challenge naive mice at the time of reconstitution. As the protection carried by the adoptively transferred Ly-4$^+$ lines was apparent in homologously challenged immunologically competent hosts (Chapter 5), it was decided to use intact recipients for the corresponding heterologous infections. Any immune reactivity transferred by the lines would be distinguishable from that transferred by a splenic T cell control population or by that background innate immunity to *P. c. chabaudi* CB challenge exhibited by naive NIH mice.

The two lines used were those which were subsequently cloned (Chapter 4), WEP 775, derived from d 16 of primary *P. c. chabaudi* AS infection, and WEP 737, taken from mice recovered from a secondary challenge. For each line, 1.77 x10^7 lymphocytes were adoptively transferred to 15 age-matched NIH female mice. These were then boxed in groups of five mice, each group receiving a challenge infection of 1 x10^5 pRBC of either *P. c. chabaudi* AS or CB strain parent stabilates, or *P. c. chabaudi* AS recrudescent clone 10. Control groups receiving 2.29 x10^7 enriched naive splenic T cells were also set up. These were similarly infected with one of the three different challenge parasites available. In addition, three normal, untransferred NIH mice were challenged with each of the three pRBC populations to act as a control of the normal course of primary

infection of these P. c. chabaudi parasites in this model system.

Figure 6.4.1 shows the courses of infection upon homologous challenge with the AS strain of P. c. chabaudi, i.e. those parasites against which the cell lines were raised. Both lines exhibited enhanced protection compared to a control of unprimed splenic T cells. This was observed as a shortened primary parasitaemia, significantly depressed peaks of both primary and secondary patencies (both $p < 0.01$), extended subpatent periods and the presence of only two patent waves of infection (Fig. 6.4.1). Thus, by all of the criteria used to determine immune protection, reconstitution with either AS-primed Ly-4^+ line was more effective than with a slightly larger inoculum of naive T cells. The latter, in fact, did give some but not very much protection, compared to the normal course of AS strain infection. These results are essentially similar to those described previously (5.2) for adoptive transfer of these lines with homologous challenge (Figs. 5.2.1 & 2). This stresses the reproducibility of the adoptive protection conferred by each of these stable Ly-4-bearing T cell lines. As described before, although the WEP 775 and WEP 737 lines each engendered considerable protection upon adoptive transfer, mice receiving these lines differed in their onset of recrudescence. WEP 737-transferred animals recrudesced 4 d ahead of those given WEP 775 cells (Fig. 6.4.1). Since this was a repetition of the data of 5.2 (Figs. 5.2.3 & 4), it would appear that this divergence in the timing of recrudescence in recipients of these Ly-4^+ populations is a feature of their transfer.

Naive mice infected with the phenotypically variant recrudescent clone of P. c. chabaudi AS were able to control infection better upon adoptive transfer of either Ly-4^+ cell line primed to the parent population of the AS strain than were controls (Fig. 6.4.2). As for homologous challenge (Fig. 6.4.1), recipients showed a shortened primary parasitaemia, clearly depressed peak acute and recrudescent parasitaemias ($p < 0.01$ in each case), and a markedly quicker final parasite clearance, each compared to mice receiving a similar dose of naive splenic T lymphocytes (Fig. 6.4.2).

The courses of infection in naive NIH mice upon challenge with the CB strain of P. c. chabaudi, i.e. heterologous strain challenge, are depicted in Fig. 6.4.3. It is apparent that there was some enhanced protection conferred by the transfer of AS-primed lymphocytes, but to a much lesser extent to that upon challenge by parasites of the same genotype (Figs. 6.4.1 & 2). Using the same parameters of protection as before, there was a slightly reduced peak primary parasitaemia, a significantly depressed recrudescence ($p < 0.01$) and a shorter total infection time, again with respect to naive

spleen cell recipients (Fig. 6.4.3). However, there was no quickened remission to subpatency compared even to the P. c. chabaudi CB challenge alone. Furthermore, for all four groups, the subpatency was very shortlived, recipients of either WEP 775 or WEP 737 recrudescing at the same time, 3 d after remission, as did naive splenic T cell recipients (Fig. 6.4.3), and this only 24 hr after the untransferred controls began to recrudesce.

Evidently, some protection was engendered by the Ly-4$^+$ lines primed to the parent population of P. c. chabaudi AS upon challenge of recipient mice by antigenically variant AS and CB strain pRBC, yet the greatest protection was against the homologous AS parent infection (Figs. 6.4.1-3). It may be that of the late trophozoite/schizont pRBC Ags against which the Ly-4$^+$ were raised, some are unique to the AS parent population whilst others are common to all strains of P. c. chabaudi. Although no direct comparison can be made between the protection conferred by AS semi-immune splenic T & B lymphocytes (6.2) and the AS-specific Ly-4$^+$ lines used here (since in the former study, the recipient mice were sublethally irradiated prior to infection), it would appear that the homogeneous Ly-4-bearing lines do give greater protection at heterologous challenge than do the freshly prepared spleen cells. This is because P. c. chabaudi CB-challenged recipients of the in vitro-cultured cell lines exhibited a pattern of parasitaemia broadly similar to that shown by mice challenged with the homologous AS strain (Figs. 6.4.1 & 3); i.e. two waves of parasitaemia separated by a distinct subpatent period. For the analogous AS and CB strain challenges to mice inoculated with AS-primed splenic lymphocytes (Fig. 6.2.1), the course of infection in P. c. chabaudi CB-infected animals was very different to that in AS strain-infected animals, as characterised by a chronic acute infection but no recrudescence. It would appear that either the larger inoculum of primed Ly-4$^+$ lymphocytes present in the cell line gives a considerably enhanced protection to heterologous strain challenge over that of a mixed lymphocyte population, or, alternatively, that the mechanisms involved in mediating control of infection are different. This is a possibility because of the seemingly more effective immunity given by the splenic T & B cells after remission of the primary P. c. chabaudi CB parasitaemia (Fig. 6.2.1). Alternatively, this latter characteristic may be caused by an immunosuppressive effect at homologous AS challenge, which is not observed upon infection with genotypically variant pRBC (Fig. 6.2.1).

One interesting aspect of this experiment was that the dichotomy observed during the recrudescent phase of infection with the AS parent pRBC in WEP 775- and WEP 737-

transferred mice (Fig. 6.4.1) was not evident upon either AS recrudescent clone 10 (Fig. 6.4.2) or CB strain (Fig. 6.4.3) challenge. For these infections, both the timing and levels of parasitaemia were similar in mice receiving either Ly-4$^+$ line. The simultaneous appearance of recrudescent parasites appeared to be due to the earlier onset of recrudescence in mice given the WEP 775 cell line at heterologous challenge (Figs. 6.4.2 & 3), compared to homologous challenge (Fig. 6.4.1). This is appreciated more easily by examination of Fig. 6.4.4. For transfer of WEP 737 to mice infected with each of the three P. c. chabaudi parasite types (Fig. 6.4.5), it can be seen that the recrudescence of AS parent pRBC-infected animals actually occurred later than did those for heterologously challenged mice. This delayed onset of recrudescence of 1-2 d was not thought to be significant, and reflected the longer subpatent period observed upon homologous infection before the appearance of a breakthrough parasite population. Moreover, the observed lag did not detract from the fact that upon infection with the AS parent pRBC to which the Ly-4$^+$ lines were sensitised, recipients of WEP 737 recrudesced significantly ahead of those of WEP 775 ($p < 0.01$) (Fig. 6.4.1). That the difference between the transfers of WEP 775 and WEP 737 was apparent only upon homologous challenge may reflect a change in the underlying mechanisms of immunity employed to control challenge infection. At heterologous challenge, the pattern of parasitaemia shown by WEP 775-inoculated mice reverted to that shown by mice given WEP 737 under conditions of homologous infection. It may be that under the pressure of infection by antigenically variant pRBC, the mechanism by which immunity was mediated by WEP 737 cells was better equipped to control infection, and thus the functional dichotomy seen at homologous challenge was not apparent. The differences in the mediation of resistance of these Ly-4$^+$ lymphocyte lines to P. c. chabaudi AS infection are dissected further in the proceeding chapters (7-9).

Regardless of the differences between the immunity transferred by each of the AS-primed Ly-4$^+$ lymphocyte populations at homologous challenge (Fig. 6.4.1), and the similarities at heterologous challenge (Figs. 6.4.2 & 3), for each cell line the strain specificity of protection was obvious. It is clear that there is a strain-transcending element of immunity conferred by these P. c. chabaudi AS-primed lines that, for example, enabled recipients to withstand infection with the heterologous CB strain of this malaria species (Fig. 6.4.3). However, it is equally evident that the protection given to mice infected with the AS parent type was greater than that given to those infected with the AS recrudescent clone, which, in turn, was greater than that given to

mice challenged with the CB strain parasites (Figs. 6.4.4 & 5). This strain-specific element of immunity is most elegantly revealed by examination of the length of the recrudescent periods of infected mice. For both WEP 775 and WEP 737 recipients, the homologous recrudescence lasted less (9 and 10 d, respectively) than did that for the variant phenotypic challenge (12 and 11 d) or that for the variant genotypic challenge (17 and 16 d) (Figs. 6.4.4 & 5). These differences were significant at the 0.01 level for both lymphocyte lines. Furthermore, for each line, the protection transferred, as determined by the length of recrudescence, was greater than that which naive splenic T cells could transfer to similarly challenged mice (Fig. 6.4.6). There was some immunity conferred by these unprimed cells, however, and this was most striking after remission of the primary parasitaemia, as the courses of the acute infection were similar to those observed in untransferred control groups (Fig. 6.4.7). For splenic T cell recipients, the mean recrudescence time was 12, 15 and 18 d for mice challenged with AS parent, AS recrudescent clone and CB parent P. c. chabaudi pRBC, respectively. Although significantly longer than the secondary parasitaemias exhibited by mice infected with either WEP 775 or WEP 737 (Figs. 6.4.4 & 5), these patencies represented a shortening by 4, 3 and 4 d, respectively, of those recrudescences in the negative control mice (Fig. 6.4.7).

Also noteworthy was the fact that the peak parasitaemia during acute infections of mice given either Ly-4$^+$ line (Figs. 6.4.4 & 5) was significantly lower ($p < 0.01$) for recipients of homologous rather than heterologous parasites. In both cases, the level of parasitaemia reached by AS parent-infected mice was higher than that for mice challenged with the AS recrudescent pRBC (Figs. 6.4.4 & 5). It would appear per se that the protection conferred early in infection by these lines was greater against challenge by the phenotypic variant. However, analysis of the normal courses of infection of these parasite populations (Fig. 6.4.7) shows that the peak of primary parasitaemia in naive NIH mice was innately lower in mice infected with the recrudescent population rather than the parent clone of P. c. chabaudi AS.

The strain specificity of protection conferred by the Ly-4$^+$ cell lines was evident from examination of the serum Ab titres for recipient animals infected with each of the three different parasite populations (Figs. 6.4.8 & 9). For both WEP 775 and WEP 737, the peak Ab levels were for homologous AS parent challenge, whilst the lowest levels were recorded for heterologous CB strain infection. The maximal Ab titre, 1:1028, was maintained for a longer period for recipients of WEP 737 (Fig. 6.4.9) than for WEP

775-transferred mice, which exhibited a transient serum Ab peak at this titre (Fig. 6.4.8). The different serum Ab profiles may reflect the fundamental differences in mediation of anti-P. c. chabaudi protection shown by these two T lymphocyte lines (Chapters 7-9). Irrespective of the titres measured, for both cell lines the patterns of the Ab titre profiles for different infections were similar (Figs. 6.4.8 & 9). The general pattern observed was of a rising serum Ab titre during acute infection which levelled out before increasing substantially at the time of recrudescence. This pattern was also reproducible in recipients of splenic T cells (Fig. 6.4.10), suggesting that the immune system of the immunocompetent host is inher ently capable of controlling infection by Ab-mediated means, and that, to some degree at least, the kinetics of serum Ig production in response to P. c. chabaudi infection are independent of the transferred AS-primed lymphocytes. However, it would be misleading to infer that the immune response, as gauged by the Ab levels during infection, is not inextricably linked to the reconstituted lymphocyte populations. This is because for infection with pRBC of AS parent (Fig. 6.4.11), AS recrudescent clone 10 (Fig. 6.4.12) or CB parent (Fig. 6.4.13), the peak Ab responses were detected in recipients of WEP 775 or WEP 737 significantly ahead of those of splenic T cells. This indicates that the transfer at the time of challenge of a population of Ly-4^+ lymphocytes previously primed to P. c. chabaudi AS aided the Ab-mediated response mounted by the host animals, demonstrable as a quicker maximal Ab titre (Figs. 6.4.11-13). What is interesting is that although recipients of unprimed T cells took longer to show peak peripheral blood anti-parasite Ig levels, when they were reached, they were of the same magnitude as those observed for primed cell recipients (Figs. 6.4.11-13). The 1:1028 Ab titre attained for AS parent-challenged mice was equal to that for the anti-P. c. chabaudi AS hyperimmune serum IFAT control. Thus, it is clear that in the P. c. chabaudi/NIH mouse model used, the adoptive transfer of AS-primed Ly-4^+ cells was not necessary to control infection, but their transfer lowered the parasitaemia and quickened parasite clearance. Moreover, anti-P. c. chabaudi serum Ab levels were elevated at the peak of recrudescence, d 35-38 p.i., when Ab-mediated mechanisms of immunity are considered to predominate (Figs. 6.4.8 & 9). Ab levels were also raised in control mice but at a time corresponding to the late remission of recrudescence, d 45-48 p.i. (Fig. 6.4.10). It would appear, therefore, that there was a degree of synergism between the AS-specific Ly-4^+ lines inoculated into challenged animals and the background primary immune response to malaria infection. This was manifested during the recrudescent phase of infection as a quickened

humoral response, but may equally have been effected by Ab-independent cellular mechanisms; these may have accounted for the lower primary parasitaemia and quickened remission to subpatency that were hallmarks of protection in WEP 775 and WEP 737 recipients upon AS parent and AS recrudescent clone challenge (Figs. 6.4.1 & 2). That the protection conferred by the transfer of Ly-4^+ cell lines to P. c. chabaudi CB strain-infected mice was not so nearly as clearly defined during the acute infection as for challenges with the other two parasite populations (Fig. 6.4.3) is suggestive of a lesser role for cell-mediated immunity in the control of infection with malaria parasites of a heterologous genotype. This would, in turn, suggest that Ab-independent mechanisms of immunity are strain-specific in this system, whilst Ab-dependent immune responses are, at least in part, capable of transcending interstrain barriers.

6.5 Discussion

The results detailed in this chapter in general concur with previously reported findings of a limited variant specificity of protective immunity to malaria in rodents. Using splenic lymphocytes taken from donor animals at peak peripheral lymphocytosis, cells primed to either the AS or CB strains of P. c. chabaudi were found to be better at conferring protection to homologous than to heterologous challenges. Indeed, most mice infected with a genotypically variant strain to the priming strain of parasite died at crisis. Jarra & Brown (1985) examined the interstrain specificity of immunity induced by infection with P. c. chabaudi. Mice previously infected with either the AS or CB strain were able to control heterologous reinfection but less well than their homologous challenge counterparts. However, only in naive control mice were fatalities observed. For the heterologous challenges described herein, mice undergoing a primary infection but receiving a semi-immune lymphocyte population were usually incapable of controlling acute parasitaemia. Only AS-primed CB-challenged animals overcame crisis to clear parasitaemia to subpatent levels. The differences noted between these two experiments using very similar systems, P. c. chabaudi AS and CB in NIH and CBA/Ca mice, are not thought to be due to any variation in the degree of cross-immunity detected. They are more likely to be caused by the relative ineffectiveness of transfer of lymphocytes primed to P. c. chabaudi pRBC for only 12 d of a primary infection compared to priming mice through a complete course of infection. No comparable studies on the adoptive transfer of protection to interstrain or intrastrain variant P. c. chabaudi have, to my knowledge, been published. It is possible that the variance seen here may

also reflect the effectiveness of adoptive transfer to syngeneic recipients, since, in other studies, hyperimmune splenic lymphocytes collected from donor animals infected at least twice failed to generate adequate levels of immunity to heterologous species challenge in naive recipients (Cox 1970, Favila-Castillo et al 1990). However, in these instances, adoptive protection had to transcend genotypic variability between malaria parasites. It is not surprising, therefore, that in mice immunologically suppressed before infection, no cross-protective activity was observed (Grun & Weidanz 1981, 1983, Cavacini et al 1986). Moreover, Falanga & Pereira da Silva (1989) showed that mice recovered from a primary P. c. chabaudi infection after a transfusion of normal blood did not have the capacity to resist challenge with P. yoelii 17X pRBC. As the mice protected following RBC transfusions exhibited a strong immunity to the homologous parasite, these results indicate that the destruction of P. c. chabaudi is due to a specific immune response that shows a lack of cross-reactivity. Paradoxically, Murphy & Lefford (1979) demonstrated that mice given a primary infection with the nonvirulent 17X strain of P. yoelii acquired a state of immunity which provided them with a prolonged heightened resistance against challenge with either the homologous parasite or highly virulent strains of P. yoelii. Furthermore, it has since been shown that cross-protective immunity could be transferred against virulent challenge by spleen cell populations taken early after infection of donor animals (Fahey & Spitalny 1986). This difference in the efficiency of transfer of protection by immune or semi-immune lymphocytes to heterologous strains of P. c. chabaudi and of P. yoelii is hard to reconcile but may be due to the contribution of different pRBC surface Ag types to the induction of protective immunity in the two systems.

In recent years, immunogenic determinants on the surface of pRBC of varying plasmodial species have been isolated and characterised. It is now known that the 250 kD, 230 kD and 190 kD proteins of P. c. chabaudi (Boyle et al 1982), P. yoelii (Holder & Freeman 1981) and P. falciparum (Holder & Freeman 1982) are equivalent (Newbold et al 1984). This Ag is a member of a family of schizont/merozoite-associated polypeptides which have been implicated in the induction of protective immunity to these plasmodia. There exists serological cross-reactivity between polyvalent mouse sera raised against these different high MW proteins (Holder et al 1983) but not between MAbs specific for each Ag. Being non cross-reactive, the MAbs must therefore recognise species-specific antigenic determinants of this class of malaria proteins. The fact that an Ag involved in the induction of immunity shows such inter- and intra-species diversity indicates that

at least part of genotype- and phenotype-specific components of malarial immunity may be directed against this family of protein Ags common to all mammalian plasmodia examined. In a study of the protective immunity induced by immunisation with purified 250 kD polymorphic schizont Ags of P. c. chabaudi AS and CB, Bates et al (1988) showed that pre-challenge serum from AS Ag-immunised CBA/Ca mice reacted with both AS and CB preparations, as determined by both IFAT and immunoprecipitation. This cross-reactivity was manifested as a delay in the onset of parasitaemia in AS Ag-immunised mice after both AS and CB challenge, although the delay was most marked after homologous challenge. Pre-challenge serum from CB Ag-immunised mice, however, was CB-specific by IFAT and immunoprecipitation, and only after homologous infection was a significant delay in initial parasitaemia observed. These findings correlate well with cross-reactive adoptive protection transferred by AS- but not CB-semi-immune splenic lymphocytes described in this chapter. On this basis, it would be tempting to envisage a prominant role for the 250 kD Ag in induction of immunity to P. c. chabaudi in the NIH mouse model as well as that of the CBA/Ca model, but it is clear that this determinant is one of several polypeptides to which an immune response is mounted, and its precise role in this process is unknown.

A feature of the immunisation with the 250 kD glycoprotein of AS or CB strains of P. c. chabaudi at homologous and/or heterologous challenge (Brown et al 1985, Bates et al 1988) was the occurrence of recrudescent parasitaemias, which were observed upon homologous challenge only. Since the parasite lines used were clones, the breakthrough parasites were presumably phenotypic variants with regard to exposed pRBC Ags, including the 250 kD polypeptide. Similarly, in the adoptive transfers at heterologous challenge described here (6.2 & 3), in irradiated recipients of AS-primed lymphocytes, only AS-challenged mice recrudesced. Although CB-infected animals suffered a protracted primary parasitaemia, once they had resolved acute infection, no further patencies were observed. Thus, the enhanced recrudescence, both in this study and for 250 kD Ag-immunised animals, indicates a negative effect of priming on the normal development of protective immunity upon infection by P. c. chabaudi pRBC. The reduced ability of the host to control the second wave of infection appeared to have specificity in that it was detectable only after challenge with homologous parasites. This is possibly indicative of a suppressor effect, the mechanism of which is not known. However, evidence from in vitro studies of P. falciparum suggest that periods of specific immunosuppression do occur during malaria infection (Brasseur et al 1983, Troye-

Blomberg et al 1983 b, 1984). Chronicity is maintained by phenotypic antigenic variation within the infective parasite strain, leading to low grade infection controlled by a variant-transcending immunity. It is thought that temporary suppression of the effector arm of the immune response allows expansion of the pRBC population circulating at the time at which suppression occurs to give rise to a patent recrudescence (McLean et al 1982 b, 1986 a).

With regard to the adoptive transfer of the P. c. chabaudi AS-specific Ly-4$^+$ lines (6.4), there was some evidence for both strain-specific and non-specific elements of protective immunity. For this study, recipient mice were immunologically intact so that the specificity of resistance engendered by transfer of these cell lines could not be scrutinised as closely as if the challenged mice were immunologically compromised. Although the differences in protection conferred upon homologous and heterologous infection were clearly distinguishable, even CB-challenged mice suffered acute-type, non-lethal infections. In the only published report of the specificity of adoptive transfer of in vitro-propagated Ly-4$^+$ cells (Brake et al (1988), BALB/c nude recipients were reconstituted with a Ly-4$^+$ clone and infected with the homologous P. c. adami. After parasite clearance, mice were then challenged with the same parasite or with the heterologous P. yoelii 17X. It was found that mice challenged with P. c. adami developed short lived parasitaemias of < 1%, whereas those challenged with the normally avirulent P. yoelii 17X developed fulminating malaria and died. The susceptibility of the nude mice to heterologous infection indicated that the Ly-4$^+$ clone possessed immunological memory for previous exposure to P. c. adami. Moreover, nude mice reconstituted with the protective clone failed to respond immunologically to either dinitrofluorobenzene or keyhole limpet haemocyanin when these Ags were administered following the resolution of P. c. adami infection (Brake et al 1988). These findings were not unexpected since the T lymphocyte clone used for the adoptive transfer would have limited ability to recognise diverse antigenic epitopes (Brake et al 1986, Chapter 4). However, athymic mice grafted with the clone and infected with P. c. adami did develop Abs capable of recognising a large number of malarial polypeptides, as judged by radioimmunoprecipitation (Goldring et al 1989). Ab reactivities were similar to those seen in infected euthymic mice and contrasted with their absence in infected nude mice. It may be that the reconstituted Ly-4$^+$ lymphocytes had been exposed to a specific plasmodial epitope as a result of infection and had consequently secreted lymphokines capable of supporting the polyclonal differentiation of B cells. In the present study,

anti-P. c. chabaudi Ab levels were elevated throughout infection but the specificity of the antigenic determinants eliciting such a response was not established. It would be doubtful, however, that such high serum Ab titres could be induced by a humoral response to a single immunogenic pRBC cell surface Ag, such as, for example the 250 kD polymorphic schizont Ag. What is known is that P. c. adami infection of C3HeB/FeJ mice is characterised by a predominant and persistent IgM response, moderate IgG_2 and IgG_3 and little significant IgG_1 response during a primary challenge (Langhorne et al 1984). In both the adoptive transfer experiments, the Ig isotype distribution of malaria-specific Ab production during infection was not examined, but presumably they follow a similar pattern. This is not guaranteed, however, since Ags shared by P. c. chabaudi and P. c. adami may not necessarily induce similar Ab responses in the two infections. In P. yoelii infections, for example, IgG_2 is the predominant isotype (Langhorne et al 1984). The results of Goldring et al (1989) that, regardless of the nature of the Ly-4^+ cell graft, sera from recipient animals recognised an array of plasmodial Ags suggests that it will not be possible to determine the antigenic specificity of cloned T lymphocyte populations in malaria infections by their capacity to provide help to restricted populations of B lymphocytes. It does, however, stress the concept that T cells, especially those of the Ly-4^+ subset, have a helper role in the production of protective Abs. This hypothesis was first put forward by Brown (1971, 1974) who suggested that helper T cells aid B cells in the synthesis of variant-specific protective Abs. Brown proposed that during malaria infection, T cells become primed to a determinant common to all plasmodial variants characteristic of the strain producing the infection, and, further, that each antigenic variant stimulates a separate B lymphocyte population. According to this hypothesis, the sensitisation of T cells with one variant type would produce an expanded T cell population capable of acting as helpers to all B cell clones responding to phenotypic variants as they arise later in infection. This proposal for the underlying mechanism of strain-specific anti-plasmodial immunity still holds favour today and explains the requirement for B cells in recipients of T cells sensitised to P. c. chabaudi AS (McDonald & Phillips 1978, see also Chapter 7). Variant-transcending immunity controls the growth, to a greater or lesser extent, of all phenotypic variants of the infective parasite genotype and accounts for the sharp drop in parasitaemia at crisis that leads to resolution of acute infection. In addition, a non-specific generalised protection transcending interstrain boundaries also plays a role in immunity to P. c. chabaudi. At present, however, the nature of this protective response has not been

elucidated and the mechanism involved has yet to be defined.

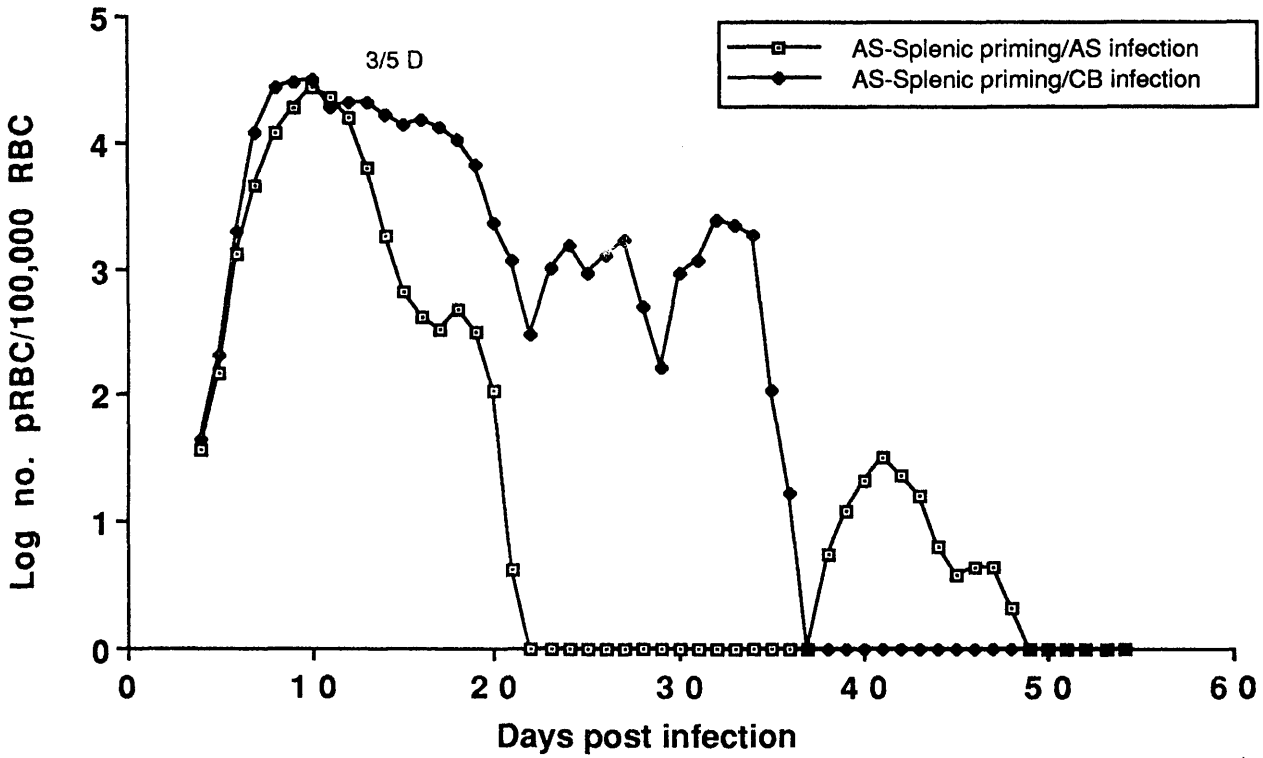

Fig. 6.2.1 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi AS-primed semi-immune splenic lymphocytes challenged with 1 x10^5 AS or CB strain pRBC i.v..

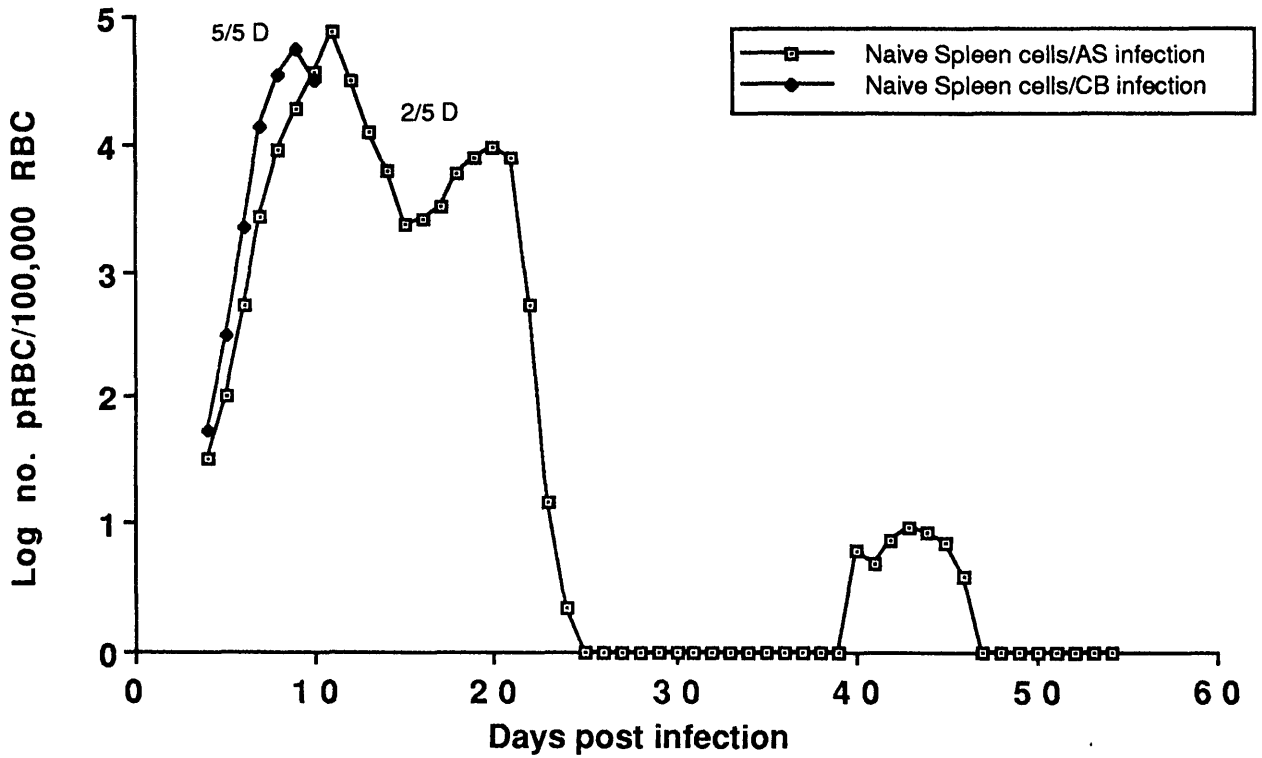

Fig. 6.2.2 Course of infection in sublethally irradiated NIH recipients of naive splenic lymphocytes challenged with 1 $x10^5$ AS or CB strain pRBC i.v..

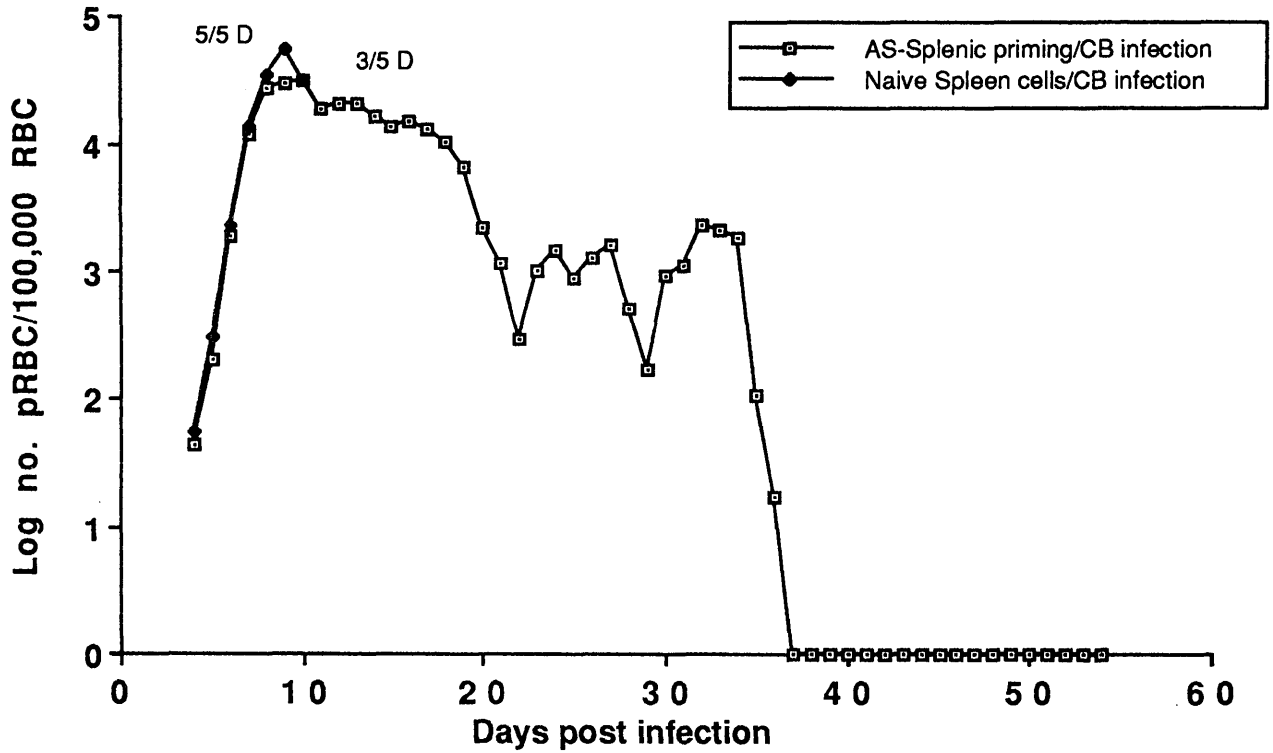

Fig. 6.2.3 Course of infection in sublethally irradiated NIH recipients of splenic lymphocytes challenged with 1 x10^5 P. c. chabaudi CB pRBC i.v..

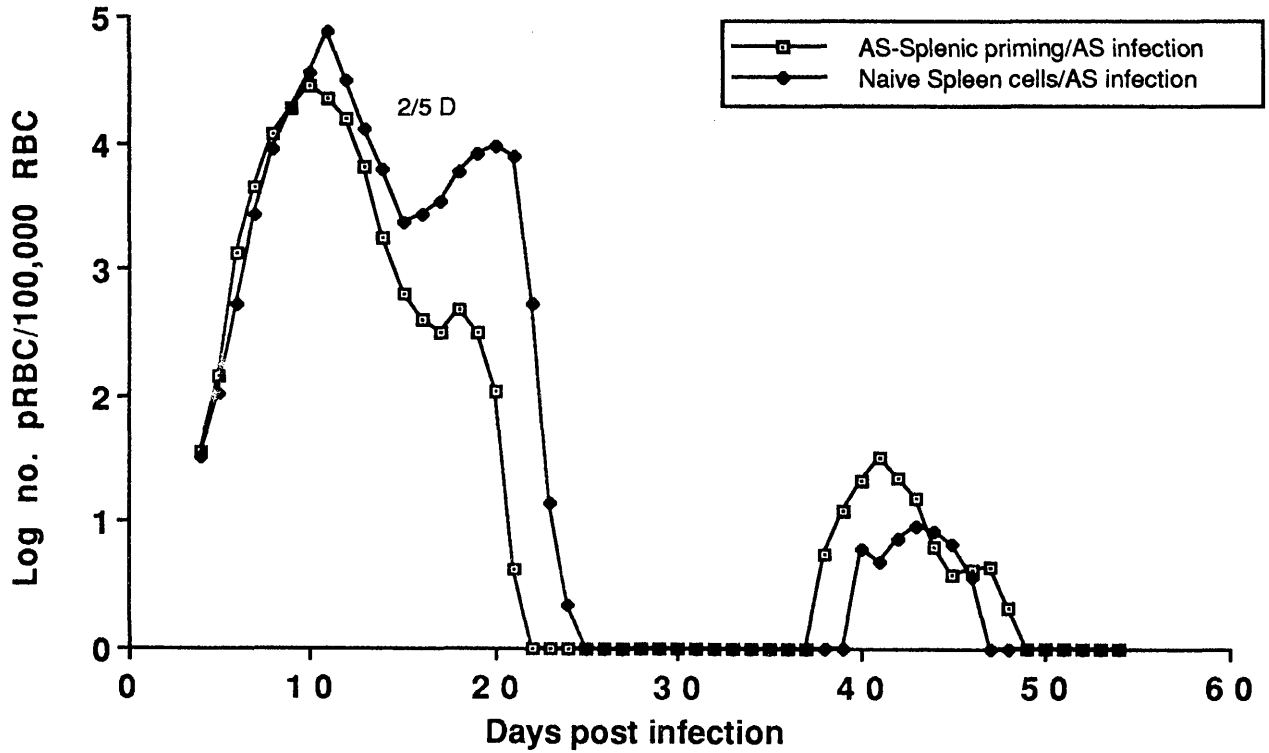

Fig. 6.2.4 Course of infection in sublethally irradiated NIH recipients of splenic lymphocytes challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

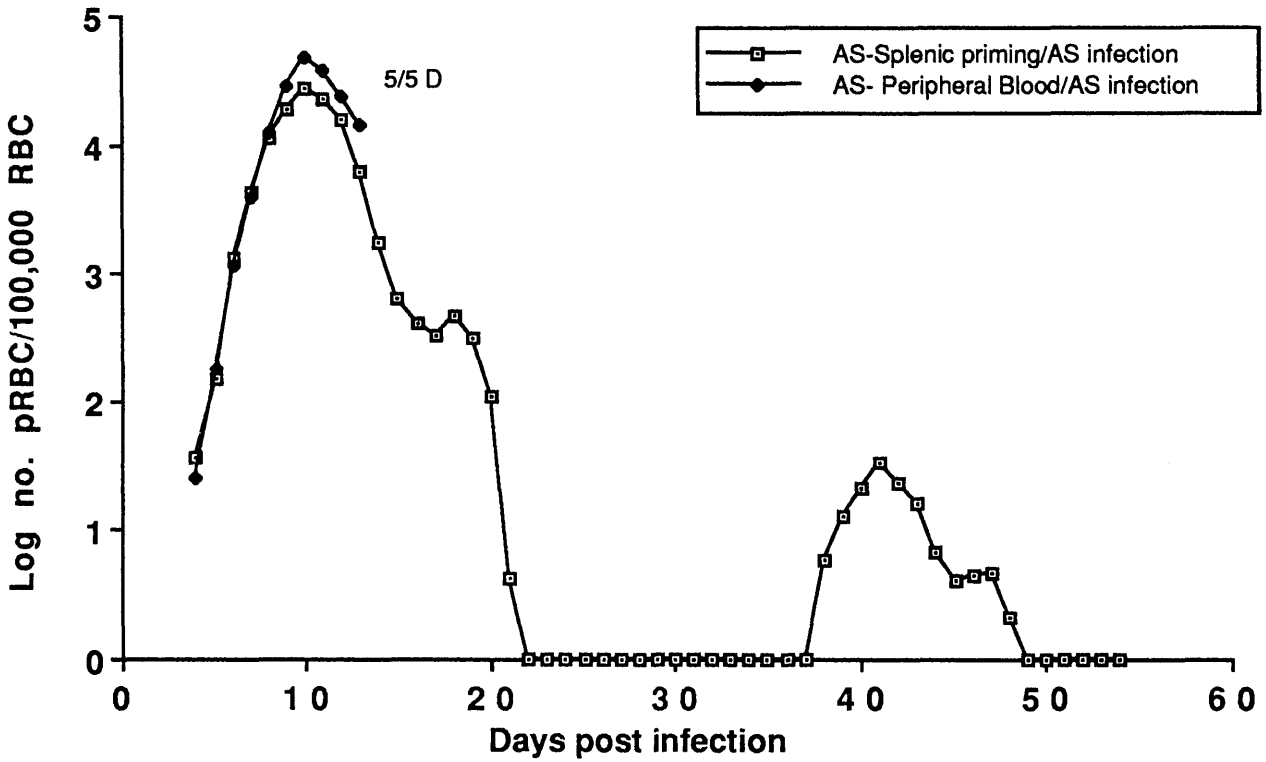

Fig. 6.2.5 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi AS-primed semi-immune lymphocytes challenged with 1 x10^5 homologous AS pRBC i.v..

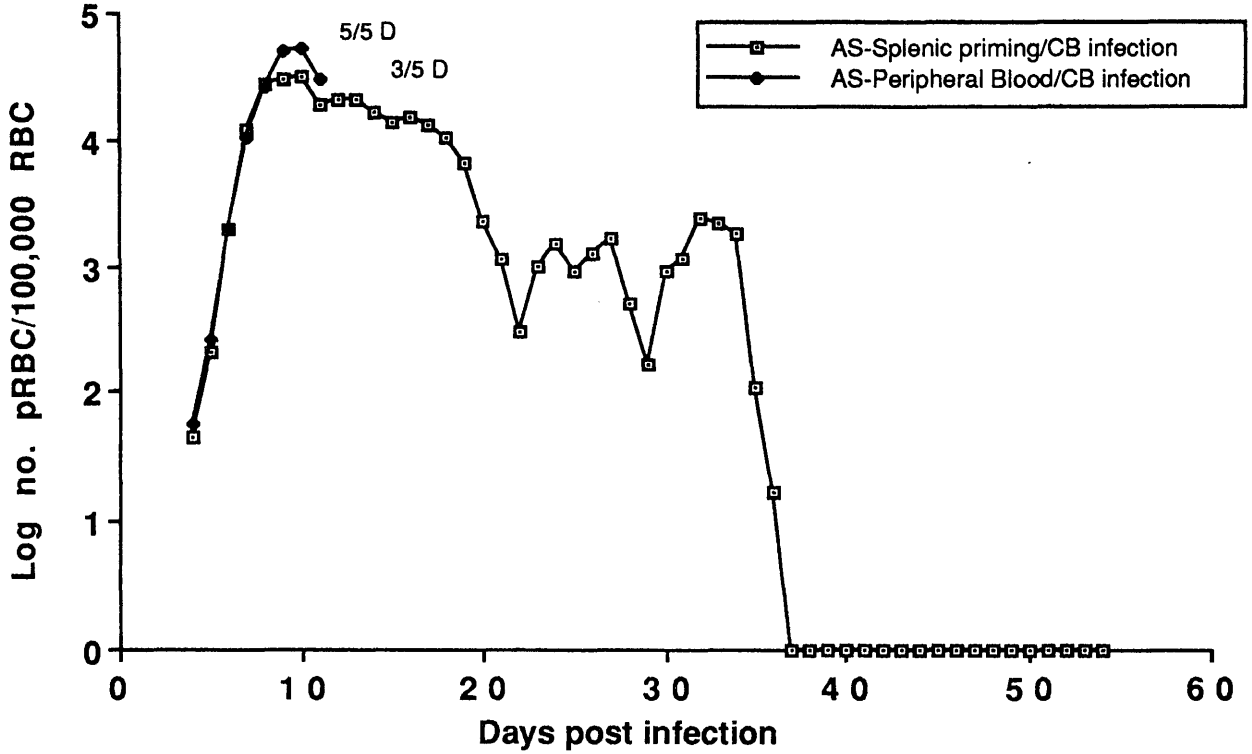

Fig. 6.2.6 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi AS-primed semi-immune lymphocytes challenged with 1 x10^5 heterologous CB pRBC i.v..

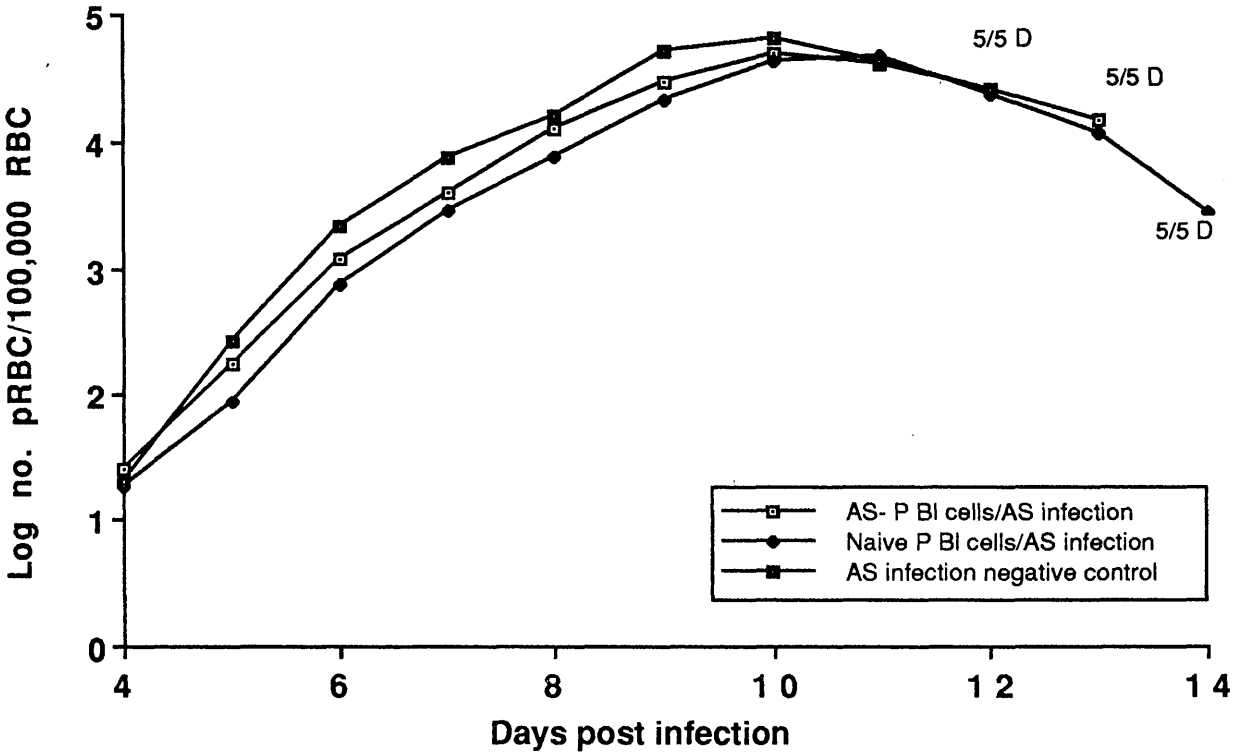

Fig. 6.2.7 Course of infection in sublethally irradiated NIH recipients of peripheral blood lymphocytes challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

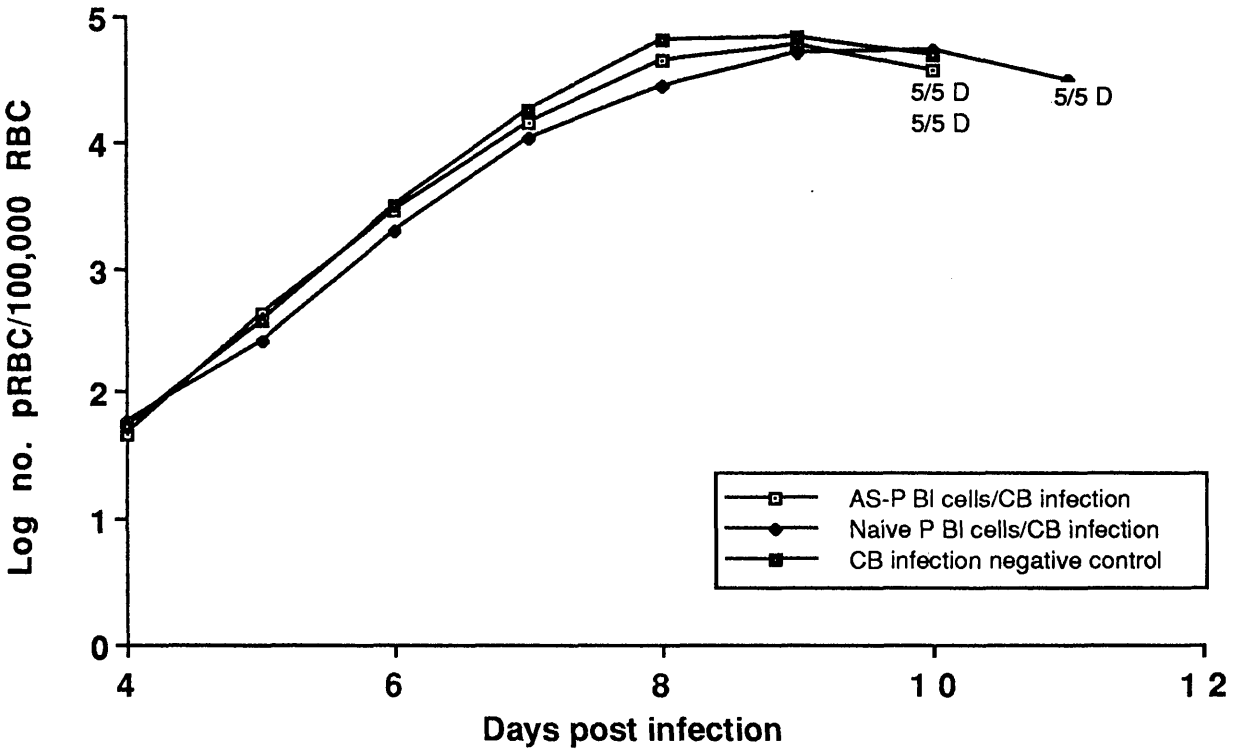

Fig. 6.2.8 Course of infection in sublethally irradiated NIH recipients of peripheral blood lymphocytes challenged with 1 x10^5 P. c. chabaudi CB pRBC i.v..

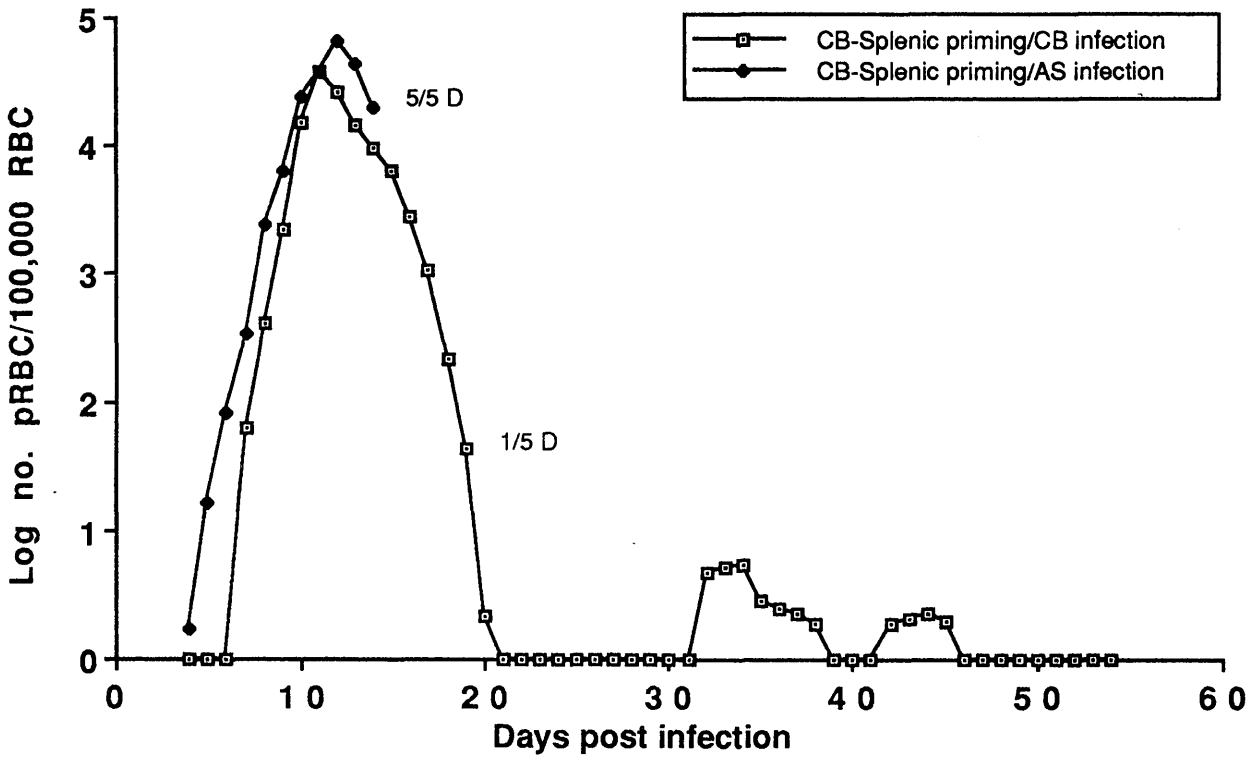

Fig. 6.3.1 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi CB-primed semi-immune splenic lymphocytes challenged with 1 x10^5 AS or CB strain pRBC i.v..

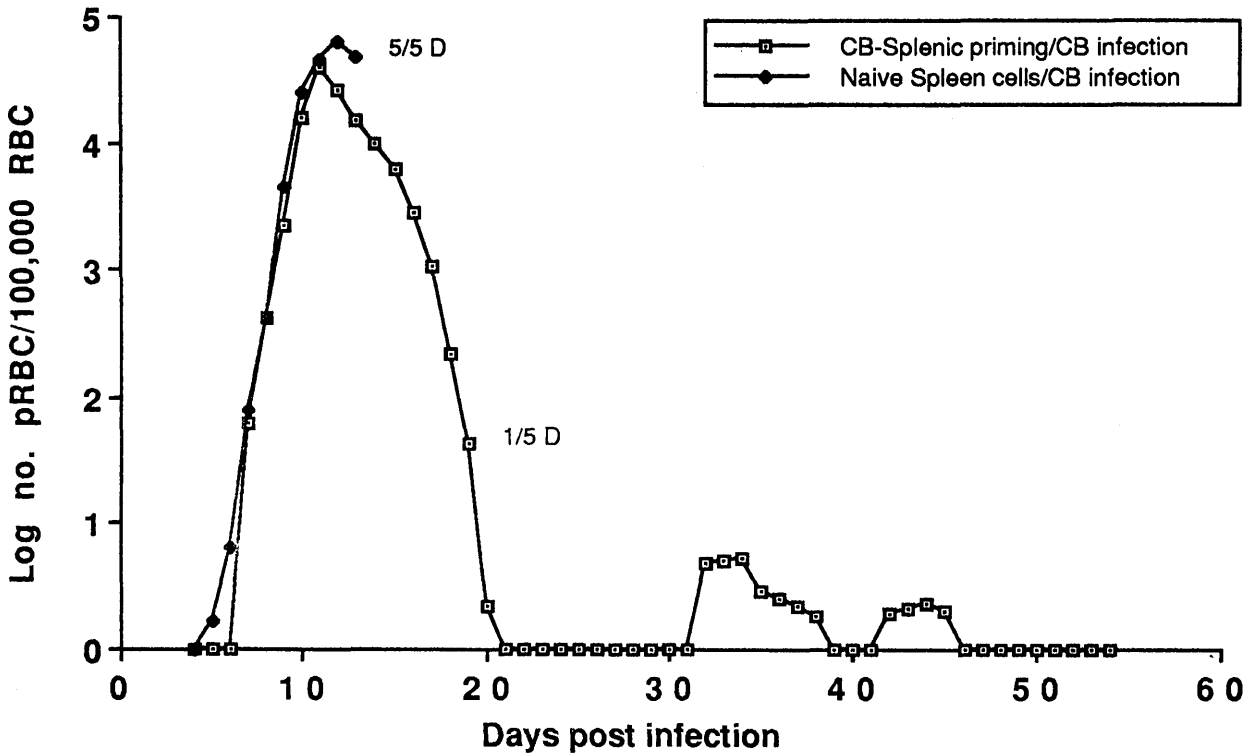

Fig. 6.3.2 Course of infection in sublethally irradiated NIH recipients of splenic lymphocytes challenged with 1 x10^5 P. c. chabaudi CB pRBC i.v..

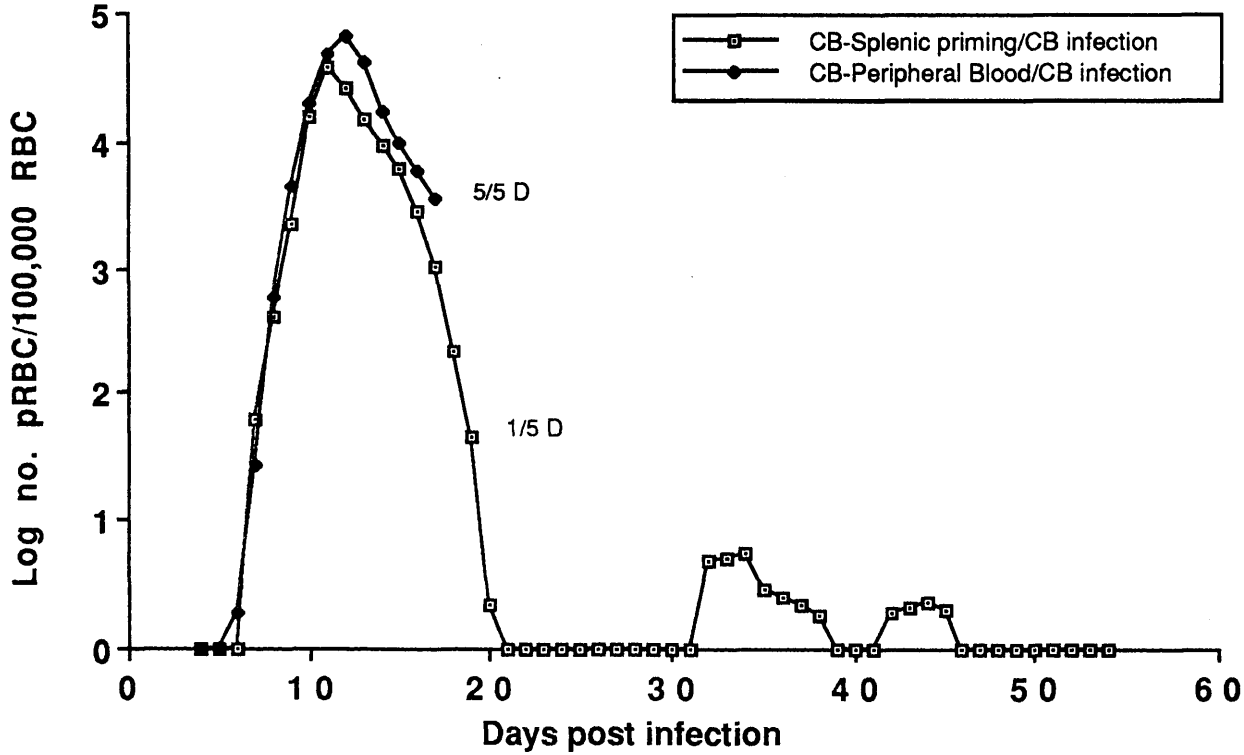

Fig. 6.3.3 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi CB-primed semi-immune lymphocytes challenged with 1 x10^5 homologous CB pRBC i.v..

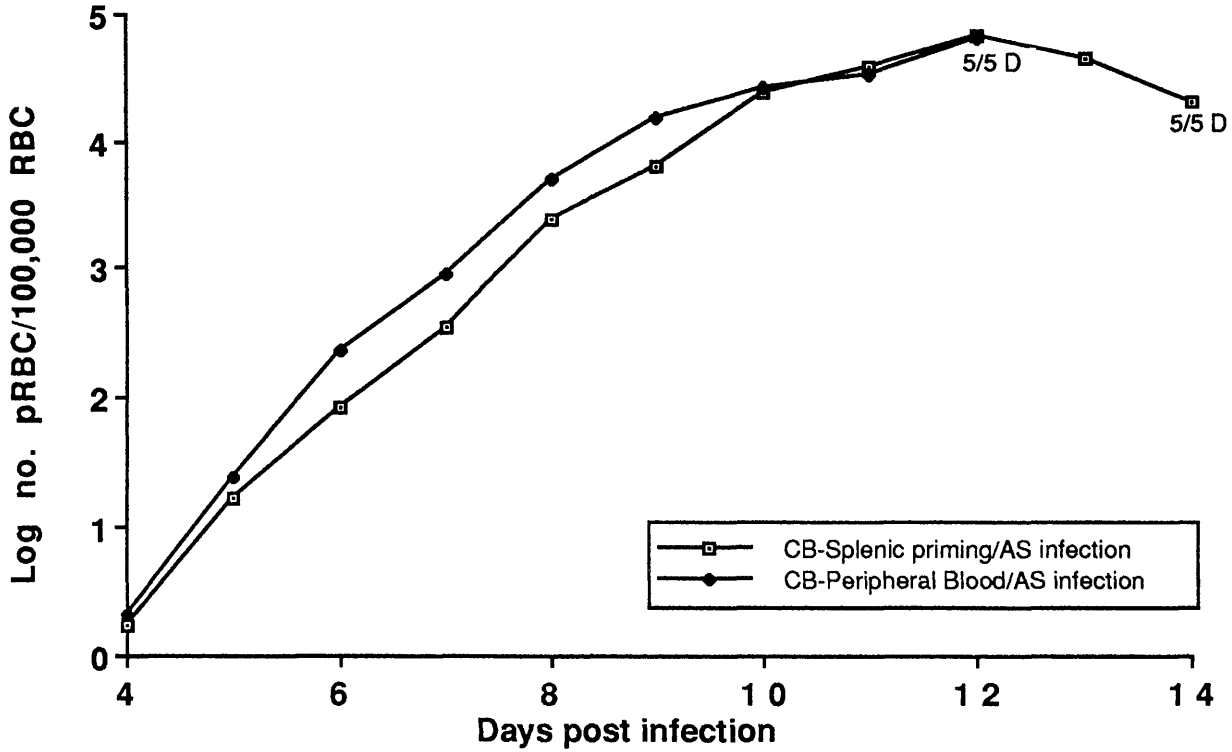

Fig. 6.3.4 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi CB-primed semi-immune lymphocytes challenged with 1 x10^5 heterologous AS pRBC i.v..

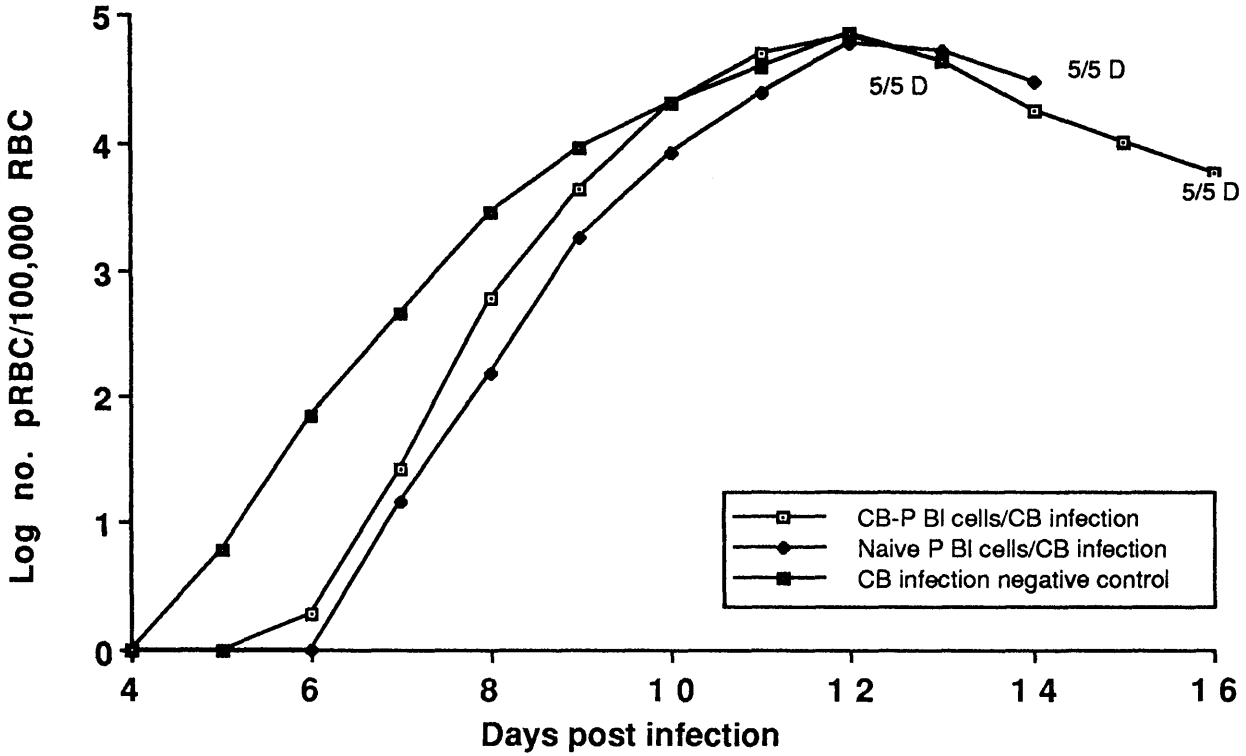

Fig. 6.3.5 Course of infection in sublethally irradiated NIH recipients of peripheral blood lymphocytes challenged with 1 x10^5 P. c. chabaudi CB pRBC i.v..

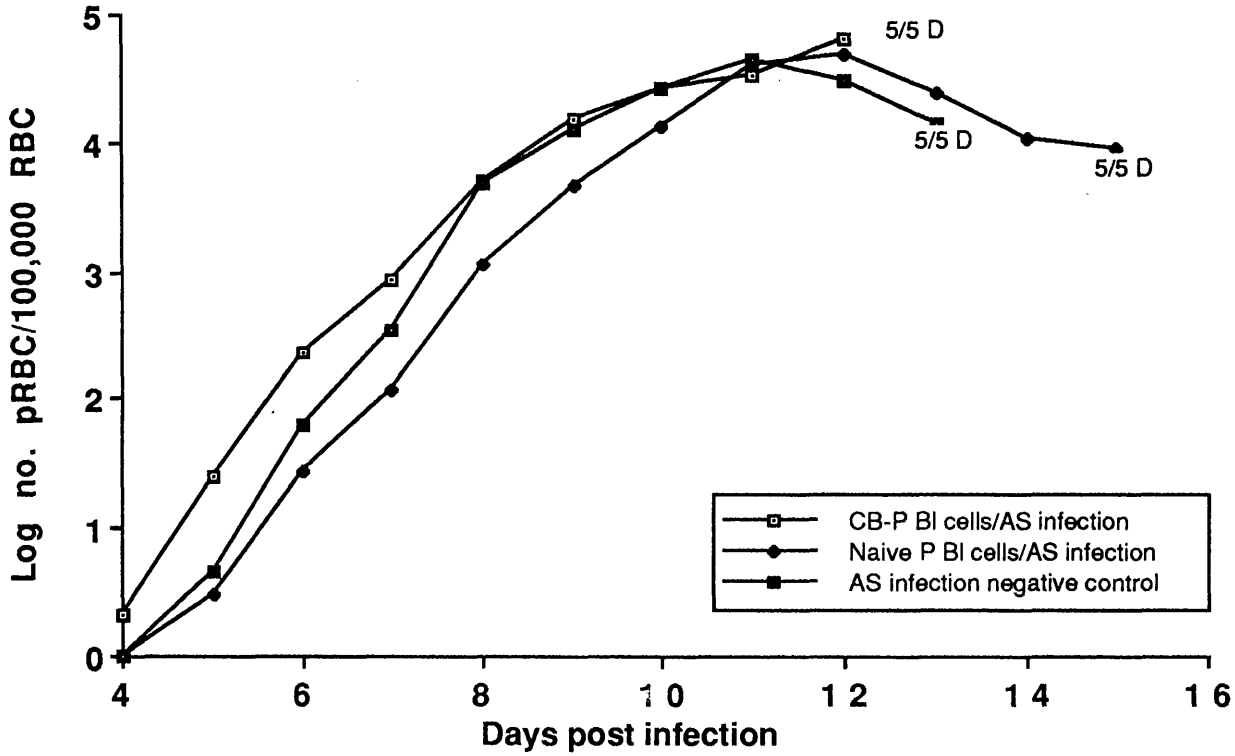

Fig. 6.3.6 Course of infection in sublethally irradiated NIH recipients of peripheral blood lymphocytes challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

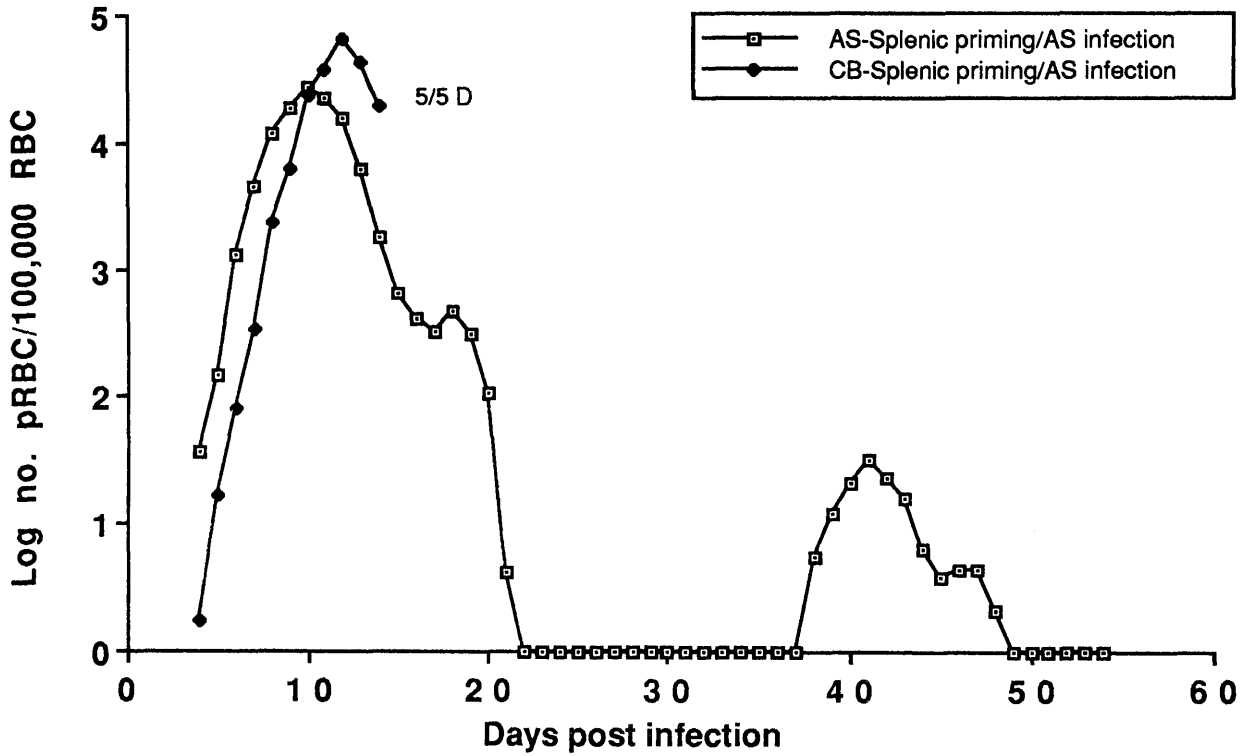

Fig. 6.3.7 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi AS- or CB-primed semi-immune splenic lymphocytes with 1 x10^5 AS strain pRBC i.v..

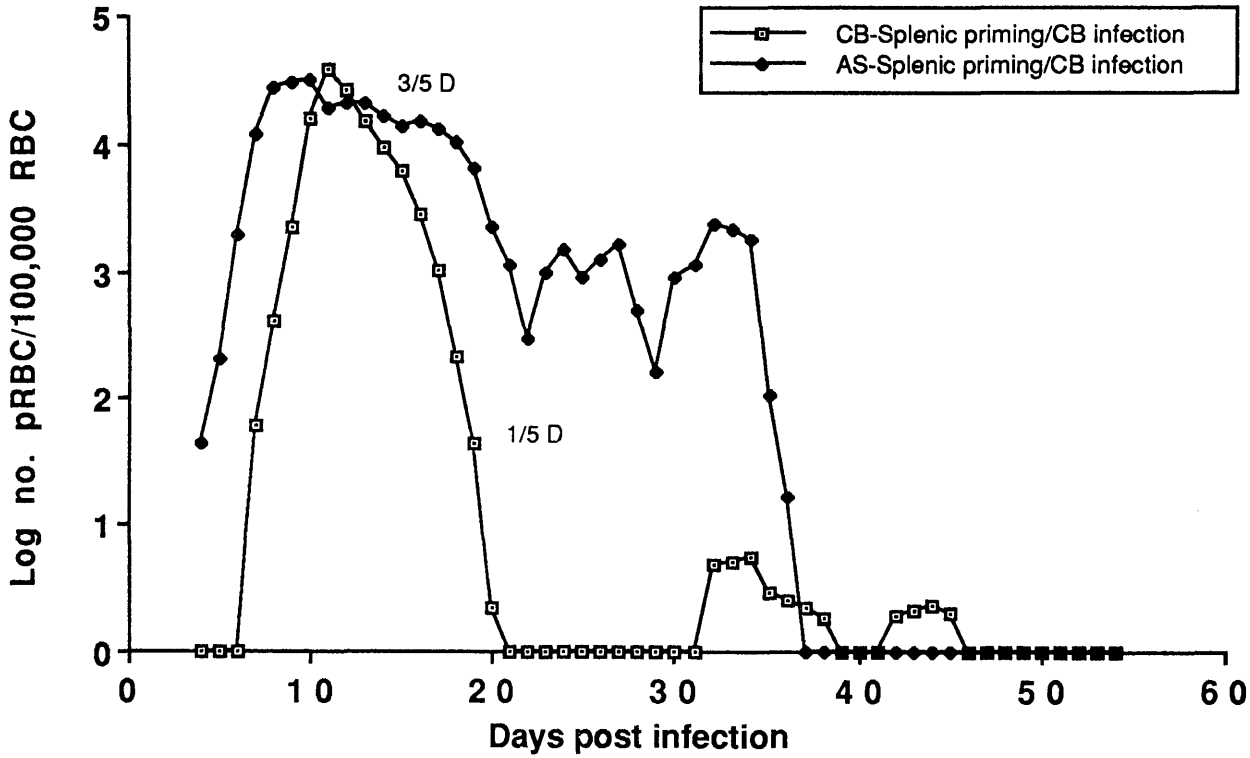

Fig. 6.3.8 Course of infection in sublethally irradiated NIH recipients of P. c. chabaudi AS- or CB-primed semi-immune splenic lymphocytes with 1 x10^5 CB strain pRBC i.v..

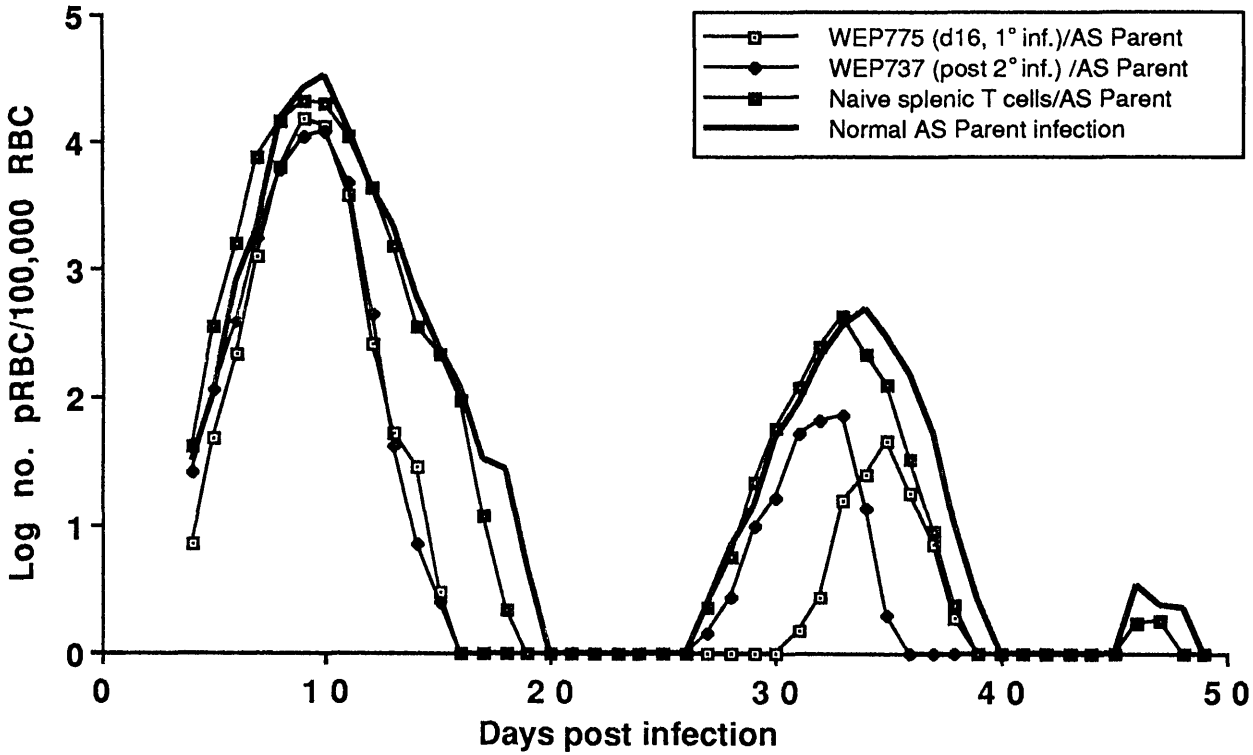

Fig. 6.4.1 Course of infection in naive NIH recipients of P. c. chabaudi AS parent-specific T lymphocyte lines challenged with 1 x10^5 AS parent pRBC i.v..

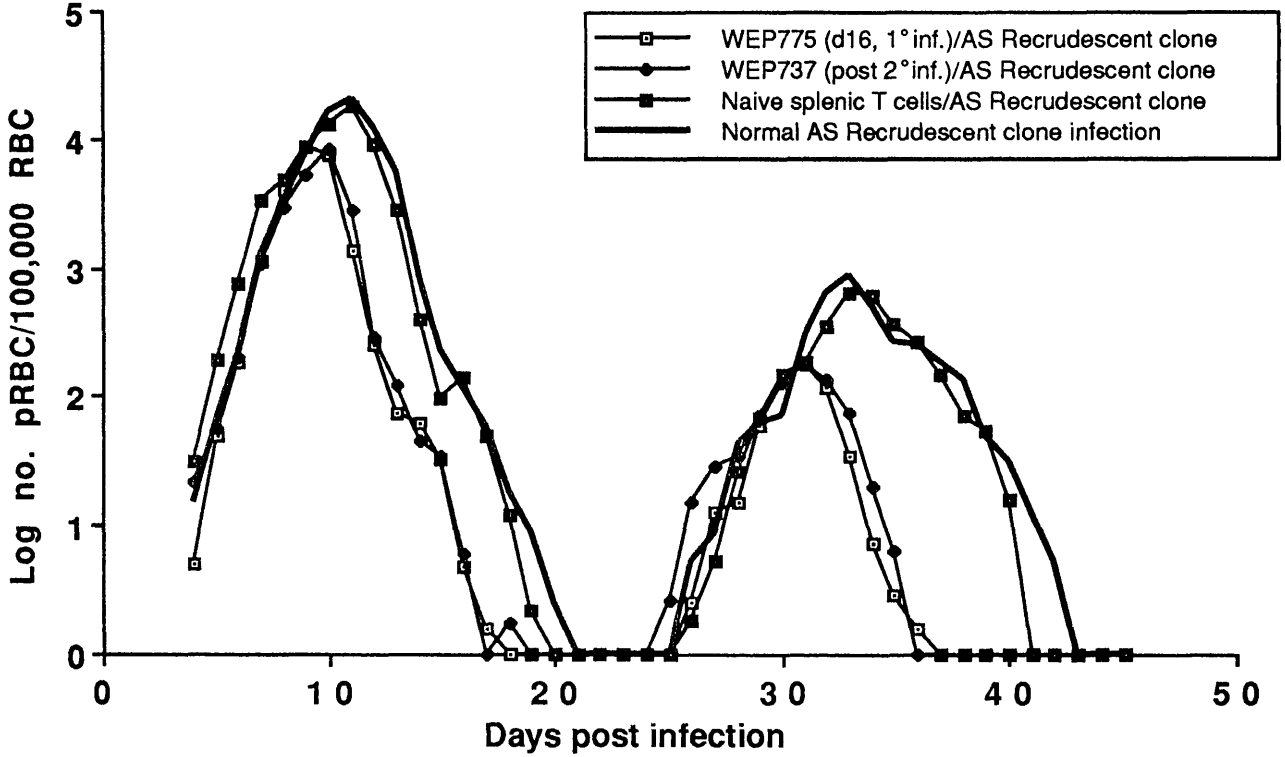

Fig. 6.4.2 Course of infection in naive NIH recipients of P. c. chabaudi AS parent-specific T lymphocyte lines challenged with 1 x10^5 AS recrudescent clone pRBC i.v..

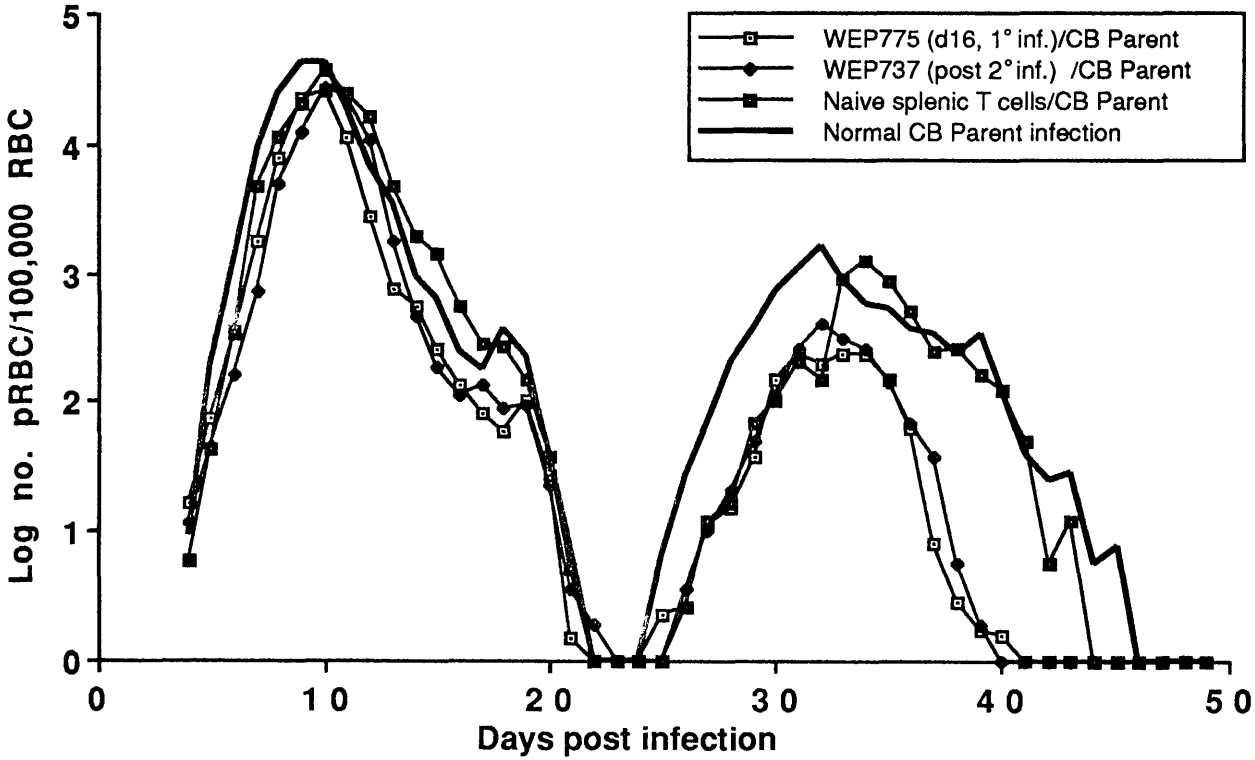

Fig. 6.4.3 Course of infection in naive NIH recipients of P. c. chabaudi AS parent-specific T lymphocyte lines challenged with 1 x10^5 CB parent pRBC i.v..

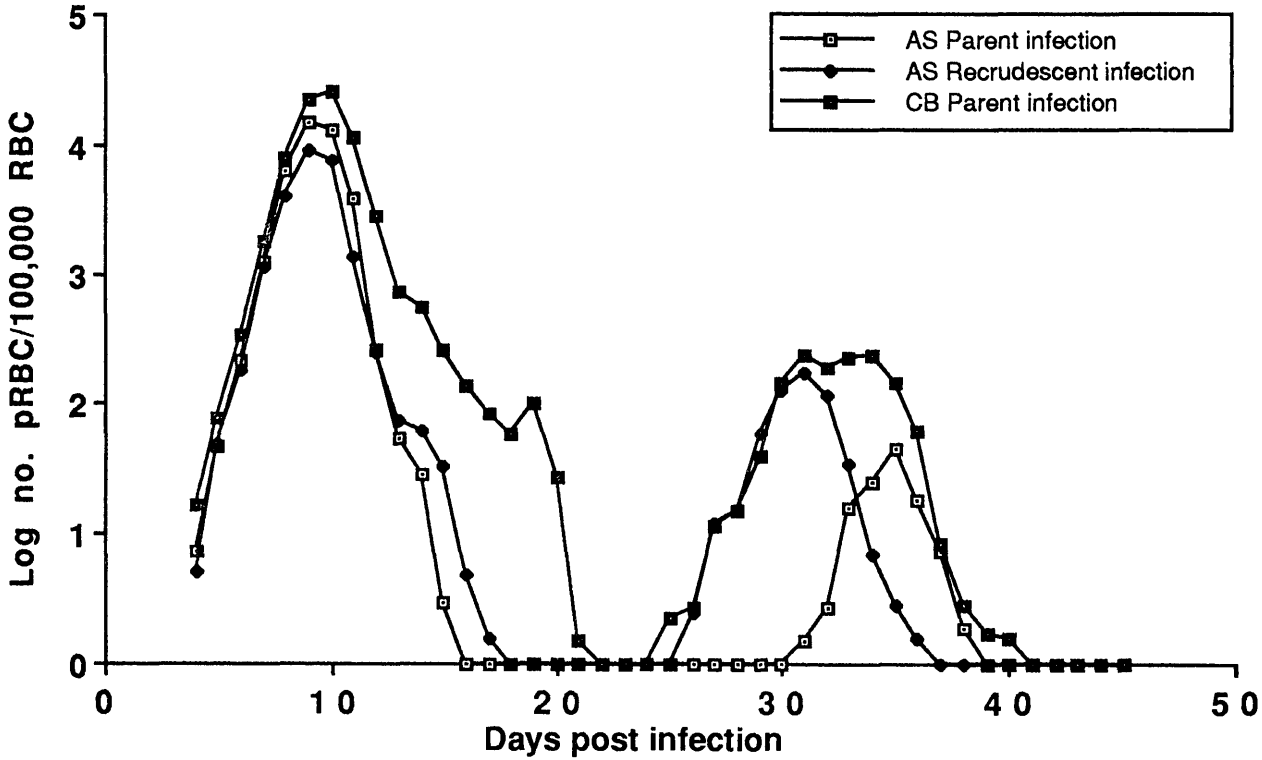

Fig. 6.4.4 Courses of infection in naive NIH recipients of the P. c. chabaudi AS-specific T lymphocyte line WEP 775 challenged with 1 x10^5 pRBC i.v..

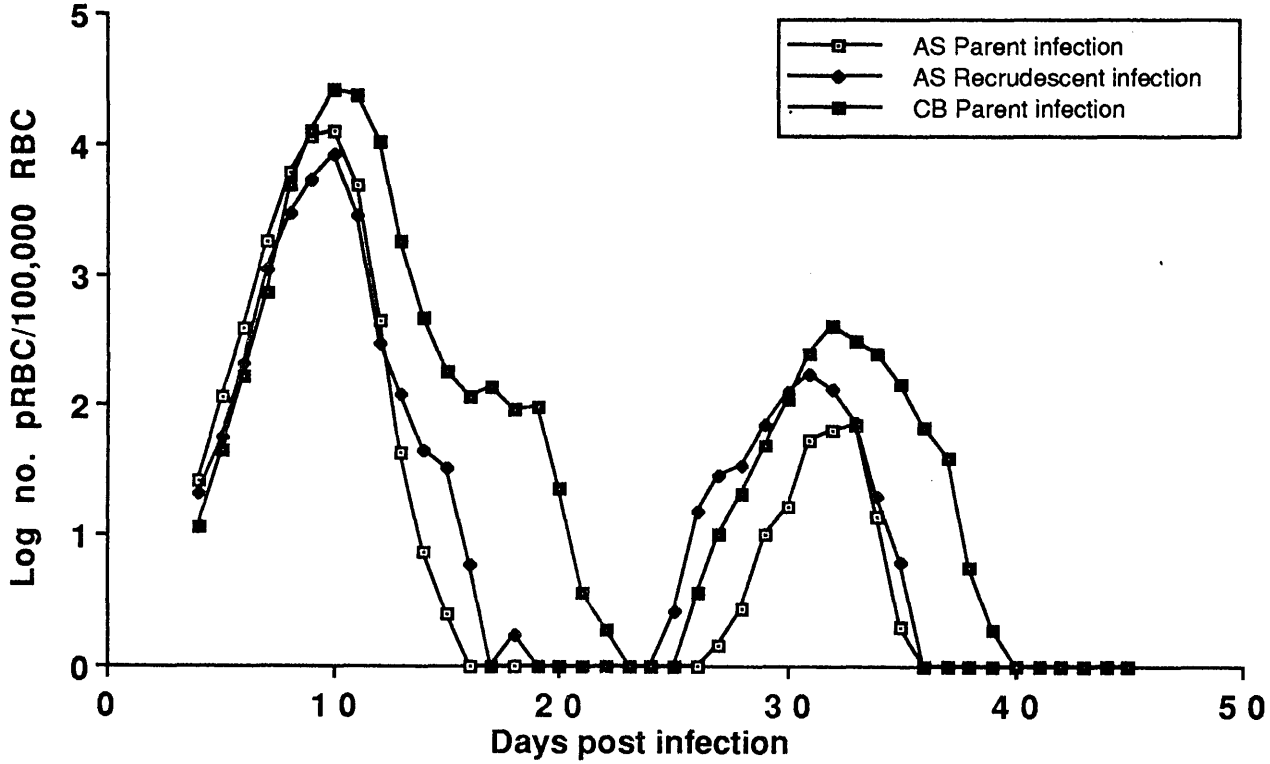

Fig. 6.4.5 Courses of infection in naive NIH recipients of the P. c. chabaudi AS-specific T lymphocyte line WEP 737 challenged with 1 x10^5 pRBC i.v..

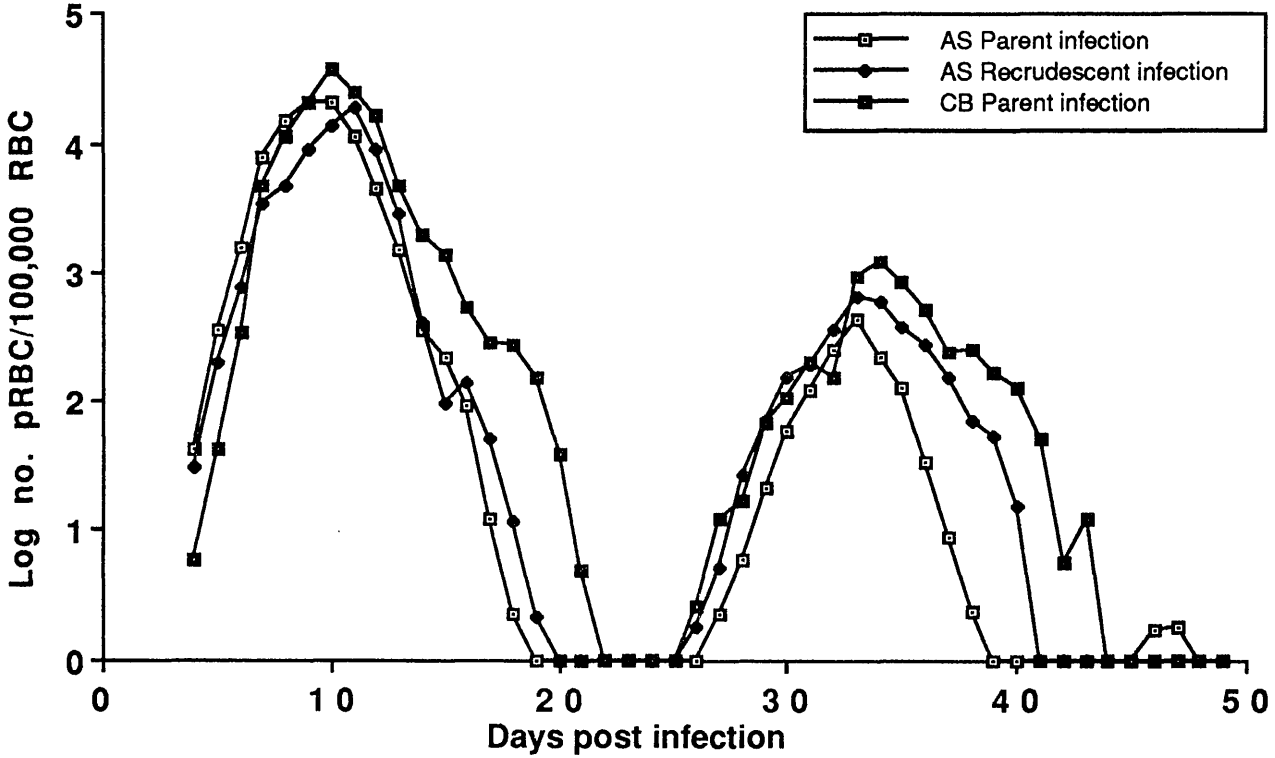

Fig. 6.4.6 Courses of infection in naive NIH recipients of naive splenic T lymphocytes challenged with 1 x10^5 pRBC i.v..

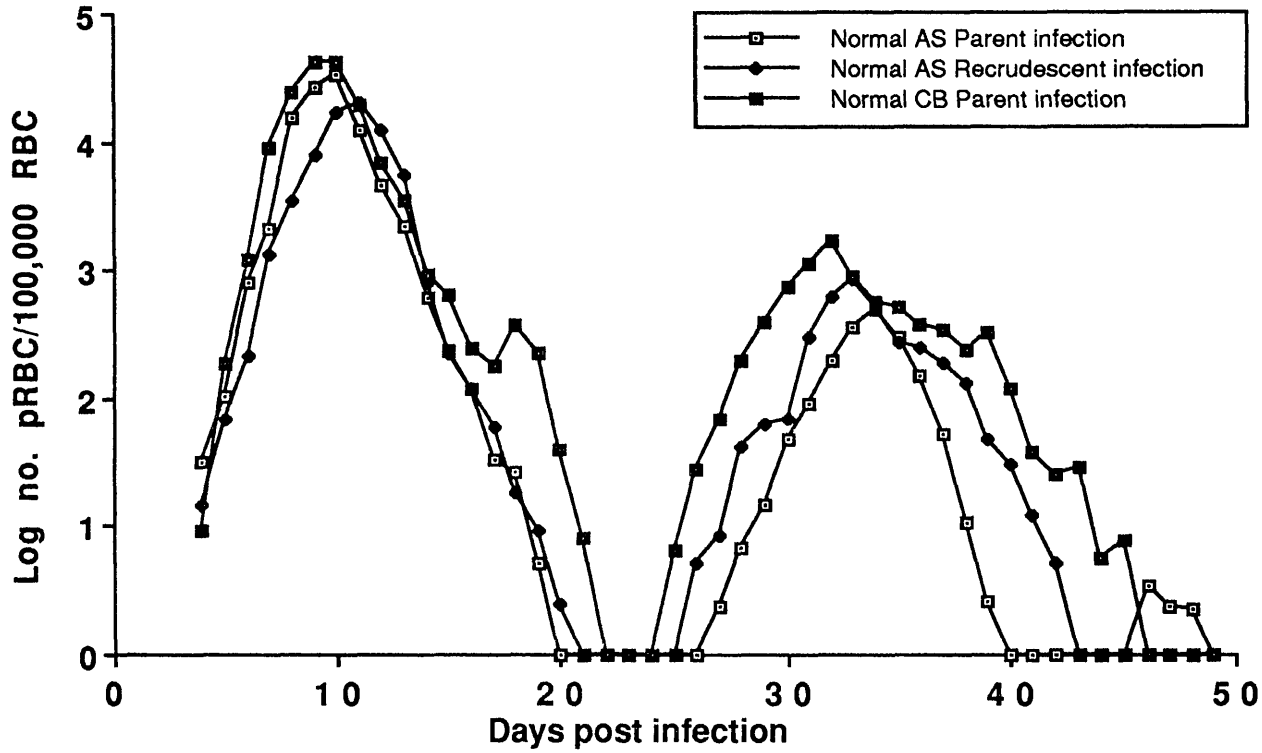

Fig. 6.4.7 Course of infection in naive NIH negative control mice challenged with 1 x10^5 P. c. chabaudi pRBC i.v..

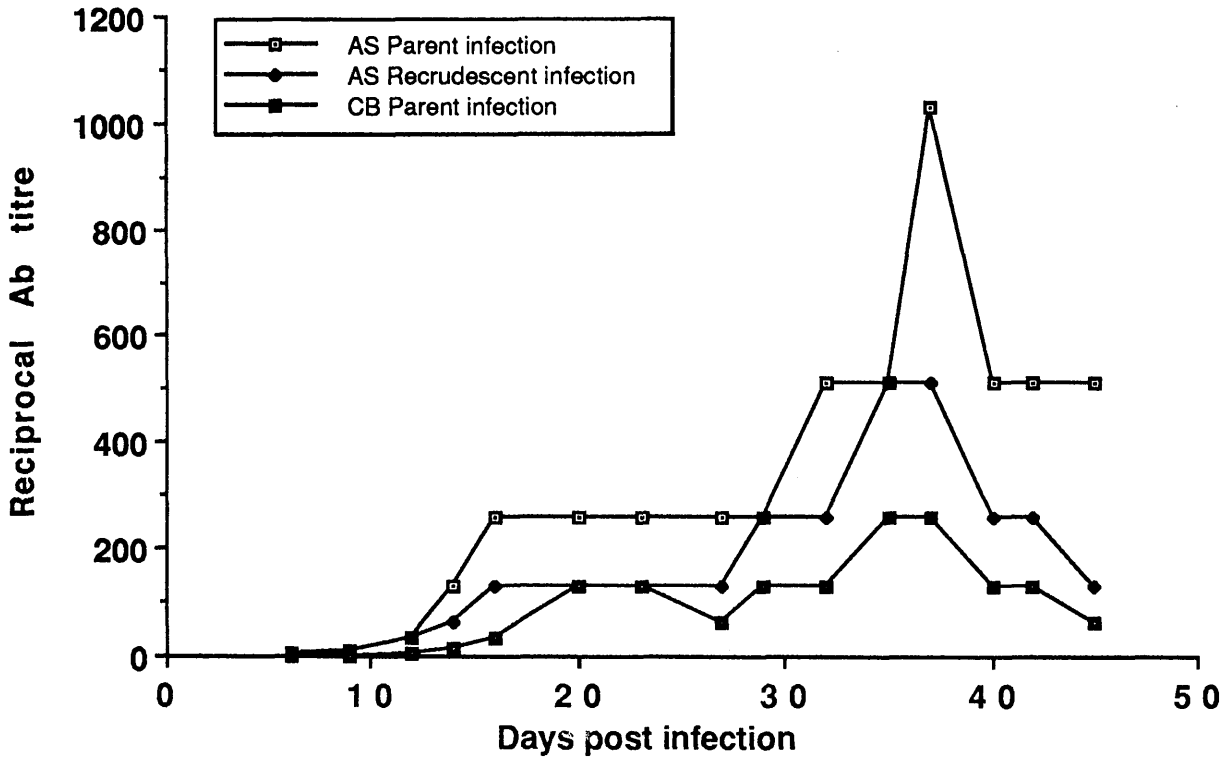

Fig. 6.4.8 Serum antibody titres during the course of infection in naive NIH recipients of WEP 775 challenged with 1 x10^5 P. c. chabaudi pRBC i.v..

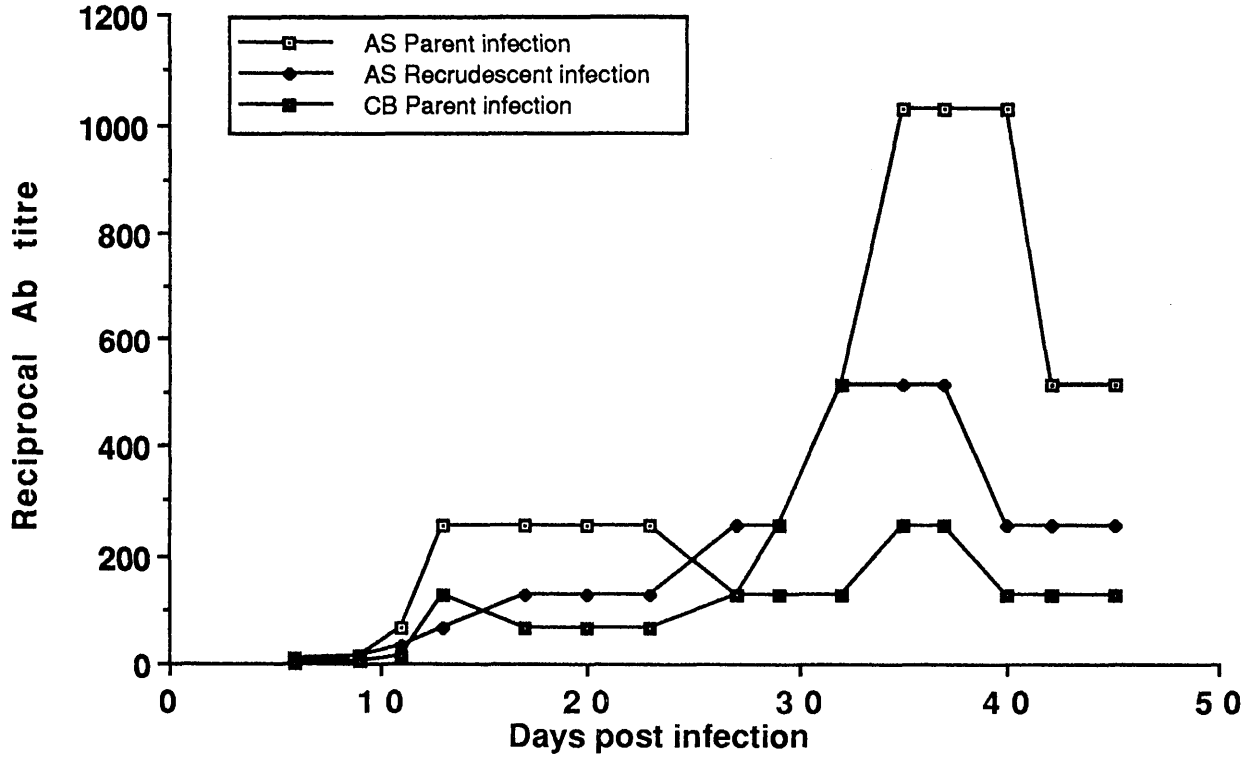

Fig. 6.4.9 Serum antibody titres during the course of infection in naive NIH recipients of WEP 737 challenged with 1 x10^5 P. c. chabaudi pRBC i.v..

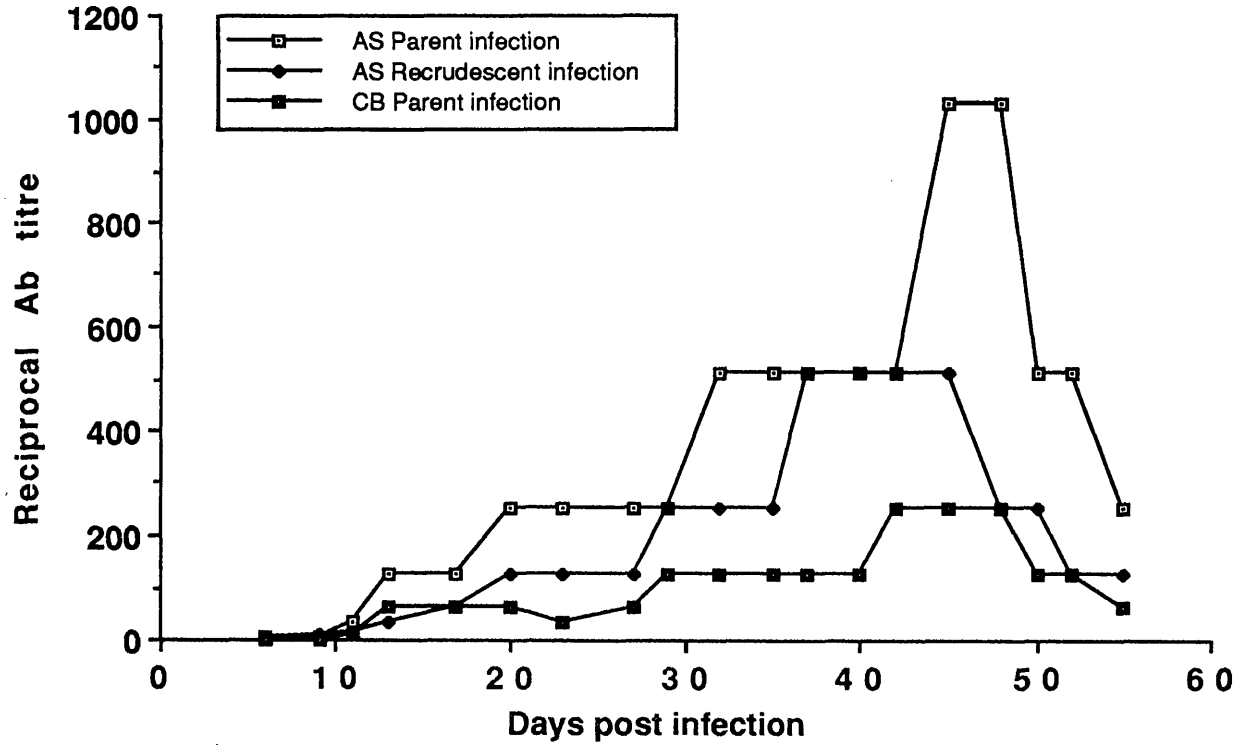

Fig. 6.4.10 Serum antibody titres during the course of infection in naive NIH recipients of naive splenic T lymphocytes challenged with 1 x10^5 P. c. chabaudi pRBC i.v..

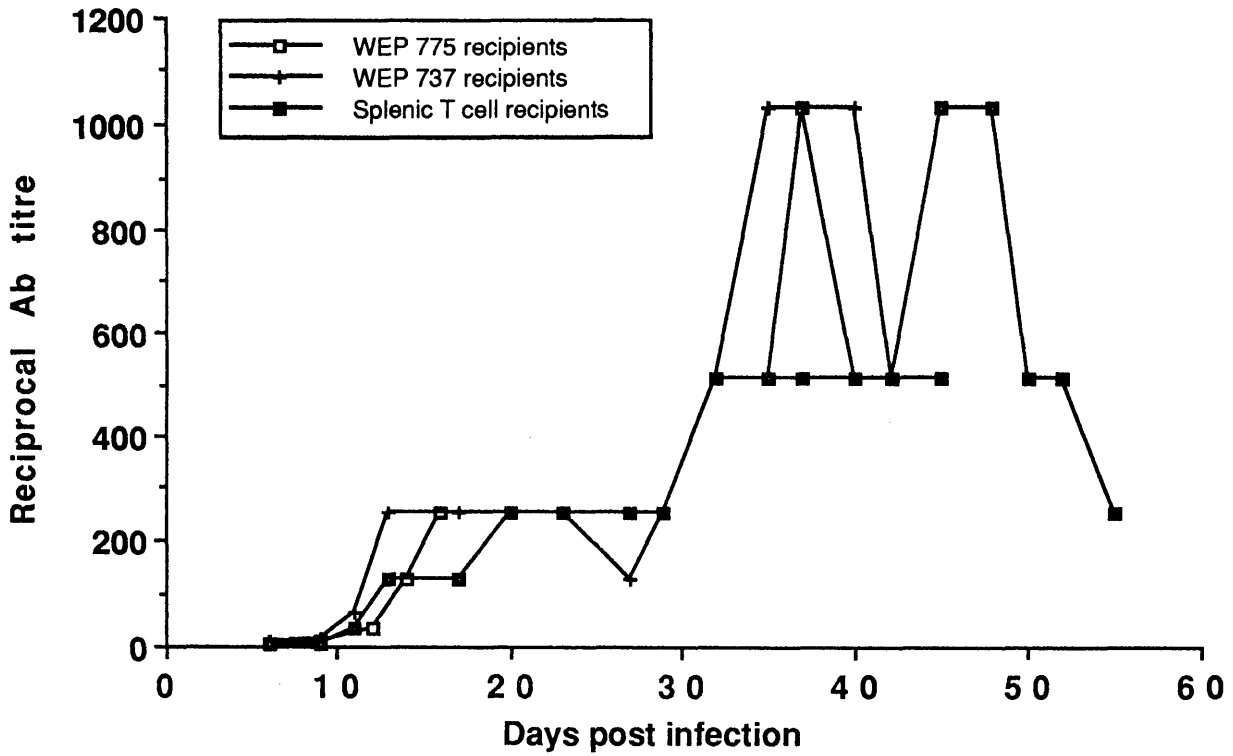

Fig. 6.4.11 Serum antibody titres during the course of infection in naive NIH recipients of P. c. chabaudi AS-specific T lymphocyte lines challenged with 1 x10^5 AS pRBC i.v..

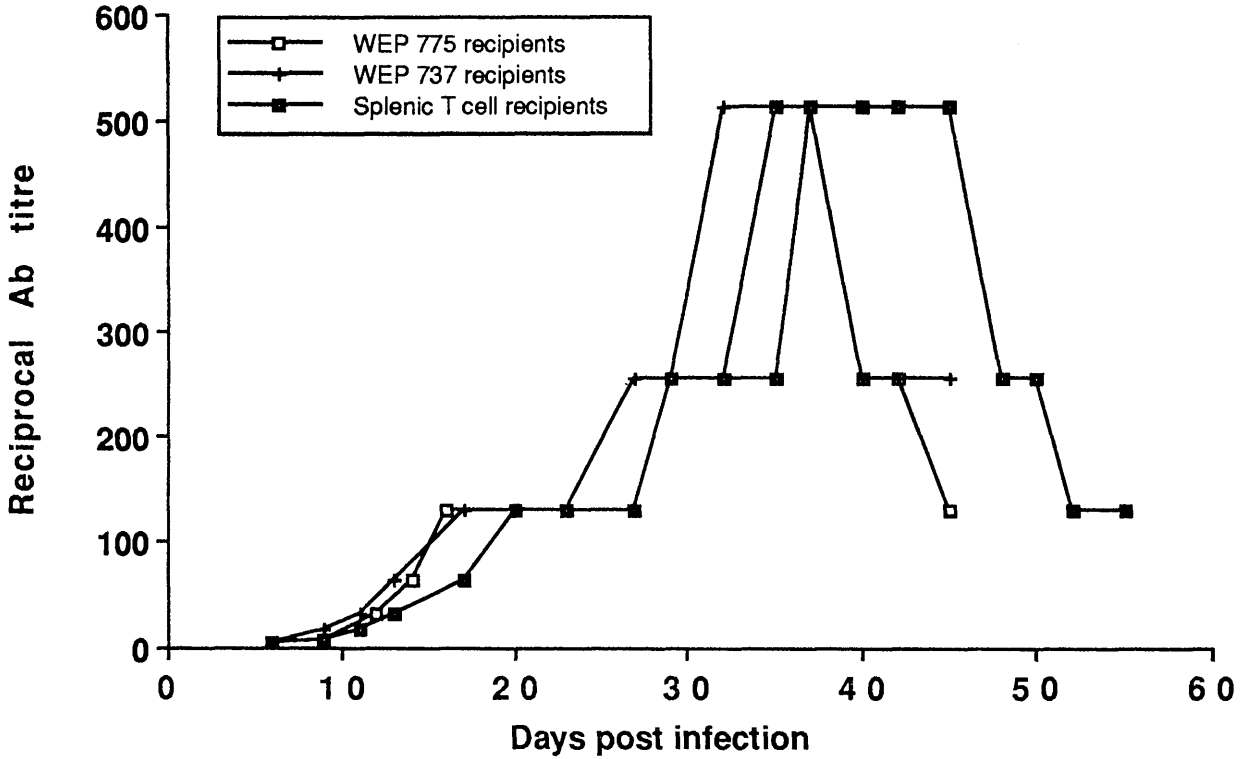

Fig. 6.4.12 Serum antibody titres during the course of infection in naive NIH recipients of AS parent-specific T lymphocyte lines challenged with 1 x10^5 AS recrudescent clone pRBC i.v..

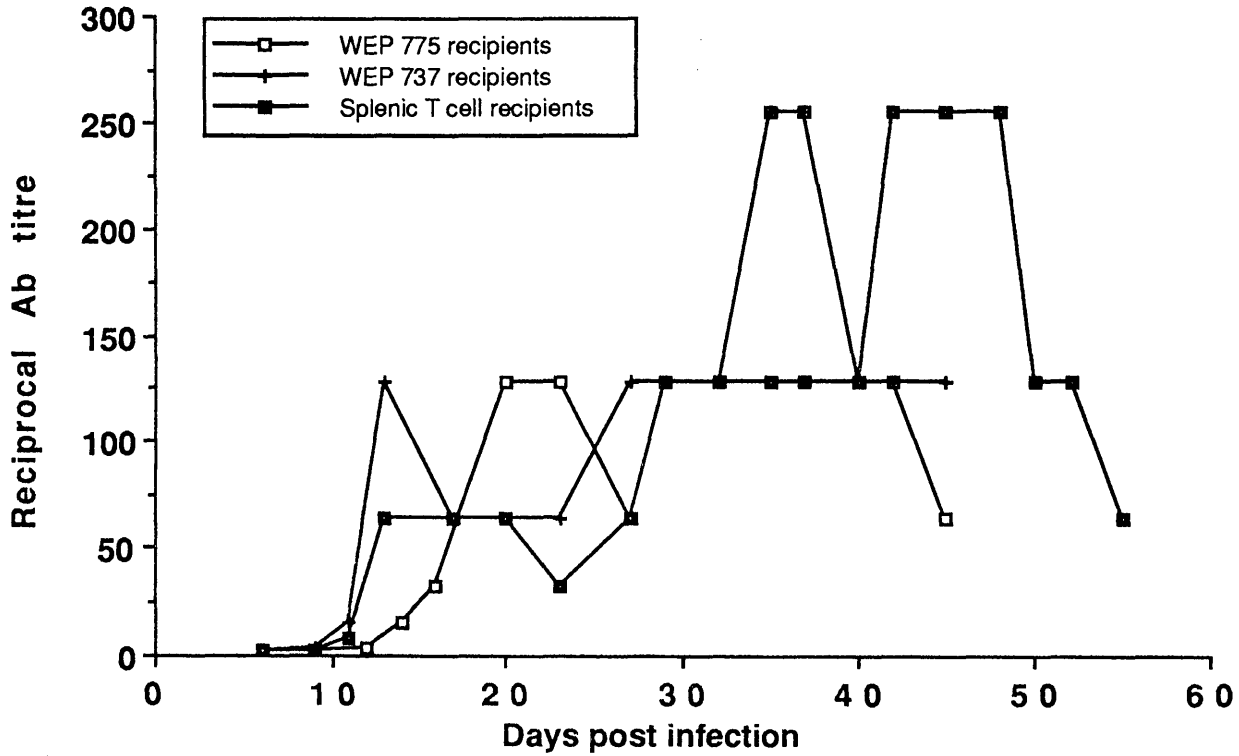

Fig. 6.4.13 Serum antibody titres during the course of infection in naive NIH recipients of AS parent-specific T lymphocyte lines challenged with 1 x10^5 CB parent pRBC i.v.

CHAPTER SEVEN

ADOPTIVE TRANSFER OF T CELL LINES TO IMMUNOCOMPROMISED SYNGENEIC RECIPIENTS

7.1 Introduction

There is now a substantial weight of evidence derived from studies utilising animal models with thymic defects either induced experimentally or congenic in origin showing that development of a protective immune response to Plasmodium requires the presence of T lymphocytes. For example, neonatal thymectomy (Brown et al 1968 a, Stechschulte 1969), treatment with anti-thymocyte serum (Spira et al 1970) or the use of athymic nude mice (Clark & Allison 1974, Weinbaum et al 1976 b) prevented the development of resistance to either P. berghei or P. yoelii. As both these species of Plasmodium appear to be susceptible to Ab-mediated mechanisms of immunity, as determined by passive immunisation studies (Diggs & Osler 1969), these findings collectively gave rise to the concept that T cells have a helper role in the production of protective Abs and led Brown (1971) to hypothesise that helper T cells aid B cells in the synthesis of asexual blood stage variant-specific protective Abs.

In addition, accumulating evidence points to additional effector roles for T lymphocytes which can act in conjunction with, or independently of, anti-malarial Abs. Observations supporting this concept include: the ability of B cell-deficient hosts to resolve malaria infection spontaneously or to resist reinfection by both avian (Ferris et al 1973, Rank & Weidanz 1976) and murine (Grun & Weidanz 1981, 1983) parasites; and the reduced effectiveness of passively transferred immune sera to protect splenectomised or T cell-deprived recipients against infection (Brown & Phillips 1974, Jayawardena et al 1977).

Another line of evidence supporting cell-mediated immunity to malaria comes from the effective transfer of immunity using specific T cells from immune animals. The earlier adoptive transfer studies mostly involved the transfer of lymphoid cells from immune to non-immune but immunologically competent recipients (Roberts & Tracey-Patte 1969, Phillips 1970, Kasper & Alger 1973, Gravely & Kreier 1976). This made it difficult to differentiate between the protective activities provided by the transferred lymphocytes alone and that resulting from the cooperation between these and the recipients' intact immune system. The contribution of the latter can be reduced by exposure to gamma-irradiation. Thus, in more recent studies, either congenitally T cell-deficient nude mice (Brinkmann et al 1985, Cavacini et al 1986) or recipients rendered T cell-deficient by adult thymectomy and irradiation (McDonald & Phillips 1978) have been used. These experiments revealed that immunity to P. c. chabaudi, P. c. adami and P. yoelii was best transferred with primed splenic T cells of the Ly-4$^+$

subset, but not with immune B cells.

Such studies have been carried further by Brake et al (1986), who established in vitro Ly-4^+ cell lines taken from mice immune to P. c. adami. When these cultured cells were adoptively transferred to nude mice, recipients were capable of surviving homologous parasite challenge. Since the cell lines possessed protective activity, they were used to develop Ly-4^+ clones, one of which transferred protection adoptively (Brake et al 1988). This reasoning was behind the similar methodology described in this thesis for the in vitro maintenance of Ly-4^+ T cell lines primed to P. c. chabaudi AS (Chapter 4) and their adoptive transfer to immunocompetent syngeneic recipient animals (Chapter 5). Having shown that each of the Ly-4-bearing populations conferred protection to naive host mice upon homologous challenge, further examination of the mechanisms of acquired immunity necessitated the use of recipients having a relative absence of endogenous T cells. The complete failure of athymic mice to develop a functioning thymus during ontogeny (Wortis et al 1971) or to exhibit T cell-mediated responses recommends this model for the study of cellular immunity to malaria. A deficiency in T lymphocytes makes these animals very susceptible to P. c. chabaudi and infections with this parasite, like those of P. c. adami, are lethal. It would be thought, therefore, that nude mice would be ideal to use as recipients for adoptive transfer experiments, as Brake et al (1986, 1988) had found. However, in this particular instance, it was not possible to use these athymic mutants because of their histoincompatability to the transferred cell lines. The MHC disparity between the H-2^q haplotype of the Ly-4^+ cells to be transferred and the H-2^d haplotype of the BALB/c nude mice would mean a failure of the allogeneic lymphocytes to survive upon injection (Kindred & Shreffler 1972, Kindred & Loor 1975). This has been illustrated amply for murine malaria by Brinkmann et al (1985), who were unable to transfer protection to C57BL/6 nude mice by injecting spleen cells from immune C57BL/10 mice. Their inability to adoptively transfer protection was thought to have been caused by the fact that the donor and recipient mice were not syngeneic, even though they were congenic for MHC genes.

In a similar manner, the recently developed murine SCID mutation would prove unsuccessful as this arose in the C.B-17 inbred strain of mouse, a congenic partner of BALB/c (Ansell & Bancroft 1989). To date, only preliminary adoptive transfers in SCID mice (lacking both functional T and B lymphocytes) with Ly-4^+ cells have been performed (Meding, S.J. & Langhorne, J., unpublished results).

In the experiments described in this chapter, immunologically crippled NIH mice were

prepared as recipients of syngeneic Ly-4$^+$ cell line inocula. Thymectomy followed by lethal irradiation and bone marrow stem cell replacement created recipients that could remain T cell-deficient indefinitely (Loveland et al 1981). This was thought a satisfactory model for T cell depletion despite the unphysiological intervention required. With care, the uncertainty regarding incomplete thymus ablation was reduced to a minimum. In such a situation, the enormous potential for a small number of T lymphocytes to expand in vivo (Bell et al 1987) in T cell-deficient animals was beneficial.

7.2 Adoptive transfer of T cell lines to adult-thymectomised, lethally irradiated & bone marrow-reconstituted syngeneic recipients

NIH female mice were thymectomised at four to five weeks of age (2.15). Four weeks later, they were subjected to 7.5 Gy gamma irradiation and injected with 5 x10^7 syngeneic bone marrow cells (2.16). The T cell-deprived animals so obtained were experimentally infected a further month afterwards when they were approximately 12 weeks old.

Each of the four established Ly-4$^+$ cell lines used for adoptive transfer was propagated in vitro after recovery from cryopreservation and harvested for reconstitution 3-4 d after subculturing. For each line, lymphocytes were adoptively transferred to five age-matched T cell-deprived NIH female mice. The inoculum used was 4.0 x10^7 viable cells, except for WEP 723, in which case 3.54 x10^7 cells/mouse were injected.

Similar sized control groups were set up to control for the in vivo manipulation of the immune system of the immunocompromised recipient hosts. These included both thymectomised and sham-thymectomised recipients of a population of syngeneic splenic T & B cells in place of a specific Ly-4$^+$ line, and also T cell-depleted and sham-depleted mice, which were reconstituted with bone marrow cells after irradiation but without further adoptive transfer. In addition, a non-thymectomised control was prepared; these mice were lethally irradiated and B cell-reconstituted but not surgically interfered. This acted as a negative control for the surgical trauma experienced by sham-thymectomised mice.

In summary, all mice receiving Ly-4$^+$ lymphocytes were T cell-deprived, whilst of control mice, some were also adult-thymectomised, lethally irradiated and bone marrow-reconstituted, some were sham-thymectomised whilst others were not thymectomised. These latter controls did, however, experience irradiation treatment

and B cell reconstitution. All mice in this experiment were inoculated i.v. with 1 $x10^5$ of a parent population of P. c. chabaudi AS pRBC.

The protection conferred by adoptive transfer of each of the four Ly-4^+ lines upon P. c. chabaudi AS challenge of T cell-depleted recipients is shown in Figs. 7.2.1 & 2. For the lines taken from infected donor mice on d 16 or d 20 of primary infection, WEP 775 and WEP 779, respectively, there was a clearly observable protective immunity given by the inoculation of these semi-immune lymphocytes (Fig. 7.2.1). This was seen as a lower peak primary parasitaemia, a significantly quickened remission to subpatency ($p < 0.01$), and a significantly lower recrudescence ($p < 0.01$), all compared to a control group of similarly T cell-depleted mice receiving naive splenic T & B cells. In contrast, for those lines derived from mice recovered from either a secondary or tertiary P. c. chabaudi AS infection, WEP 737 and WEP 723, respectively, the protection engendered was not nearly so great. Indeed, the courses of infection of recipients of these lines and of splenic T & B lymphocytes were comparable (Fig. 7.2.2). As can be seen from this figure, the pattern of acute infection was similar for all three groups with a comparable level and timing of peak parasitaemia, but a significantly quicker remission for mice inoculated with unprimed T & B cells ($p < 0.05$). Indeed, recipients of WEP 737 actually failed to clear the primary parasitaemia to subpatent levels, whilst those mice given WEP 723 did so only for 1 d. Moreover, the magnitude of the recrudescence was similar for recipients of WEP 723 and of naive splenic lymphocytes.

It would appear that the divergent patterns of parasitaemia attained upon adoptive transfer of these Ly-4^+ lymphocyte populations to immunocompetent recipients (Chapter 5) are enhanced against a background of host T cell depletion. This is most easily appreciated by comparison of the courses of infection of immunocompromised mice given either WEP 775 or WEP 737 (Fig. 7.2.3). It is evident that the Ly-4^+ lines each provided a distinct immune protection, and suggests that in vivo their behaviour fell into two classes. For WEP 737, the protection engendered under these circumstances was no greater than that given by a similar number of mixed T & B cells (positive thymectomy control). Figure 7.2.4 compares the patterns of parasitaemia of WEP 775 and of WEP 737 recipients with that in mice receiving no additional lymphocytes after B cell reconstitution (negative thymectomy control). For the latter group, four of five mice failed to control infection and in the other the chronic high parasitaemia resolved only on d 39 p.i.. This served to show the necessity for repopulation of the immune system with syngeneic T lymphocytes. These would be

ideally those with previous experience of P. c. chabaudi AS, but this was not essential as shown by the resistance to infection acquired by thymectomised recipients of unprimed spleen cells (Fig. 7.2.3).

Examination of the courses of infection in mice thymectomised before challenge compared to their sham-thymectomised counterparts is very revealing (Fig. 7.2.5). Mice that were sham-thymectomised, then lethally irradiated and bone marrow-reconstituted, but without further adoptive transfer, i.e. the sham controls for the negative thymectomy group, showed similar levels of parasitaemia as did the non-reconstituted thymectomised animals, also with some fatalities. These mice contained an intact thymus, and therefore presumably a full complement of mature T lymphocytes, yet were equally unable to clear P. c. chabaudi AS infection a month after lethal irradiation and B cell reconstitution as were the thymectomised mice. As the peripheral blood population of T cells would have recovered to normal levels during the time between immunosuppression and parasite challenge, it would suggest that the failure of the thymectomised animals (negative thymectomy control) to curb infection could not be due directly to their lack of T cells caused by thymectomy, as one may at first have suspected by studying Fig. 7.2.4. Confirmation of this comes from the sham positive thymectomy mice, i.e. the sham equivalents of those mice receiving splenic T & B cells after T cell depletion. Although the acute infection was of higher peak parasitaemia and reached subpatency 4 d later than did that for the sham-thymectomised mice, thymectomised recipients of splenic lymphocytes at the time of challenge thereafter showed identical kinetics of infection as did their sham equivalents (Fig. 7.2.5). Thus, the recrudescent phase of infection did not appear to be dependent on the presence of a full T cell complement. This is suggestive of the resolution of recrudescence leading to parasite clearance being a B cell-dependent phenomenon, and not necessarily requiring the presence of T cells to function. As there was a clear difference during the early part of the infection between thymectomised and sham-thymectomised mice (Fig. 7.2.5), it would appear that the mechanisms of immunity involved in control of the primary parasitaemia have a T cell dependency. The inability of both T cell-depleted mice that did not receive a further spleen cell reconstitution upon infection and their sham-thymectomised counterparts to control either the early or late phases of P. c. chabaudi AS infection would appear to be due to a lesion in the B cell compartment of these immunocompromised animals. The presence of T cells in sham control mice did not offset the effects of lethal irradiation, in which peripheral blood and splenic

lymphocytes were removed. In sham mice, the circulation was repopulated with thymus-derived T cells, yet this did not alter the course of the subsequent infection. Both T cell-depleted and sham-depleted mice were still immunoincompetent, even after bone marrow reconstitution. This reflected the relative radiosensitivity of B lymphocytes compared to T lymphocytes; as the 7.5 Gy irradiation dose had to be sufficient to destroy the peripheral blood T cell population, it effectively knocked out the B cell population. The repopulating inoculum of bone marrow stem cells was clearly insufficient to offset the P. c. chabaudi AS infection and mice died, presumably due to a lack of B cell-mediated immune responses.

With regard to the adoptive transfer of the Ly-4$^+$ cell lines (Figs. 7.2.1 & 2), the relative inability of WEP 737 and WEP 723 to control infection (Fig. 7.2.2) compared to WEP 775 and WEP 779 (Fig. 7.2.1) may have been caused by a lack of B lymphocytes in the challenged hosts. In naive recipients of these cell lines, the patterns of parasitaemia also fell into the same two classes, although the protection engendered was essentially similar (Chapter 5). In this case, the recipients used were fully immunocompetent and possessed both B and T lymphocytes, though with no previous exposure to the infecting parasite. Thus, the divergency in response of these lines upon transfer to T cell-depleted recipients may reflect an underlying difference in the mechanisms by which these two groups of Ly-4-bearing lymphocytes mediate anti-malarial activity in vivo.

7.3 Adoptive transfer of T cell lines and additional splenic B cells to adult-thymectomised, lethally irradiated & bone marrow-reconstituted syngeneic recipients

The results of the adoptive transfer study described in 7.2 furthered the findings of Chapter 5 in discriminating between the behaviour of the P. c. chabaudi AS-specific Ly-4$^+$ cell lines in terms of protection against homologous challenge. For the lines taken after multiple infections of donor mice, WEP 737 and WEP 723, there was less protection conferred in T cell-depleted mice than in immunocompetent mice. Analysis of appropriate control groups indicated that this effect was probably due to a lack of B lymphocytes, and, therefore, that these lines mediate protection through B cell help. In contrast, for the lines taken during primary infection of donor mice, WEP 775 and WEP 779, there were similar effects on the two different immunological backgrounds. The retention of activity upon T cell depletion suggested indirectly that these Ly-4$^+$ cells

acted by a mechanism other than B cell activation. This proposed discrepancy between the T cell-dependent mechanisms of immunity utilised by different Ly-4$^+$ populations warranted further investigation. This section describes the adoptive transfer of the Ag-reactive Ly-4$^+$ lines to T cell-deprived recipient mice either alone (as for 7.2) or together with syngeneic splenic B or T & B cells.

As before, prospective recipient NIH female mice were surgically thymectomised, lethally irradiated and reconstituted with 5 x10^7 syngeneic bone marrow cells. Four weeks after immunosuppression, these animals were infected with 1 x10^5 pRBC P. c. chabaudi AS and the course of infection followed. Three groups of six age-matched mice were each inoculated with either WEP 775 or WEP 737; the inoculum used was 4.15 $\pm$ 0.06 x10^7 viable cells/mouse. For each cell line, mice in one group were given these cells alone (as a confirmation of 7.2), whilst mice in the other two groups received additional injections of either 4 x10^7 naive syngeneic splenic B cells or of a mixed T & B population. For these investigations, WEP 775 and WEP 737 were used as representative lines of the Ly-4$^+$ populations derived during primary or after multiple P. c. chabaudi AS infections. However, for the specific case of reconstitution of enriched splenic B cells, WEP 779 and WEP 723 were also adoptively transferred to recipient T cell-deprived mice. To gauge the possibly enhanced protection conferred by these lines in the presence of sufficient B cells, mice receiving 4 x10^7 splenic T cells and 4 x10^7 splenic B cells taken from naive donors were also prepared. In addition, the protection engendered by either of these two lymphocyte populations inoculated alone was also assessed.

The protection conferred by each of the Ly-4-bearing lines upon adoptive transfer with an equal number of naive, syngeneic B cells is shown in Figs. 7.3.1 & 2. It is clear that all four populations engendered an immune reactivity upon reconstitution; this was manifested principally as a reduction of parasitaemia to subpatent levels compared to control T cell-deprived recipients of naive splenic lymphocytes. In this regard, the patterns of parasitaemia were essentially similar to those attained upon transfer to P. c. chabaudi AS-infected naive mice (Chapter 5); this was especially noticeable for WEP 737 and WEP 723, the transfer of which to T cell-deprived recipients failed to resolve acute infection to subpatency (7.2). Thus, the repopulating of the lethally irradiated recipients with mature B cells was responsible, in part, for the resolution of primary parasitaemia in these mice as for fully immunocompetent animals. As for naive recipients, the courses of infection of mice given lymphocytes from either of the two

groups did not differ significantly during the acute infection, but varied in the timing of the recrudescence (Fig. 7.3.3). All experimentally challenged mice resolved primary parasitaemia either on d 22 or d 23 p.i.. However, T cell-depleted recipients of WEP 737 and additional naive B cells showed a recrudescence ahead of similarly depleted mice given WEP 775 and B cells. These differences between the kinetics of infection upon transfer of these two Ly-4$^+$ lines to T cell-depleted mice was evident only when naive B cells were cotransferred (Fig. 7.3.3) and not in their absence (Fig. 7.2.3). This was due to a change in the pattern of parasitaemia of recipients of WEP 737 upon full B lymphocyte reconstitution (Figs. 7.2.2 & 7.3.2) and not of the parasitaemia exhibited by WEP 775-inoculated mice under these varying conditions (Figs. 7.2.1 & 7.3.1). Therefore, mice deficient of T cells but fully competent in the B cell repertoire showed the same level of resistance to P. c. chabaudi AS pRBC upon transfer of WEP 737 as did mice possessing both lymphocyte compartments. This suggests strongly a B cell dependency for the WEP 737 and WEP 723 Ly-4$^+$ lines to mediate anti-P. c. chabaudi immunity in vivo.

The B cell dependency of the Ly-4-bearing lines derived from the spleens of mice recovered from secondary or tertiary malaria infections is evidenced by the divergent patterns of immune protection elicited by WEP 775 and WEP 737 to T cell-depleted mice in the absence of B cells (confirmation of 7.2) (Fig. 7.3.4). Recipients of WEP 737 reached a transient subpatency on d 34 p.i., compared to d 25 p.i. for WEP 775-transferred mice. The magnitude of the recrudescence in the former was in fact higher than that detected in mice given a control population of unprimed splenic T cells, although parasite clearance was quicker (Fig. 7.3.4). Thus, the degree of protection conferred by WEP 737 in the relative absence of B cells was not nearly as great as that conferred in a B cell-rich environment (Fig. 7.3.3). For WEP 775, however, the presence or absence of additional B cells had a lesser effect on the level of protection engendered by this T lymphocyte line to immunocompromised host animals (Figs. 7.3.3 & 4). Thus, unlike WEP 737, WEP 775 appeared to mediate protection in vivo largely through B cell-independent mechanisms.

That the requirement of the Ly-4$^+$ lines taken from reinfected donor mice was for B cells rather than for T cells can be determined by examination of Fig. 7.3.5. These adult-thymectomised, irradiated & bone marrow-reconstituted mice received a total of 4 x10^7 naive splenic T & B cells as well as either WEP 735 or WEP 737 (the control group received 8 x10^7 spleen cells). For both recipients of either Ly-4$^+$ line, the

course of infection observed upon cotransfer with a mixed splenic population was very similar to that seen in the presence of just B cells (Fig. 7.3.3). Thus, the additional presence of unprimed T lymphocytes did not appear to have an appreciable effect on the patterns of parasitaemia in recipients of either the proposed B cell-dependent or B cell-independent T cell lines. The mediation of protection by both these lines, thought to be by different immunological pathways, therefore, did not depend upon a repopulating T cell population for functional activation in vivo. This is not to suggest that non-immune T cells do not necessarily play a part in immune protection through Ly-4$^+$ effector mechanisms, but that the numbers involved are very probably fewer, thus not demanding a substantial repopulation of thymectomised and lethally irradiated animals for significant reactivity.

The dichotomy of protection given by the adoptively transferred Ly-4$^+$ populations is appreciated best by studying the courses of infection of mice receiving these cells with or without supplementary lymphocytes (Figs. 7.3.6 & 7). The similar patterns of reactivity of WEP 737 upon transfer to T cell-depleted recipients with additional syngeneic B cells and with a mixture of naive T & B cells (Fig. 7.3.6) suggest that it is predominantly the B lymphocyte fraction of the heterologous splenic population with which the malaria-primed Ly-4$^+$ cells interact. This is highlighted by the course of infection for mice receiving the WEP 737 lymphocytes alone, thus with relatively few B cells present. In this case, the B cell/T cell interaction was limited, thereby restricting specific Ab production. Hence, the P. c. chabaudi AS-challenged mice suffered an extended primary parasitaemia and a heightened recrudescence (Fig. 7.3.6).

For the Ly-4$^+$ lines considered to be B cell-independent in their mode of action, this experiment showed qualified support for this hypothesis. WEP 775 lymphocytes transferred to T cell-depleted recipients together with an equal total number of splenic B & T cells as B cells alone gave identical courses of infection (Fig.7.3.7). Thus, a reduction by approximately 50% in the number of B cells present in the system had no effect on the behaviour of this protective Ly-4$^+$ T cell line. Animals inoculated with WEP 775 cells alone recrudesced at the same time as did those given additional non-reactive lymphocytes. However, there was a lag of 4 d in resolution of the primary parasitaemia with respect to recipients of more complex multiple transfers. This divergence in behaviour occurred at a time of infection when non-specific effects usually predominate in the anti-malarial immune response. The apparent greater protection in the presence of an excess of naive B lymphocytes, as determined by the

quicker resolution of acute infection (Fig. 7.3.7), was thought to be due to an additive effect of the considerable population of mature B cells present in those mice given B cells in addition to WEP 775 lymphocytes. This explanation of this shift of the primary parasitaemia in the absence of a B cell population was considered more likely than a specific B cell/T cell interaction between WEP 775 and those naive B cells inoculated, since, this effect notwithstanding, the patterns of parasitaemia in T cell-depleted recipients of WEP 775 were very similar in the presence or absence of naive B and/or T & B cells (Fig. 7.3.7). The only difference observed was before a specific anti-P. c. chabaudi AS response could be mounted. During the subpatent and recrudescent periods, when specific effector mechanisms are thought to offer a greater protection, the parasitaemias were essentially alike. An alternative view of the anomalous reactivity of the WEP 775 line in the absence and presence of additional B cells is that this may have been due to its not being pure, i.e. a mix of two or more Ly-4$^+$ T cell subsets or cell types. Hence, the overall effect may be the sum of the actions of both individual types. However, homogeneous cloned populations derived from the WEP 775 line showed a similar behaviour in vivo (Chapter 9), indicating that contamination of the Ly-4-bearing population was an implausible explanation for the results obtained. These findings, therefore, generally support the view that those cell lines derived from spleens taken early during primary P. c. chabaudi AS infection, WEP 775 and WEP 779, exert protective immunity by non-Ab-mediated mechanisms.

The effect of the addition of unprimed B lymphocytes could not, however, explain the difference in reactivity of the WEP 737 Ly-4$^+$ line with or without B cells or non-specific T cells (Fig. 7.3.6). This T cell line is thought to behave in a synergistic helper role with those B lymphocytes present, and this hypothesis gained strength from this adoptive transfer study. The immunity conferred by WEP 737 was greatly enhanced by the concurrent inoculation of splenic B cells, reducing the pre-subpatent period, the height of the recrudescence and the total time for parasite clearance. For the first 20 d p.i. the patterns of parasitaemia were very similar in all recipients of WEP 737 (Fig. 7.3.6), and it was only during the remission of the primary infection that a discrepancy in the protection conferred by WEP 737 cells in the presence or absence of additional B cells was first noticeable. The relative lack of protection engendered by the WEP 737 line in the absence of sufficient repopulating B cells would indicate a helper role for these T cells in variant-specific anti-P. c. chabaudi AS Ab synthesis. Hence, when cotransferred with a large population of mature B cells, lymphocytes of the WEP 737

line were able to control infection, presumably through classical Ab-mediated mechanisms. When insufficient B cells were inoculated into recipient mice at the time of challenge, during the second phase of infection a humoral immune response could not be mounted and phenotypically variant asexual stage parasites were able to multiply unchecked, thereby causing a protracted infection (Fig. 7.3.6).

Different groups of naive spleen cells inoculated into immunosuppressed mice without Ly-4-bearing T cell lines were set up as controls for this experiment. Transfer of either unfractionated T & B splenic lympocytes or T- or B-enriched populations did give some protection compared to a negative control of the normal course of infection of P. c. chabaudi AS observed in non-reconstituted T cell-deprived NIH mice (Fig. 7.3.8). Each group of naive lymphocytes was capable of reducing the level of parasitaemia, but their adoptive transfer affected neither the pattern nor the total length of infection. Thus, recipients of unprimed cells alone suffered a chronic primary parasitaemia lasting not less than 38 d. By comparison, the Ly-4$^+$ lines were effective in conferring immune protection, seen as a reduced level and shortened duration of acute infection, remission to subpatency, and as a significantly quicker final clearance of parasites (Fig. 7.3.3). The inability of naive lymphocytes to control the course of challenge infection in immunosuppressed mice was especially significant in the case of recipients of 8 x10^7 unprimed B cells (Fig. 7.3.8). This showed that the ability of WEP 737-transferred recipients to control challenge in the presence of additional B cells (Fig. 7.3.6) was caused by a synergism between Ly-4$^+$ lymphocytes and the naive B cells and not due to the mediation of the latter alone.

7.4 Discussion

The work presented in this chapter attempted to explore the pathways by which each of the four Ly-4-bearing T cell lines specific for P. c. chabaudi AS used throughout this study mediated protection to homologously challenged recipients. It had been established that each line gave some protection when adoptively transferred to naive, syngeneic mice (Chapter 5). To elucidate the nature of this protection further, recipient animals were previously surgically thymectomised, gamma-irradiated and bone marrow-reconstituted. This in vivo manipulation, from which the mice were allowed to recuperate fully, produced an immunocompromised state effectively devoid of mature T lymphocytes. Initial studies revealed that the patterns of reactivity in vivo were not all the same but fell into two distinct groups. Those Ly-4$^+$ lines derived originally during

primary P. c. chabaudi AS infection, WEP 775 and WEP 779, were able to control challenge upon reconstitution of T cell-deprived mice in a similar way to adoptively transferred immunocompetent, naive mice. These findings concurred with those of Brake et al (1986), who showed that in vitro-propagated Ly-4$^+$ lines were effective in transferring protection adoptively to athymic nude mice challenged with P. c. adami. However, similar P. c. chabaudi AS-specific lines taken from mice after a secondary (WEP 737) or tertiary (WEP 723) infection showed a lesser protection in T cell-deprived mice than in fully competent mice. From this information, it would appear that thymus ablation had a deleterious effect on the conferment of protection by these two lines. Examination of control mice showed that animals challenged without receiving an inoculum of T cells exhibited a fulminating lethal infection regardless of whether they had been adult-thymectomised or only sham-thymectomised. Therefore, the presence of an intact thymus appeared to be irrelevant to the mediation of protection by WEP 737 or WEP 723 to immunosuppressed animals. Assuming that the deficiency in the host mice was in the B cell rather than the T cell compartment, another adoptive transfer experiment was performed to explore this possibility. That the greater parasitaemia in immunosuppressed mice transferred Ly-4$^+$ lines taken from reinfected mice was due to a lack of B lymphocytes was not an unreasonable suggestion in view of their greater sensitivity to the acute effects of radiation than T lymphocytes (Kataoka & Sado 1974). It may be that either or both the number of bone marrow stem cells inoculated after lethal irradiation and the time between reconstitution and challenge were insufficient to produce a population of circulating mature B cells sufficiently large to interact with the adoptively transferred Ly-4-bearing lymphocytes to give rise to an Ab-dependent cellular response to infection. When a large repopulating inoculum of mature splenic B cells was cotransferred with WEP 737 cells, an effective immune response was elicited, for a level of protection was engendered sufficient to control challenge as for immunocompetent recipients. The behaviour of this Ly-4$^+$ line was, therefore, similar to that exhibited by cells of the Ly-1$^+$23$^-$ phenotype (mostly Ly-4$^+$ cells) upon adoptive transfer to T cell-deprived mice challenged with P. yoelii (Jayawardena et al 1982). In this study, it was shown that Ly-1$^+$ T cells reconstituted the ability of T cell-deprived mice to produce anti-malarial Abs. Furthermore, there was a clear synergism between inoculated Ly-1$^+$ cells and B cells, for mice injected with a mixture of the two cell types mounted an earlier and more vigorous Ab response than mice receiving either cell type alone. It would be expected that any cell-mediated immune

response to P. yoelii would be in a helper capacity for Ab synthesis, since, for this particular plasmodial species, resolution of infection is through humoral immunity. Adoptive transfer of the Ly-4$^+$ lines into P. c. chabaudi AS-infected T cell-deprived recipients with or without additional B or T & B cells demonstrated clearly that the lines from reinfected mice, WEP 737 and WEP 723, gave greater protection when reconstituted with syngeneic B cells, whereas the lines from mice recovering from primary parasitaemia, WEP 775 and WEP 779, showed similar levels of protection in the presence or absence of naive B cells and/or non-reactive T cells. This dichotomy of the protection engendered in vivo by these T cells, all of which were phenotyped to the Ly-4$^+$ T cell subset (Chapter 9), correlated with a functional heterogeneity of these lines at a cellular level. The secondary and tertiary infection-derived cells appeared to act by Ab-mediated mechanisms, i.e. as helper T cells for specific Ig production. The two lines taken early in primary infection did not behave in this way and were considered B cell-independent in their reactivity in vivo. It was thought that they may cause protection through alternative effector mechanisms, such as, for instance, the induction of activated macrophages. To dissect the underlying mechanisms responsible for the mediation of protection conferred by each of the Ly-4$^+$ lines upon adoptive transfer, every line was subjected to a detailed characterisation in vitro , as described elsewhere (Chapter 9).

The findings reported here match very closely those of Langhorne et al using the same P. c. chabaudi AS parasite in C57BL/6 mice. These workers have investigated the type of T lymphocytes involved in immunity to P. c. chabaudi AS by application of a limiting dilution assay system on splenic lymphocytes taken from mice during the course of an infection (Langhorne & Simon 1989, Langhorne et al 1989 a), and by using rat MAbs against murine Ly-2 and Ly-4 determinants to deplete mice of these T cell subsets prior to or during a P. c. chabaudi AS infection (Süss et al 1988, Langhorne et al 1989 b). They found that Ly-4$^+$ cells had a major role in controlling challenge infection whereas cells of the Ly-2$^+$ subset were concluded to be of marginal importance. During the first 14 d of a primary infection, the predominant Ly-4$^+$ T cell response was of the T_H1-type, i.e. Ab-independent (Langhorne et al 1989 a). From d 14 p.i., the frequency of this cell type declined and by d 21 p.i. the Ly-4$^+$ response was predominantly of T_H2 cells, characteristic of an Ab-dependent activity. These data provided indirect evidence that the effector mechanisms controlling the primary patent parasitaemia of a P. c. chabaudi AS infection may be Ab-independent, but that this is superceded by Ab-

mediated immunity. The functional dichotomy of the Ly-4-bearing T cell subset described for host immunity to P. c. chabaudi AS in C57BL/6 mice was revealed again by the experiments described herein using NIH mice. In essence, therefore, the results of these studies using two different approaches were alike. The two lines recovered from donor mice on d 16 and d 20 of a primary infection, WEP 775 and WEP 779, respectively, were determined to be B cell-independent in their reactivity in vivo and could thus be nominally termed T_H1 cells, although evidence for this classification required characterisation of lymphokine secretion profiles (Chapter 9). These lines represented the majority of Ly-4^+ cells present in the spleens from which they were taken at the time of splenectomy, in that T_H1 lymphocytes are more frequent during the acute phase of infection of P. c. chabaudi AS and shortly thereafter. As there is thought to be a shift in the balance of Ly-4^+ cells towards those of the T_H2 subset later in infection to coincide with the appearance of detectable levels of serum Ig, the lines taken from multiply-infected mice, WEP 737 and WEP 723, were likewise representative of the majority of Ly-4-bearing lymphocytes present in the host immune system after prolonged exposure to P. c. chabaudi AS.

One interesting aspect of the present study was the presence of recrudescent parasitaemias in the T cell-reconstituted immunosuppressed challenged mice. For the second experiment (7.3), animals received an inoculum of naive B cells in addition to lymphocytes of a Ly-4^+ cell line, and all recipients managed to control infection. The parasitaemia showed a typical biphasic pattern similar to that seen in immunocompetent mice. It has been suggested that for P. knowlesi infection of rhesus monkeys the switch from one parasite variant to another under host immune pressure may be Ab-induced (Brown 1973, Brown & Hills 1974). Although the selective pressure for induction of antigenic variation in P. c. chabaudi AS has not been elucidated, these data do not suggest other than an Ab-mediated mechanism. Paradoxically, however, in the earlier experiment in which a large repopulating dose of B cells was not cotransferred with each Ly-4^+ line, recrudescent parasitaemias were still observed (7.2). As it is known that the number of mature B lymphocytes present in these immunosuppressed animals was not sufficient to interact with the B cell-dependent Ly-4^+ cells to synthesise variant-specific Ab, presumably the low level of B cells would also have affected the induction of antigenic variation. However, both for recipients of these lines and for those given the lines thought to be B cell-independent, pronounced recrudescences were observed, indicative of the presence of phenotypically variant parasites in the peripheral

circulation. This would indicate a selection pressure for induction of antigenic variation by P. c. chabaudi AS pRBC that does not involve variant-specific Ab for its expression. If this is the case, as for P. c. adami infection, protection cannot be principally Ab-mediated. Grun & Weidanz (1981) showed that P. c. adami infection in B cell-deficient mice resulted in an activation of a T cell-dependent immune mechanism which terminated acute malaria in a similar way to that seen in immunologically intact mice. In contrast, B cell-deficient mice and chickens died of fulminant malaria when infected with P. yoelii and P. gallinaceum, respectively (Rank & Weidanz 1976, Weinbaum et al 1976 b, Roberts et al 1977), but when their acute infections were controlled with subcurative chemotherapy, B cell-deficient hosts developed chronic low grade infections and resisted challenge with homologous parasites (Roberts et al 1977, Roberts & Weidanz 1979). Responses to infections by the two rodent plasmodial species P. yoelii 17X and P. c. adami appear to represent extremes in the repertoire of host immune responses to malaria, one predominantly cell-mediated and one predominantly humoral. It remains unclear why resolution of infections by these two rodent parasites should require different types of responses by the host, but it may relate to the phylogenetic distance between the two plasmodia and the fact that they parasitise preferentially different types of RBC. It is plausible, however, that both Ab-independent and humoral immune mechanisms may participate in resolution of all murine malarial infections, but that one or the other may predominate in particular circumstances. Indeed, the current thinking concerning immunity to P. c. chabaudi AS perceives a host resistance to primary infection involving both Ab-dependent and Ab-independent mechanisms (Langhorne 1989). If this is the case, the selection of two Ly-4^+ lines which collaborate in the synthesis of anti-P. c. chabaudi AS Ab and two other lines which appear to participate in or activate other cell-mediated mechanisms of immunity is of particular relevance.

In the case of P. knowlesi and P. yoelii infections, the detection of antigenically variant pRBC in the blood stream is thought to be caused by a selective pressure enforced by the presence of variant-specific Ig, whereas for P. c. adami challenge, resolution of acute infection is primarily independent of Ab. The results of this study suggest that both cellular and humoral immunity play a role in acquired resistance to P. c. chabaudi AS, and, therefore, induction of parasite antigenic variation may not be critically dependent upon either for its expression in vivo. For P. c. chabaudi AS, there is also the possibility that phenotypic variation in the expression of parasitic antigenic

determinants is not induced by immunological selection at all, but is instead an innate property of the asexual blood stages of this species. There is no precedence for this proposal, but as direct evidence for host immunological induction of antigenic variation is still lacking, it cannot be excluded. Indeed, the ubiquitous presence of recrudescent parasitaemia in mice immunologically crippled in both T and B cell functions, as shown in this study, would not detract from this possibility. In light of evidence in favour of immunological selection inducing antigenic variation in other murine plasmodia, it is unlikely that the appearance of recrudescent pRBC would occur without immunological intervention but this remains unproven. To address this question, well-defined immunologically deficient models such as nude or SCID mice could be used, to which P. c. chabaudi AS-specific T cell or B cell lines may be transferred adoptively. If, for instance, specific Ab responses, as measured by increased levels of serum Ig, titred out in parallel with protective immunity, it would show variant-specific Ab activity in the presence of a particular T cell line, which would thereby be performing in an Ab helper capacity. In the experiments described herein, it was not a primary objective to examine the nature of the mechanism of induction of antigenic variation; it was coincidental that the results touched on this subject. The findings reported were not unequivocal, however, since the immunodeficient mice used were not totally B cell-deprived, but only lacking a mature B cell compartment. To analyse whether or not variation in parasite phenotype amongst P. c. chabaudi AS pRBC is a B cell-mediated phenomenon, it would be best to use host animals rendered B cell-deficient by lifelong treatment with goat anti-mouse IgM serum (anti-μ), or alternatively to use mice suffering from severe combined immunodeficiency (SCID), from which both functional B and T lymphocytes are lacking. Unfortunately, in conjunction with such studies, at least in congenitally deficient mice, the Ly-4^+ T cell lines raised against P. c. chabaudi AS may not be used due to histocompatibility restriction, which would cause their non-functioning upon transfer to recipients of a different H-2 haplotype, as found by Brinkmann et al (1985). These difficulties would not, however, prevent dissection of the roles of different lymphocyte subsets in protective immunity to P. c. chabaudi AS infection by specific T or B cell depletion of immunocompetent host animals through MAb therapy. Such a technique has been used to circumvent partially problems of both histocompatability and effective lymphocyte depletion and will be described in Chapter 8.

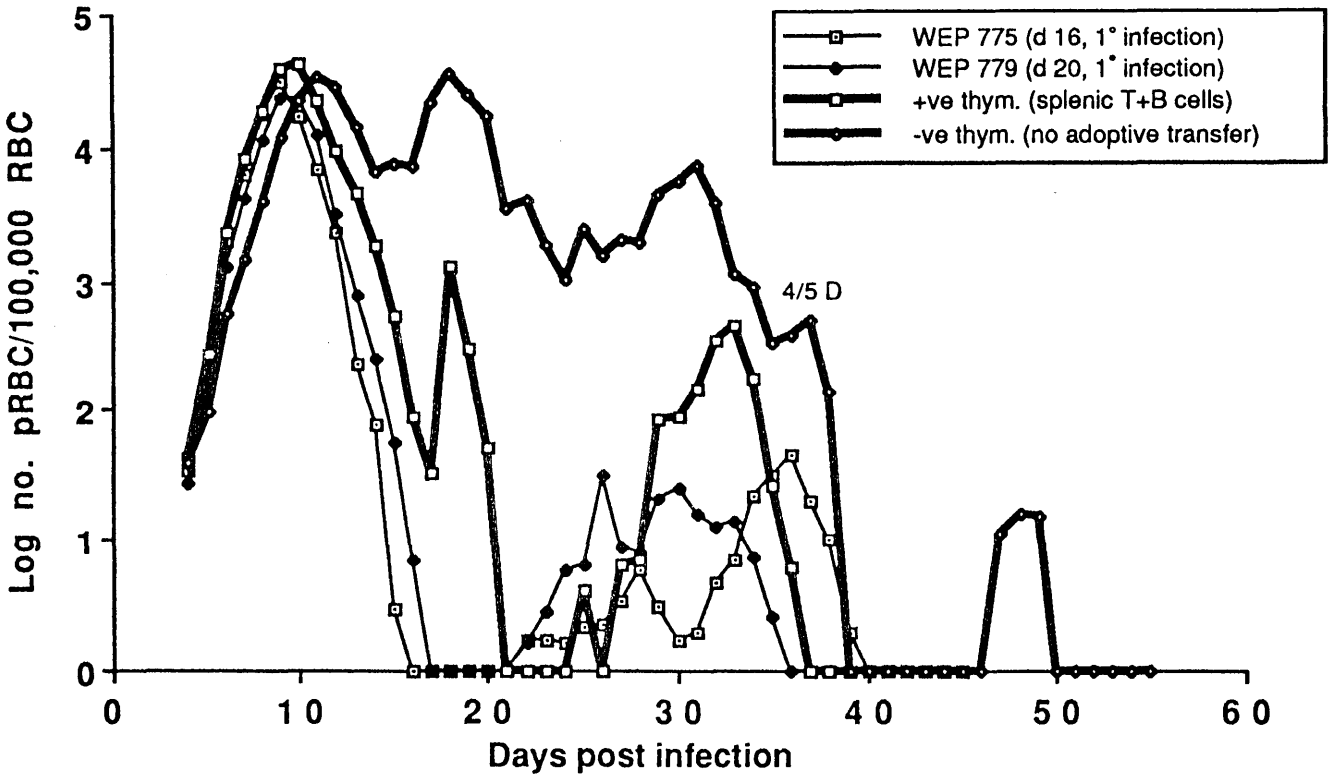

Fig. 7.2.1 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 & WEP 779 upon challenge with 1 x10^5 pRBC i.v..

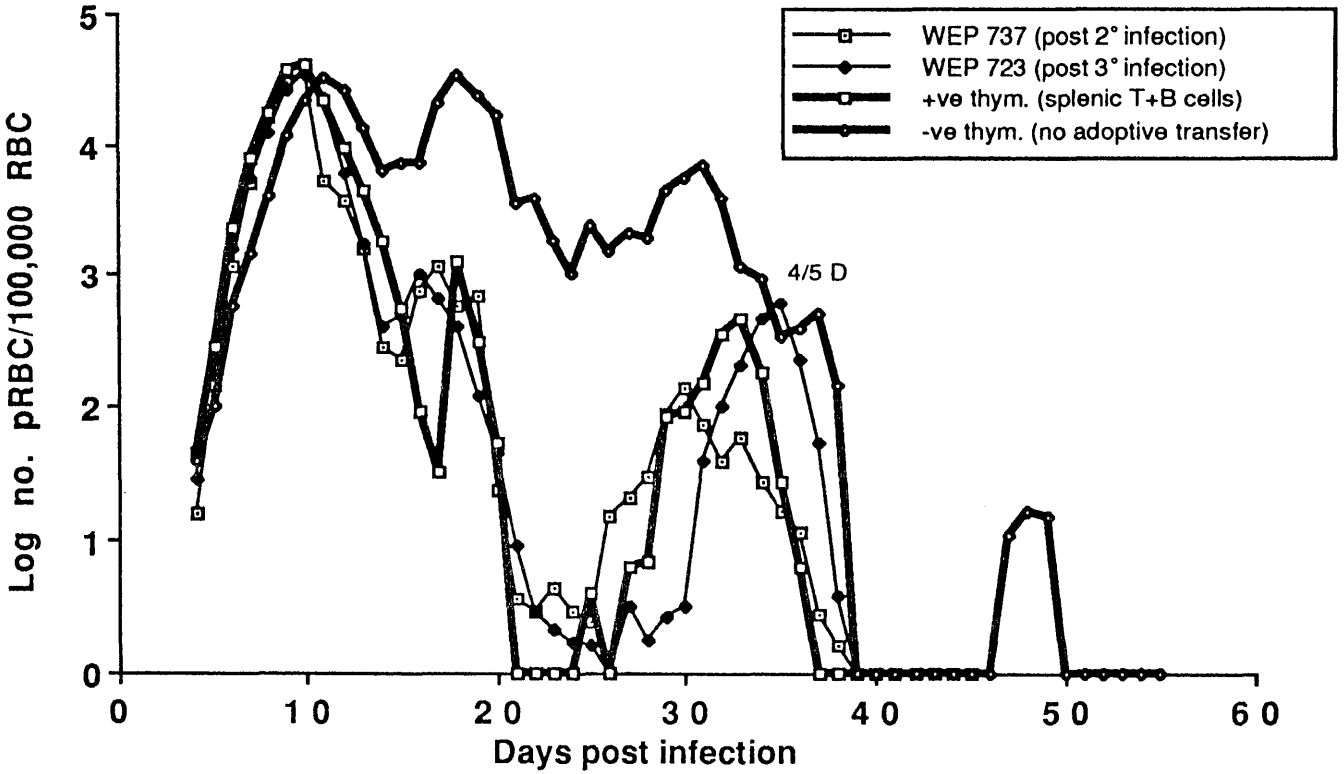

Fig. 7.2.2 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 737 & WEP 723 upon challenge with 1 x10^5 pRBC i.v..

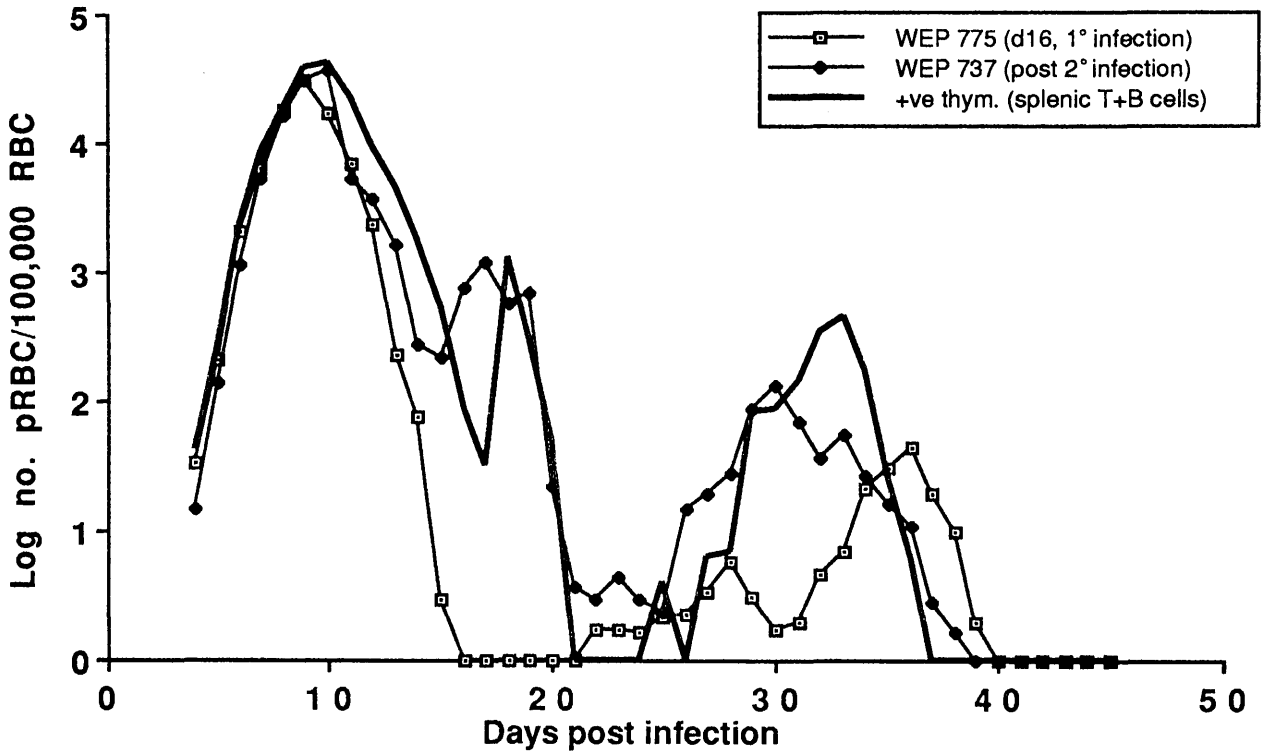

Fig. 7.2.3 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 and WEP 737 upon challenge with 1 x10^5 pRBC i.v..

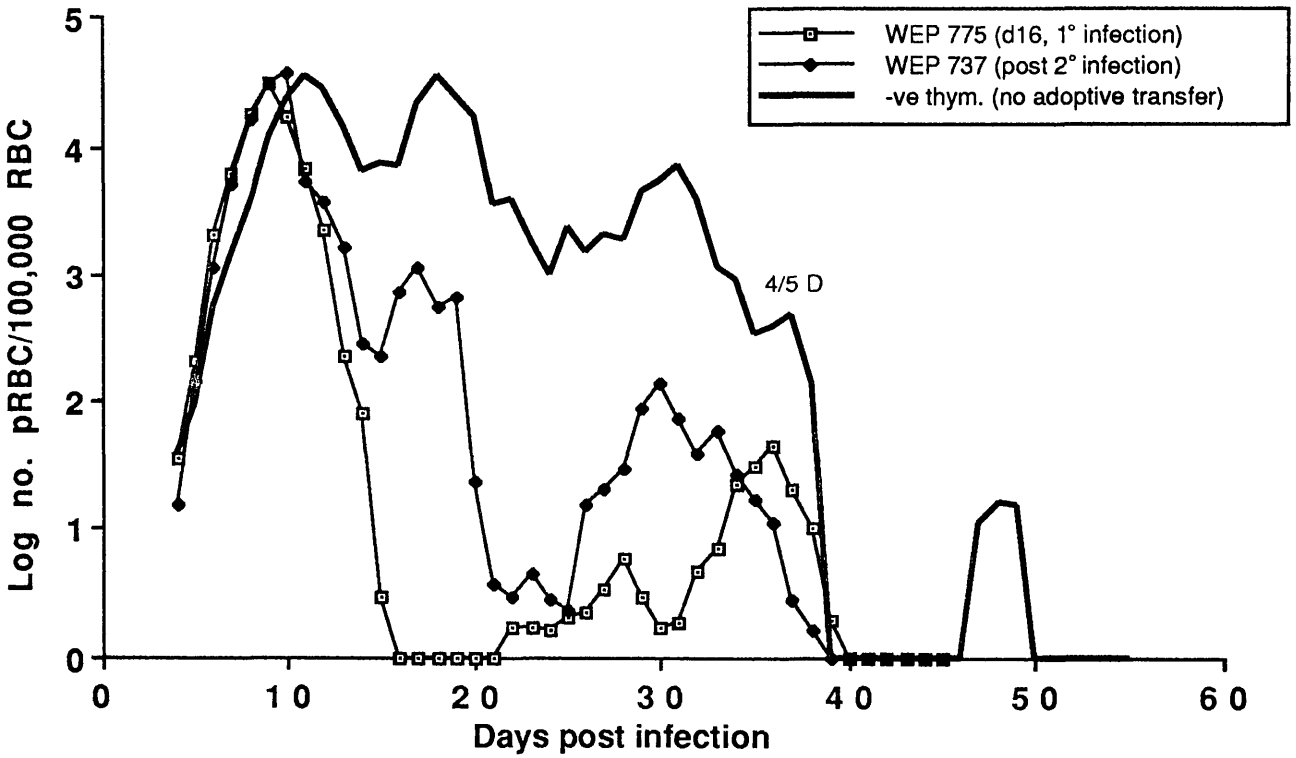

Fig. 7.2.4 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 and WEP 737 upon challenge with 1 x10^5 pRBC i.v..

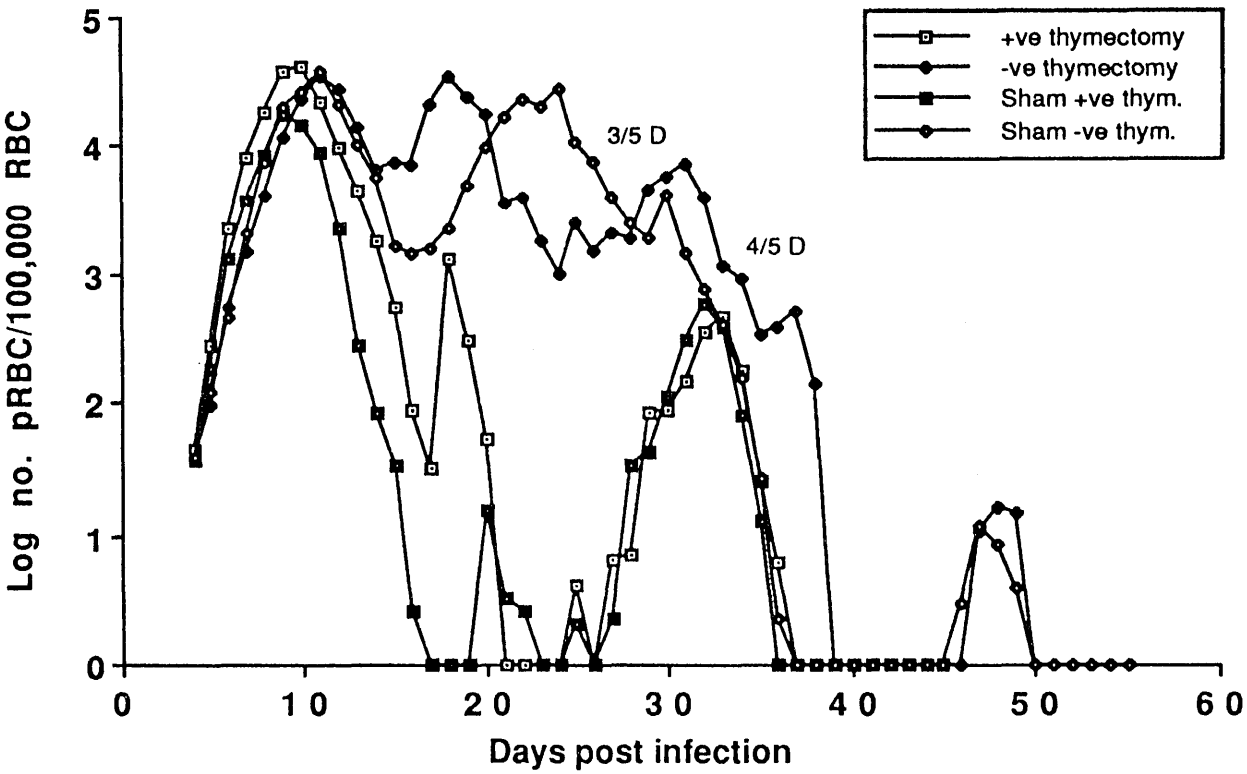

Fig. 7.2.5 Courses of infection in adult- & sham-thymectomised, lethally irradiated & bone marrow-reconstituted NIH control mice upon challenge with 1 x10^5 pRBC i.v..

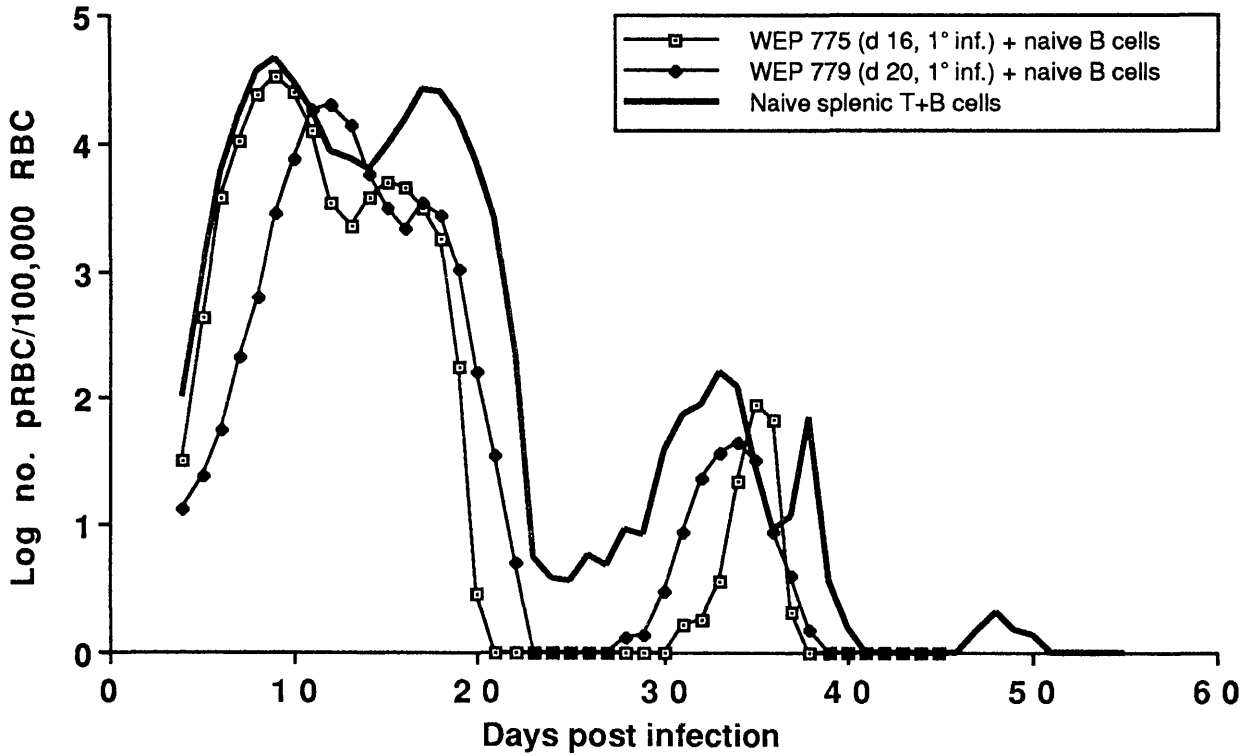

Fig. 7.3.1 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 and WEP 779 with additional naive B cells upon challenge with 1 x10^5 pRBC i.v..

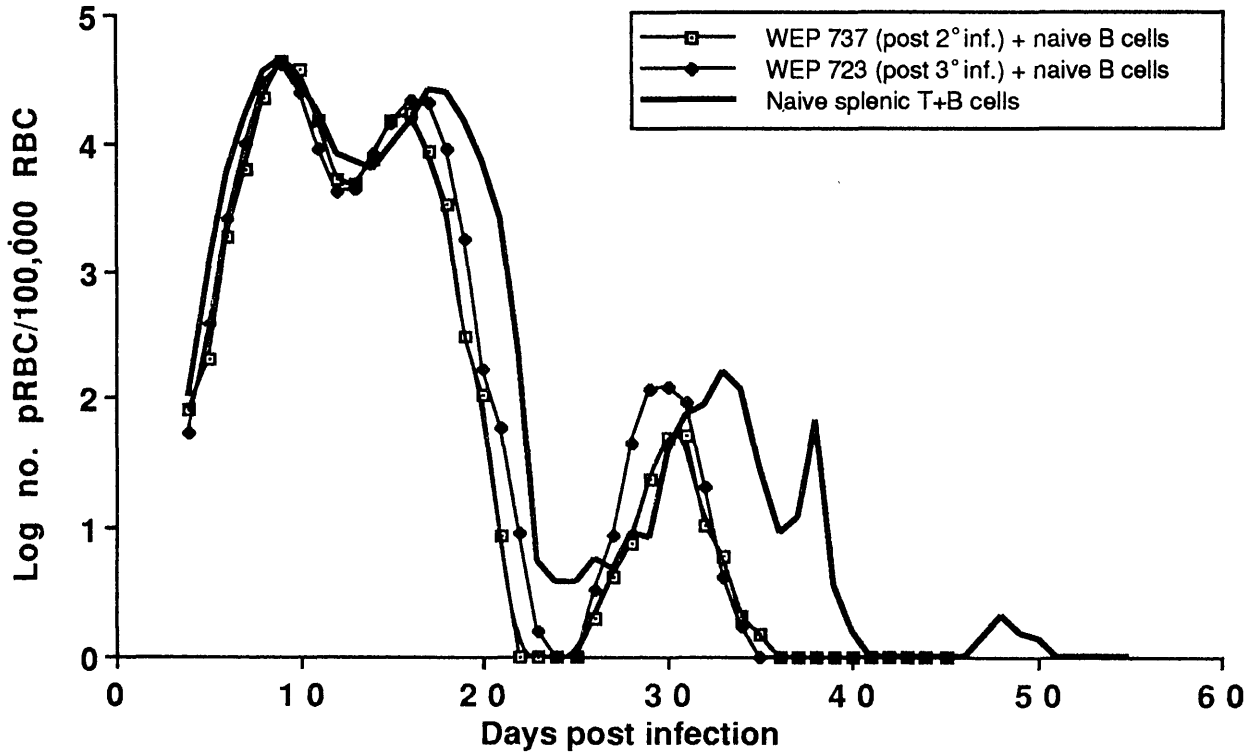

Fig. 7.3.2 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 737 and WEP 723 with additional naive B cells upon challenge with 1 x10^5 pRBC i.v..

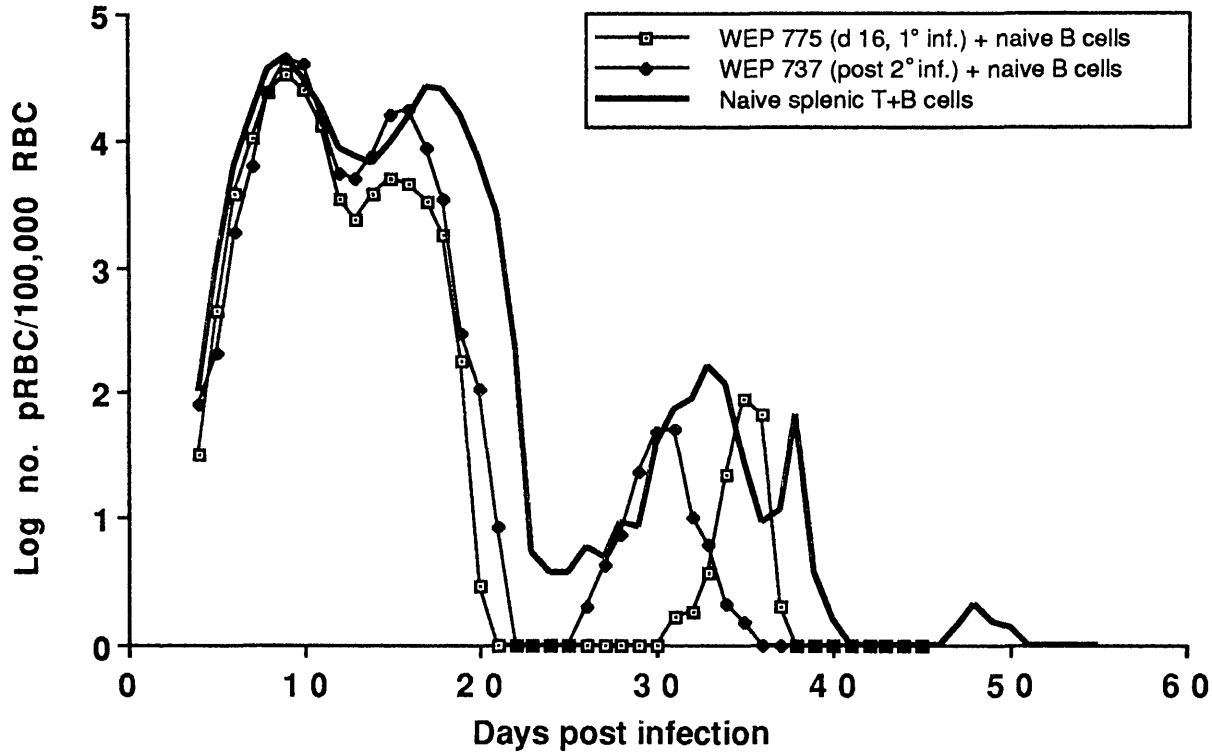

Fig. 7.3.3 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 and WEP 737 with additional naive B cells upon challenge with 1 x10^5 pRBC i.v..

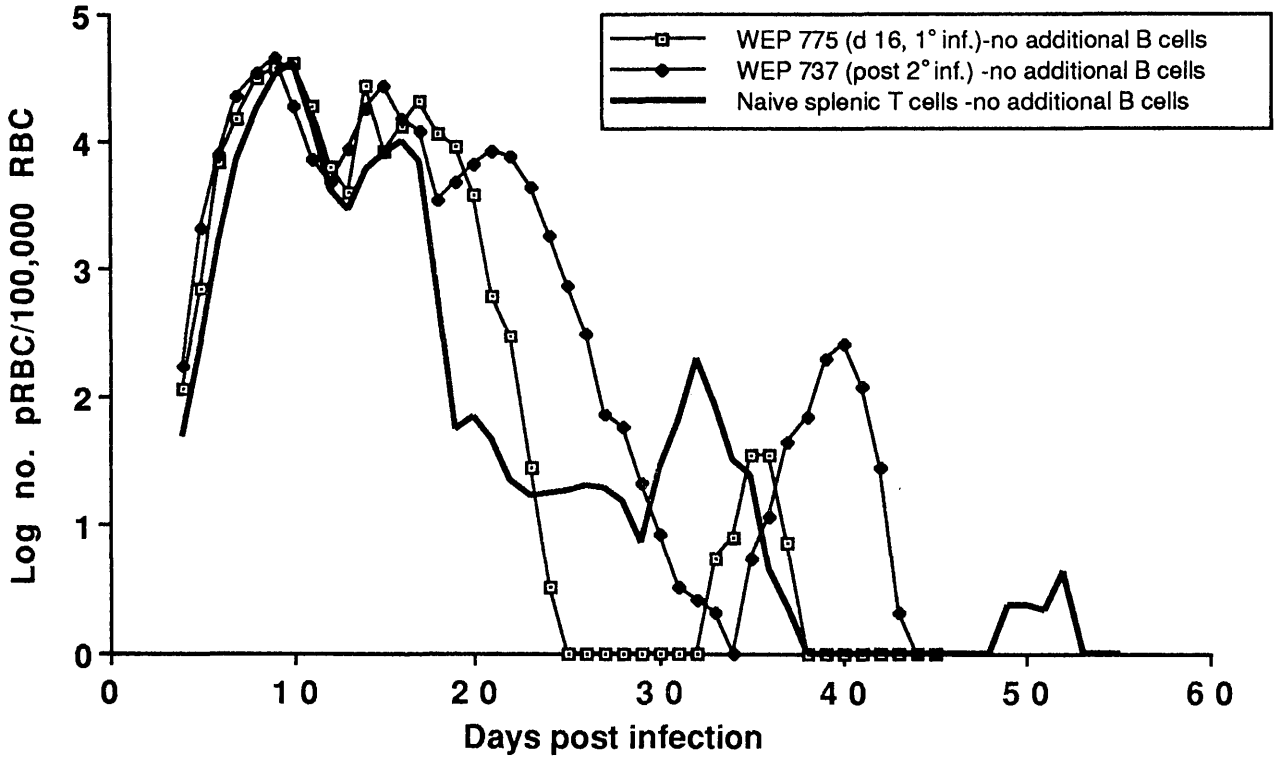

Fig. 7.3.4 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 and WEP 737 (without additional naive B cells) upon challenge with 1 x10^5 pRBC i.v..

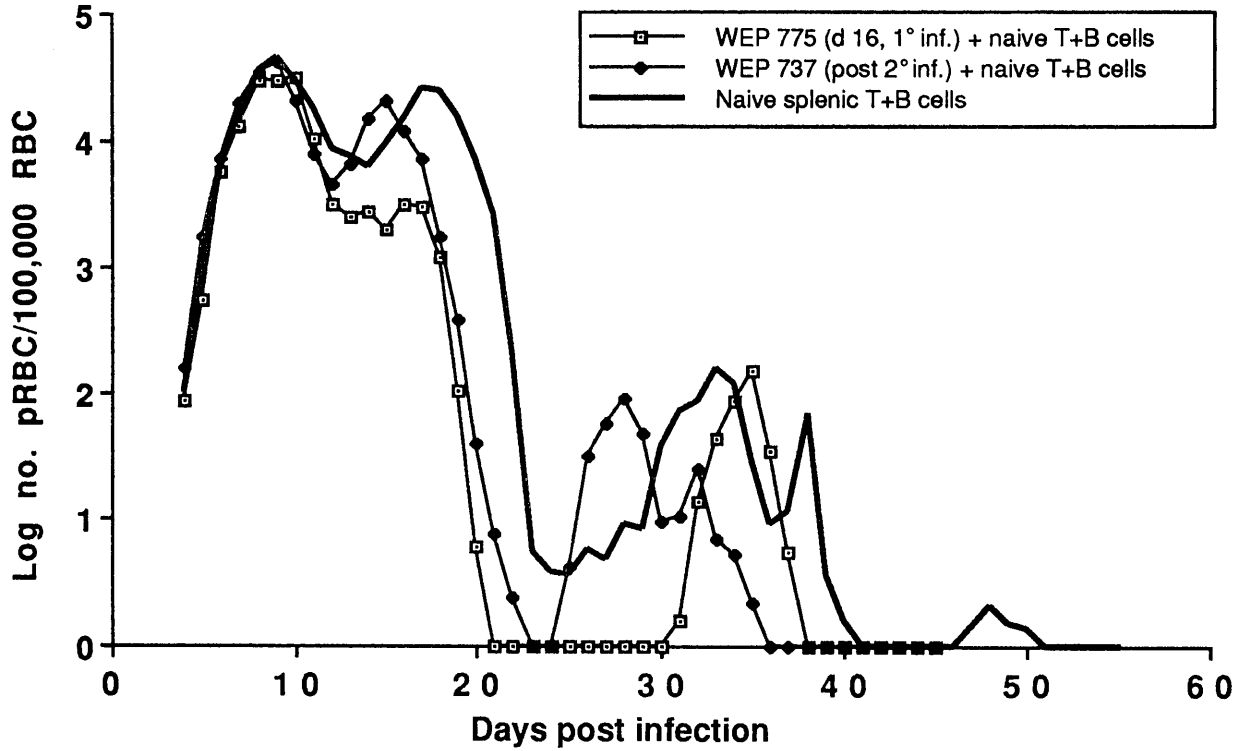

Fig. 7.3.5 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 and WEP 737 with additional naive T & B cells upon challenge with 1 x10^5 pRBC i.v..

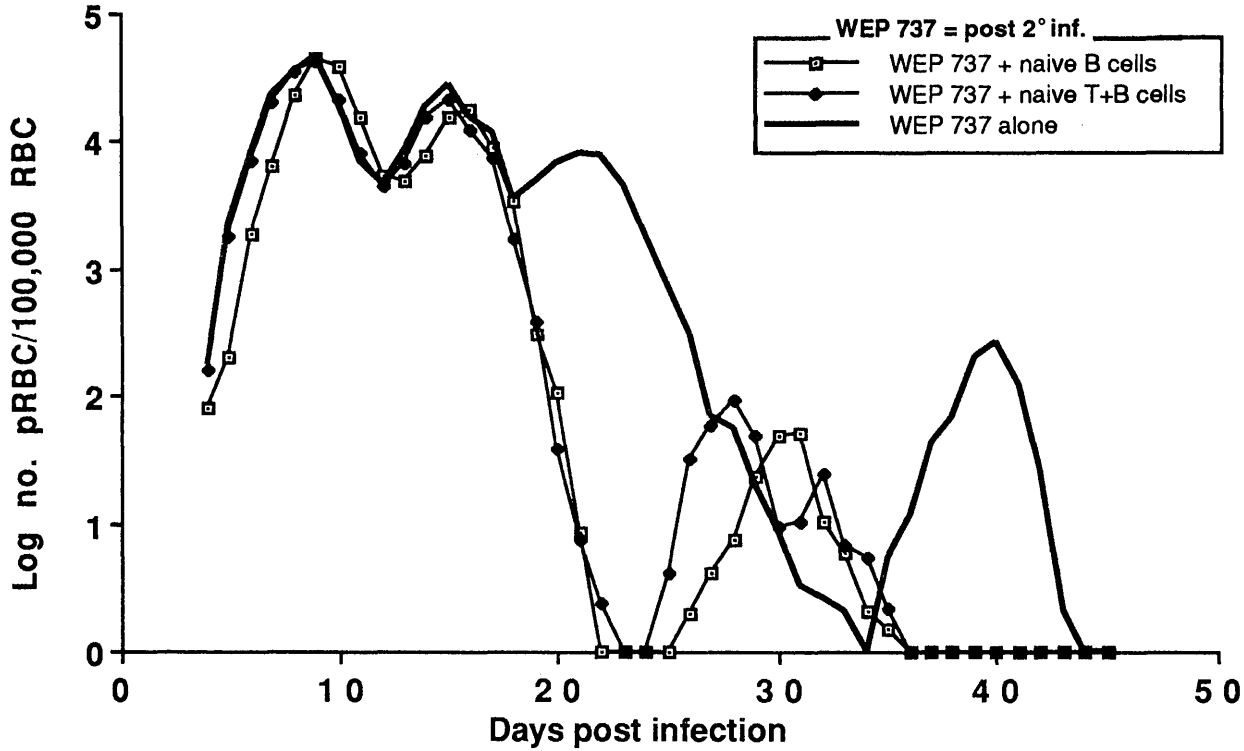

Fig. 7.3.6 Course of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 737 challenged with 1 x10^5 pRBC i.v..

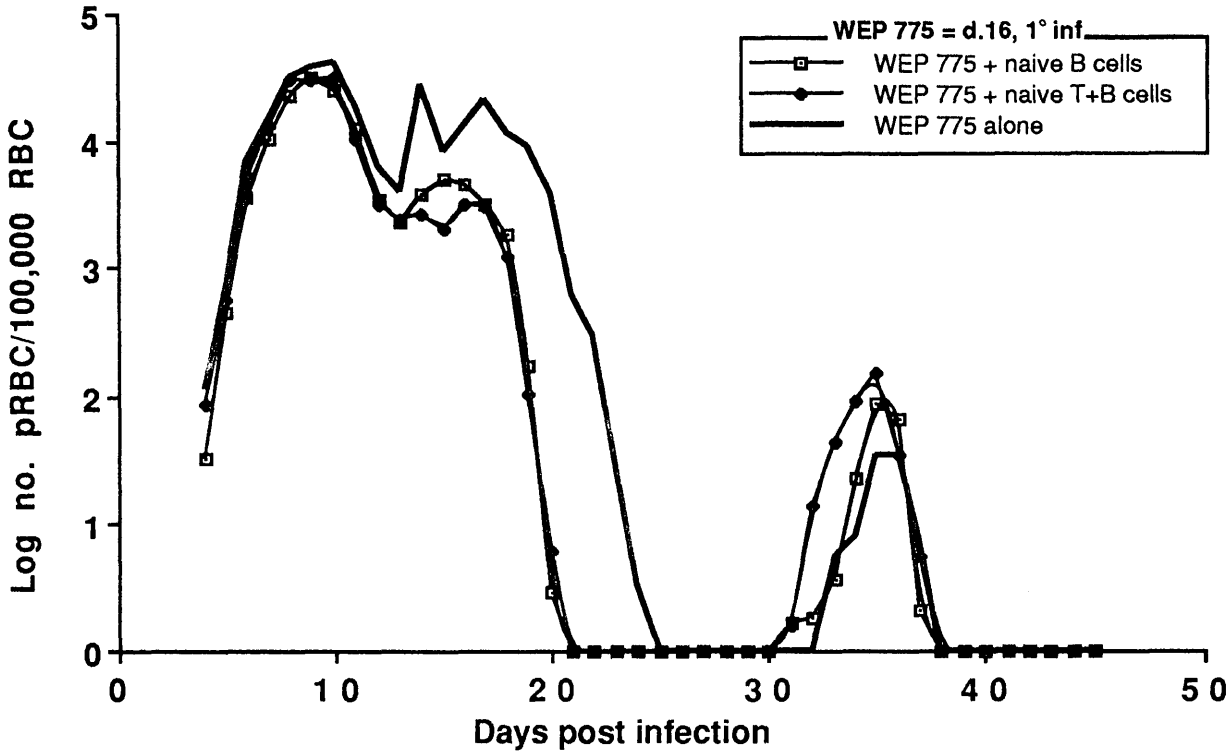

Fig. 7.3.7 Course of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of WEP 775 challenged with 1 x10^5 pRBC i.v..

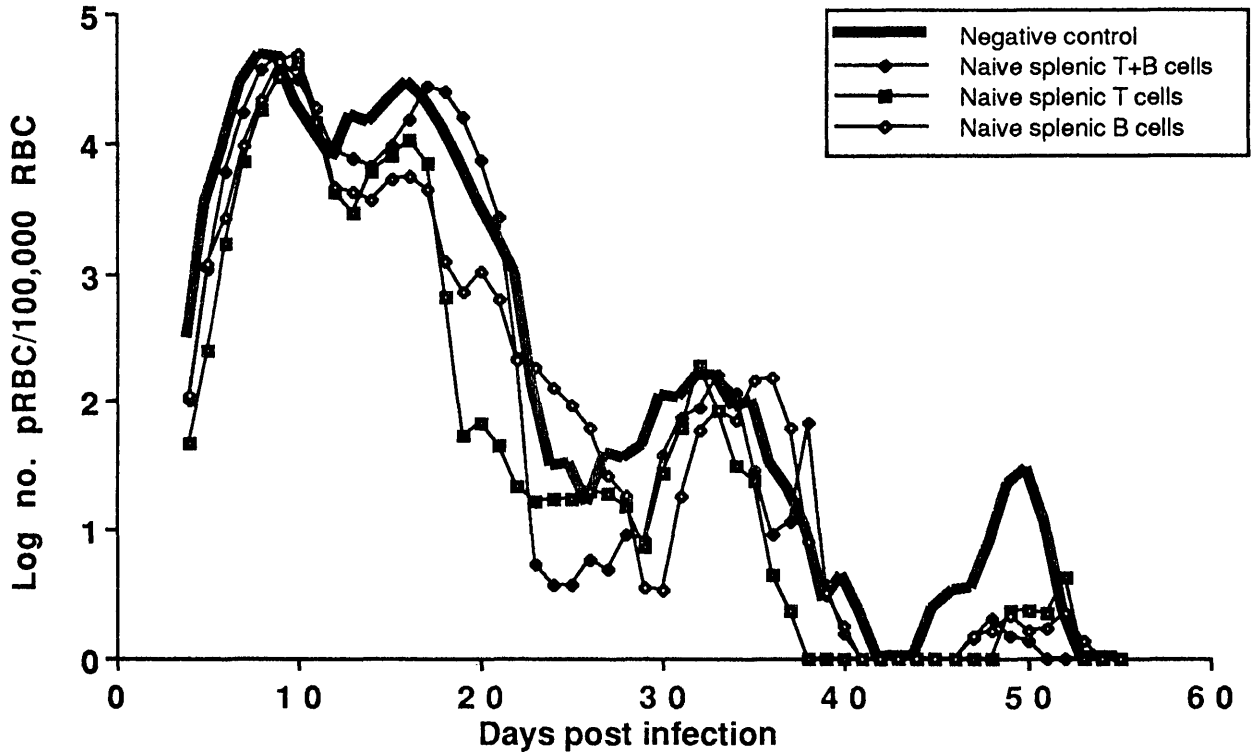

Fig. 7.3.8 Courses of infection in adult-thymectomised, lethally irradiated & bone marrow-reconstituted NIH recipients of naive splenic lymphocytes challenged with 1 x10^5 pRBC i.v..

CHAPTER 8
ADOPTIVE TRANSFER OF T CELL LINES TO SELECTIVELY T CELL-DEPLETED SYNGENEIC RECIPIENTS

8.1 Introduction

A pivotal role for $CD4^+$ or Ly-4^+ T lymphocytes in the protective immune response to malaria infection is now undisputed (Weidanz & Long 1988, Long 1988, Langhorne 1989). Some of the most elegant studies demonstrating the importance of T cells in effector stage immunity to the asexual stages of Plasmodium were performed using manipulative techniques to deplete the host immune system artificially of mature B lymphocytes. Thus, bursectomised birds and μ-suppressed mice, which did not contain B cells or circulating Ig, could be immunised by natural infection and shown to be protected against subsequent parasite challenge (Ferris et al 1973, Rank & Weidanz 1976, Grun & Weidanz 1981, 1983).

In recent years, the adoptive transfer of immune spleen cells (e.g. Brown et al 1976 a, McDonald & Phillips 1978, Cavacini et al 1986) and of T cell lines (Gross et al 1984, Brake et al 1986) was shown to transfer immunity to various murine malaria parasites. Most recently, Brake et al (1988) demonstrated that a T cell clone transferred protection against P. c. adami infection in nude mice; moreover, this clone was of the Ly-4^+ phenotype.

Major efforts are now being directed towards delineating the anti-malarial T cell response and the relevant Ags which stimulate it. It is necessary to determine the protective effector mechanisms involved and the subset(s) of T lymphocytes which mediates these effects in order to design a vaccine which incorporates epitopes which are appropriately presented to the given T cells. By demonstrating that a single Ly-4^+ T cell clone could transfer protection against malarial challenge adoptively, Brake et al (1988) indicated the possibility that a single T cell site on one parasite Ag could be a target for a protective immune response and gave credence to the T cell immunity approach for subunit vaccine research.

A major caveat of this work was that only one of 10 clones tested was protective. This begs the question whether or not all T cell epitopes on malaria proteins are equally effective in mediating protection, or if the numbers are strictly limited. In view of this latter possibility, the value of analysis of anti-malarial immunity through alternative means of dissection of the host immune response has become apparent.

One such approach used to manipulate the immune system exploits the ability of MAbs against cell surface differentiation Ags to eliminate or block functional subsets of T cells in vivo (Cobbold et al 1984). This lymphocyte elimination results in a significant immunosuppression more specific than that seen following previously used methods (e.g.

anti-lymphocyte serum, thymectomy, splenectomy or irradiation) and without the concomitant disturbance of the experimental system experienced with surgery or whole body irradiation. The precise specificity of MAbs makes them ideal probes for elucidating the lymphocyte sub-population(s) involved in elimination of various infectious agents, including viruses (Leist et al 1987) and trypanosomes (House & Dean 1988). Regarding malaria, the advent of MAb technology realised the correlation between the occurrence of cerebral malaria and Ly-4^+ cells (Grau et al 1986). This new tool has also been deployed to examine immunoprotective rather than immunopathological effects of T cell subsets at challenge infection.

The experiments described in this chapter utilised the immunodepletive ability of MAbs directed against Ly-2- and Ly-4-bearing T cells to examine the roles of these T lymphocyte subsets in the development of a protective immune response to a primary infection of P. c. chabaudi AS in the NIH mouse. The MAbs employed were chosen for their ability to lyse selectively, in the presence of endogenous complement, the target lymphocyte of interest. Through a course of continuous treatment, it was possible to reduce to minimal levels during infection the host complement in naive mice of Ly-4^+ and/or Ly-2^+ T cells. In vivo depletion of one T cell subset enabled analysis of the nature of the remaining subset in relative isolation. Essentially, this methodology represented the negative corollary of the previous adoptive transfers of enriched or cloned T cell populations, and was similar to the study of Süss et al (1988).

An extension of the application of anti-T cell MAbs was to deplete selectively mice of a T cell subset prior to P. c. chabaudi AS infection. Rather than continuing the treatment after challenge in order to ensure Ly-2^+ or Ly-4^+ depletion, infected mice had been previously adult-thymectomised. The procedure of thymectomy , which removed all T cell precursors, followed by peripheral blood T cell subset removal effectively gave rise to animals permanently lacking a T lymphocyte of a given phenotype. The state of immunosuppression achieved by this technique was the same as that reached through regular administration of MAbs during the course of infection. However, in this experimental system, it was possible additionally to examine the degree of protection conferred by any T cells introduced by adoptive transfer. By this methodology, immune or naive splenic T cells enriched for Ly-2^+ or Ly-4^+ lymphocytes by in vitro MAb treatment were transferred adoptively to similarly depleted recipient mice and the protection engendered by the repopulating inocula observed upon challenge with P. vinckei (Kumar et al 1989) or P. yoelii (Vinetz et al 1990). In the present study, this

procedure was taken a step further by the use of stable, in vitro-propagated P. c. chabaudi AS-specific Ly-4^+ T cell lines. The populations chosen for reconstitution of MAb-treated mice were two of the four Ly-4^+ lines previously shown to confer immunity to P. c. chabaudi AS in vivo (Chapters 5-7); these were WEP 775 and WEP 737, putatively classed as T_H1- and T_H2-type T cells on the basis of their reactivity in vivo (Chapter 7). Using the more precise treatment of MAb inoculation rather than lethal irradiation followed by bone marrow reconstitution, in the experiments described herein it was possible to achieve a T cell-depleted or -deficient background without concommitant reduction of the host B cell compartment. This treatment thereby gave conditions suitable for the observation of the maximal possible protective effects of the adoptive transferred P. c. chabaudi AS-primed Ly-4^+ lines upon homologous challenge of such Ly-4^+- and/or Ly-2^+-depleted murine recipients.

8.2 Effects of selective T cell subset depletion on host immunity to Plasmodium chabaudi chabaudi AS

Four groups of 10 NIH naive female mice in each were used for this T cell subset depletion study. Individual mice were immunologically modified by a series of injections of a specific rat anti-mouse MAb prepared from ascitic fluid (2.40). Experimental groups were as follows: mice treated with either anti-Ly-2 or anti-Ly-4 MAbs, or both, or a control group receiving normal rat serum (2.42 a). From each group of 10 mice, all were challenged with 1 $x10^5$ pRBC P. c. chabaudi AS and five were retained to determine the parasitaemia throughout infection. Of the remaining five mice per group, individuals were sacrificed at various times after infection to determine the efficacy of depletion of the relevant T cell subset; this was assessed by immunofluorescence (2.42 c).

The phenotypic characterisation of peripheral blood lymphocytes taken from individual immunodepleted mice sacrificed at weekly intervals after P. c. chabaudi AS challenge is shown in Table 8.2.1. These data indicate that mice treated with monoclonal anti-Ly-2 and -Ly-4 Abs in vivo were depleted of the appropriate subsets of T lymphocytes. Both rat IgG_{2b} reagents used, when administered i.v., gave a substantial and long term depletion. For either anti-Ly-2 or anti-Ly-4 treatment, the level of depletion attained at the time of parasite challenge was approximately half of the maximal values attained thereafter. This showed that the three MAb treatments prior to infection took some time to take effect. That they did was shown for anti-Ly-4 and anti-Ly-2 treatment,

respectively, by 92% and 95% depletion levels recorded in mice 5 d p.i., i.e. 2 d before the next MAb inoculum was to have been administered (Table 8.2.1). Thereafter, weekly inoculation of either or both MAbs, depending on the elimination required, was sufficient to maintain levels of T cell subset depletion at 79% throughout the 60 d time course of this experiment. Thus, treatment in vivo with MAbs recognising Ly-2 or Ly-4 cell surface determinants never reached 100%. This was presumably due either to the presence of Ly-2$^-$ Ly-4$^-$ double negative cells (for double depletion), or possibly the emergence of cells that had lost the expression of either marker (for single MAb depletion).

One interesting aspect of this immunosuppression was that elimination of either Ly-2$^+$ or Ly-4$^+$ T lymphocytes from the peripheral blood of treated animals was accompanied by a slight but significant compensatory increase in cells of the other T cell phenotype (Table 8.2.1). Hence, the level of Ly-2$^+$ lymphocytes detected by immunofluorescence rose from 2.8% to 4.4% upon Ly-4$^+$ depletion; conversely, in Ly-2$^+$-depleted mice, the % of Ly-4-bearing cells rose from 1.5% to 3.5% throughout the course of infection. These marginal increases were, however, not of significance compared to the number of lymphocytes lysed by specific Ab therapy, and were not thought to have affected the immunological status of the depleted mice thus attained. In the case of double-depleted mice, this compensatory increase in either Ly-2$^+$ or Ly-4$^+$ cell numbers obviously did not occur. In mice rendered Ly-2$^-$ Ly-4$^-$, the degree of lymphocyte elimination remained constant from shortly after challenge to the termination of the experiment (Table 8.2.1). In addition, the number of Thy-1$^+$ lymphocytes was also correspondingly reduced. For individual T cell subset depletion, the proportion of those Thy-1-bearing lymphocytes lost reflected the proportion of all T cells bearing either the Ly-2 or Ly-4 cell surface Ag. Thus, although in Ly-2$^+$-depleted animals, up to 97% of the Ly-2$^+$ lymphocytes were removed from the peripheral circulation, this represented only approximately 31% of all Thy-1 cells. This was in accordance with the accepted ratio of Ly-4$^+$: Ly-2$^+$ T cells of 2.6 in naive mice (Goronzy et al 1986).

The effect of different T cell subset depletions on the outcome of P. c. chabaudi AS infection in the NIH mouse is shown in Figs. 8.2.1 & 2. These two figures illustrate identical data but using different scales for quantification of parasitaemia; $\log_{10}$ (8.2.1) and % (8.2.2). They have both been included since each emphasises different features of the course of infection with blood stage malaria parasites. The figures show that there was a marked difference in the patterns of parasitaemia of mice receiving varying T cell

subset depletion treatments upon pRBC challenge. For the control group, for which mice were inoculated with normal rat serum in place of a purified MAb, the course of infection was essentially similar to that seen in normal, immunocompetent host animals (Fig. 1.2). Parasites could be observed in the peripheral blood 4 d after injection and the acute infection reached a peak parasitaemia 9 d p.i., and was then cleared to subpatency by 17 d p.i.. A recrudescent patency characteristic of P. c. chabaudi AS in this murine model took place before final parasite clearance 38 d p.i. (Figs. 8.2.1 & 2). In Ly-2^+-depleted mice, the peak parasitaemia was significantly greater than that observed in the control group ($p < 0.05$) (Fig. 8.2.2). However, after remission of the primary patency to subpatent levels by 19 d p.i., the course of infection of mice deficient of a Ly-2^+ T cell compartment resembled closely that seen in the controls (Fig. 8.2.1). This was characterised by the same length of subpatency (8 d) and similar spans and peak levels of recrudescence (14 d and 0.399% for Ly-2^- mice, and 13 d and 0.266% for NRS-transferred mice). From these data, it can be inferred that Ly-2^+ T cells are not critical for development of a protective immune response to P. c. chabaudi AS. Further, they suggest that lymphocytes of the Ly-2^+ phenotype are only of marginal importance to host acquired resistance to infection, since in their absence the pattern of parasitaemia is broadly similar to, and the degree of parasitaemia only slightly higher than for, undepleted control animals (Figs. 8.2.1 & 2).

In contrast to the negligible effects of Ly-2^+ depletion on the resolution of P. c. chabaudi AS infection, elimination of Ly-4^+ lymphocytes had a profound effect on the outcome of infection. Mice depleted of Ly-4^+ cells developed a significantly higher peak of acute infection than for either Ly-2^+-depleted or undepleted mice ($p < 0.01$) (Figs. 8.2.1 & 2). The parasitaemia was reduced to 18% by d 11 p.i. but did not fall below this level thereafter. Indeed, parasitaemia rose again to 33% (d 18 p.i.) before consistently recording values of between 25-22% from d 24 after challenge until the termination of the experiment (Fig. 8.2.2). This intriguing observation clearly shows the inability of mice lacking Ly-4^+ lymphocytes to clear infection, but the invariability of the parasitaemia sustained and the lack of fatalities sustained also reflects the possibility that an equilibrium had been reached between parasite burden and the host immune response. In Ly-4^+-depleted mice chronically infected with parasitaemia of approximately 25% it may be that rate of parasite invasion of RBC matched the rate of erythropoiesis, so leading to a saturating parasitaemia. In this proposed steady state, the parasitaemia could not rise unless the rate of RBC synthesis and maturation outgrew

the rate of pRBC uptake. Conversely, the parasitaemia could fall only if the supply of available RBC to be infected declined or if an effective immune response was mounted. Since these mice were Ly-4$^+$-deficient, the Ab-dependent response that is predominant in the latter stages of a normal course of P. c. chabaudi AS infection would be severely hampered by the lack of helper T cells that this depletion would cause. Daily examination of blood smears revealed that the rate of erythropoiesis did fall, but not drastically, to give mildly anaemic mice. Under these circumstances, the number of RBC were so few that it forced merozoite invasion of reticulocytes under host immune pressure (P. c. chabaudi AS will infect reticulocytes but usually shows a preference for the older normocyte (Jarra & Brown 1989). Thus, although parasitaemia was maintained at approximately 25% for at least 36 d (certainly longer if the time course were to have been extended), the actual absolute total number of pRBC at this time was substantially less than that recorded at peak primary parasitaemia. Despite the anaemic condition of Ly-4$^+$-deficient mice, free merozoites were observed in thin blood smears quite infrequently, suggesting that those merozoites that were not able to infect RBC upon schizont rupture were effectively eliminated by host immune mechanisms; such mechanisms of parasite clearance were obviously not dependent on Ly-4$^+$ lymphocytes and were probably non-specific in nature. It thus appears that acute P. c. chabaudi AS infection could be partially reduced in mice deficient of a Ly-4$^+$ T cell compartment, but that the parasitaemia could not be cleared.

For mice depleted of both Ly-2$^+$ and Ly-4$^+$ subsets of T lymphocytes, the course of infection was identical to that of Ly-4$^+$-depleted animals. From this group, as for all others, there was a similar prepatent period of 3 d, after which the kinetics of infection were virtually indistinguishable from those of mice lacking Ly-4$^+$ but possessing Ly-2$^+$ cells (Figs. 8.2.1 & 2). The peak parasitaemia recorded during acute infection was 47% on d 8 p.i. (c.f. 51% on d 8 p.i. for Ly-4$^+$-depleted mice), but this insignificant deviance ($p > 0.05$) apart, the levels of parasitaemia recorded in double-depleted mice and those depleted of only the Ly-4$^+$ subset were not significantly different ($p > 0.05$). This similarity between the courses of infection of P. c. chabaudi AS in mice treated with MAbs against cells of both T cell phenotypes and against cells of the Ly-4$^+$ subset alone confirmed the finding from Ly-2$^+$-depleted animals that Ly-2$^+$ T cells play a minimal role in the protective immune response to the erythrocytic stages of this murine malaria parasite. The fact that no greater protection was conferred by the presence of Ly-2-bearing lymphocytes in mice depleted of the Ly-4$^+$ subset over that exhibited by

double-depleted mice also indicates that there was no synergistic activity between populations of the two different T cell subsets towards a protective immune response, and moreover, suggested that they acted independently of each other in vivo. Another finding apparent from examination of the data collected from the double depletion group was the observation of an initial reduction in the degree of parasitaemia at or around crisis (d 9-11 p.i.) in the relative absence of T lymphocytes (Fig. 8.2.2). Results showed that any further resolution of acute infection necessitated the presence of Ly-4^+ cells, but this transient reduction of peripheral blood parasitaemia may be due to the mediation of activated macrophages, the products of which may be directly parasiticidal.

8.3 Adoptive transfer of T cell lines to selectively T cell-depleted syngeneic recipients.

For each of three categories of adult-thymectomised, sham-thymectomised and non-thymectomised NIH female mice, every animal was immunosuppressed through a series of treatment of MAbs prior to malaria challenge. As for 8.2, the MAbs used were specific for Ly-2 or Ly-4 T lymphocyte surface markers and groups were set up to include either Ly-4^+- or Ly-2^+-depleted, as well as double-depleted, mice. Adult-thymectomised mice were boxed in groups of 10 to allow sacrifice at weekly intervals of individuals to monitor for effective T cell subset depletion by the methodology used (2.42 b & c); sham- and non-thymectomised animals were arranged in groups of five mice, each of which was examined daily following infection for peripheral blood parasitaemia. In addition, control groups of undepleted adult-thymectomised, sham-thymectomised and immunocompetent naive mice were prepared.

A further nine groups of five mice each were set up; these consisted of three groups of either adult-, sham- or non-thymectomised mice. Within each category of recipient mouse, animals were adoptively transferred either WEP 775, WEP 737 or naive splenic T cells. In each instance, the inoculum used was 4.0 $x10^7$ viable lymphocytes inoculated i.v.. Shortly after adoptive transfer, recipient mice were challenged with 1 $x10^5$ pRBC P. c. chabaudi AS, this being the standard parasite dose to infect all experimental mice used in this study.

As can be seen from Table 8.3.1, the depletion of subsets of T lymphocytes from mice by MAb treatment following adult thymectomy was completely satisfactory. The levels of depletion achieved by this technique were comparable with those attained through a series of MAb inoculations both before and after pRBC challenge (Table 8.2.1). Indeed,

the degrees of elimination reached at the time of challenge, d 0, were near maximum levels, and certainly higher than those observed in mice treated with MAbs alone (Table 8.2.1 & 8.3.1). The satisfactory depletions attained showed that this alternative methodology employed to prevent the elimination of Ly-4$^+$ cells upon adoptive transfer to mice containing circulating anti-Ly-4 MAbs was effective in maintaining T lymphocyte subset levels at a minimum up to 60 d after P. c. chabaudi AS challenge (when the experiment was ended), i.e. 72 d after the latter of two MAb injections (2.42 b). The fact that the number of lymphocytes of either Ly-2$^+$ or/and Ly-4$^+$ population remained the same despite the lack of continuous MAb therapy showed that once the complement of mature peripheral circulatory T cells had been eliminated, this could not be replaced by thymic progenitors, as would normally be the case, due to the surgical removal of the thymus from mice at five weeks of age (2.15). The combination of adult thymectomy and peripheral blood T cell elimination thus produced mice with a permanent deficiency of mature Ly-4$^+$ and/or Ly-2$^+$ lymphocytes, but without affecting the B cell compartment of the host immune system. The methodology described therefore appeared very suitable for the T cell deprivation of host animals prior to the adoptive transfer of P. c. chabaudi AS-specific Ly-4$^+$ lines at homologous challenge.

The efficacy of adult thymectomy and MAb pretreatment was shown not only by phenotypic characterisation of those lymphocytes remaining (Table 8.3.1), but also by the courses of infection upon P. c. chabaudi AS challenge of depleted animals (Fig. 8.3.1). The parasitaemias attained in thymectomised mice were remarkably similar to those observed in MAb-treated mice (Fig. 8.2.1). Hence, Ly-2$^+$-depleted animals had the same kinetics of parasite clearance as did adult-thymectomised mice which did not receive MAb treatment. For the latter control group, peripheral blood T cells of both phenotypes had not been eliminated, but lacking thymic replenishment, they appeared not to provide any observable protection; indeed, in these mice, a secondary recrudescence was detected, which was not present in animals devoid of Ly-2$^+$ cells. Thus, a possible immunosuppressive role for those relatively few remaining circulatory Ly-2-bearing lymphocytes in non-MAb-treated mice is suggested. For mice given anti-Ly-4 MAbs following thymus ablation, the resulting infection showed an identical pattern to that seen in similarly thymectomised animals that received both anti-Ly-4 and anti-Ly-2 MAbs (Fig. 8.3.1). In both cases, the ascending primary parasitaemia was the same as for the other two groups studied, but after a significantly higher peak parasitaemia ($p < 0.01$), these animals failed to clear the post-crisis pRBC from the

peripheral blood. The chronic infection that ensued was very similar to that seen previously (Fig. 8.2.1). The identity between Ly-2$^+$-deprived animals and those lacking both T cell subsets confirmed both the critical part played by T cells of the Ly-4$^+$ phenotype in clearance of P. c. chabaudi AS, and also the apparent inability of Ly-2$^+$ cells to contribute to the anti-parasitic immune response, at least towards asexual blood stages of malaria.

In sham-thymectomised recipients of various depletive treatments (Fig. 8.3.2), the patterns of parasitaemia were each like that described previously for P. c. chabaudi AS infection of thymus-ablated animals (Fig. 8.3.1). Thus, Ly-4$^+$-depleted or double-depleted mice were also incapable of clearing infection, which again reached a plateau of parasitaemia. In addition, animals rendered deficient of the Ly-2$^+$ T cell compartment resolved both acute and secondary waves of infection with the same characteristics as sham controls (Fig. 8.3.2). Furthermore, for non-thymectomised mice challenged with the same parasite (Fig. 8.3.3), the courses of infection for each of the four groups showed the same features as for the corresponding animals in the adult- or sham-thymectomy categories (Figs. 8.3.1 & 2). The similarity between the parasitaemias observed in mice receiving identical MAb treatments in sham- and non-thymectomised situations indicated that the trauma suffered by mice undergoing sham thymectomy had a negligible effect on the outcome of the subsequent malaria infection. Hence, mice that had been anaesthetised and surgically manipulated without thymus removal suffered no ill effects and showed the same levels of protective immunity to P. c. chabaudi AS challenge as found in non-thymectomised mice, before or after T cell subset depletion (Figs. 8.3.2 & 3). This was a pleasing finding, since surgical procedure per se was not intended to have any serious effects on the immunological state of the host animals. Sham-thymectomised mice were only acting as a negative control for the adult-thymectomised group. The similarities between the courses of infection not only in non- and sham-thymectomised mice (Figs. 8.3.2 & 3), but also in sham- and adult-thymectomised mice (Figs. 8.3.1 & 2) challenged with P. c. chabaudi AS, showed that thymus removal had little or no adverse effect on the outcome of infection. In each case, the protective immunity engendered by the host immune system appeared to be dependent solely on the subset of T cells surviving MAb depletion. Thus, Ly-4$^+$-depleted mice were incapable of clearing blood stream parasites whilst Ly-2$^+$ depletion had no such adverse effects on the kinetics of pRBC destruction. It is apparent that MAb treatment to remove peripheral blood T lymphocytes was sufficient to effectively eliminate the

mature T cell populations of either Ly-2$^+$ or Ly-4$^+$ phenotype, and that an intact thymus was incapable of reconstituting the depleted mature circulatory lymphocytes, at least during the time course of a primary P. c. chabaudi AS infection. Moreover, this study showed that two quite large inocula of MAb suitably in advance of parasite challenge (the latter injection being 12 d before infection) were sufficient to deplete to residual levels peripheral circulatory populations of either T lymphocyte subset by the time of challenge (Table 8.3.1), thus negating the necessity for continuous MAb treatment during infection (Table 8.2.1). Prior to this study, neither the long-lasting effect of MAb therapy on the constitution of the peripheral blood, or the slowness of repletion of each eliminated T cell population by thymic precursors had not been appreciated fully.

As well as confirming that adult thymectomy had little effect upon mature immune function, direct comparisons of each MAb depletion for each of the three immunologically manipulated conditions (Figs. 8.3.4-7) emphasised the roles of each T lymphocyte subset in the mediation of a protective response to P. c. chabaudi AS in immunologically intact host animals. There was no observable difference between the effects of anti-Ly-4 and of combined anti-Ly-4 and -Ly-2 treatments (Figs. 8.3.4 & 5), indicating that it was the loss of the Ly-4$^+$ population that prevented the mediation of an effector immune function. The dependence of host immunity upon cells of the Ly-4$^+$ T lymphocyte subset was confirmed by the lack of protective activity shown by Ly-2-bearing lymphocytes towards blood stage malarial infection. The course of P. c. chabaudi AS infection in Ly-2$^+$-depleted mice (Fig. 8.3.6) was essentially no different from that in untreated mice (Fig. 8.3.7). A consistent feature, however, was a slight secondary recrudescence in mice possessing a complete mature lymphocyte population (Fig. 8.3.7), but not in mice deficient of Ly-2$^+$ lymphocytes (Fig. 8.3.6). The extension of infection at patent levels through a second breakthrough parasite population observed in adult-thymectomised or competent mice (Fig. 8.3.7) was thought to be caused by an immunosuppressive effect of peripheral Ly-2$^+$ cells, as when these were removed (Fig. 8.3.6), pertebation of infection ceased.

Concerning the reconstitution of T cell-depleted mice by adoptive transfer of P. c. chabaudi AS-raised Ly-4$^+$ lymphocyte lines, the inoculation of both WEP 775 and WEP 737 cells provided an immune protection; this was apparent as reconstitution of the activity provided in undepleted host animals by peripheral blood lymphocytes belonging to the Ly-4$^+$ T cell subset. In mice depleted of either the Ly-4$^+$ population or of both T

lymphocyte subsets, similar patterns of ascending primary parasitaemia were observed for all experimental groups (Figs. 8.3.8 & 9), showing that the kinetics of parasite multiplication prior to the mounting of an immune response were identical in immunodepressed and competent mice alike. However, thereafter, profound differences occurred in the parasitaemias of T cell-depleted mice and those depleted and then reconstituted. These differences were equally apparent in animals deficient of Ly-4-bearing lymphocytes (Fig. 8.3.8) and those lacking both Ly-4^+ and Ly-2^+ T cell subsets (Fig. 8.3.9), due to the absence of immunity to the asexual stages of P. c. chabaudi AS resident within the Ly-2^+ compartment of the murine immune system. The peak of acute infection was reached at the same time, d 10 p.i. for all groups, but the level of parasitaemia attained was significantly lower ($p < 0.05$) for mice receiving either WEP 775 or WEP 737 cells than for their non-recontituted counterparts (Figs. 8.3.8 & 9). Mice depleted of the Ly-4^+ population, either alone or with concurrent Ly-2^+ cell depletion, were incapable of clearing P. c. chabaudi AS to subpatent levels, as noted previously. Likewise, challenged mice receiving naive splenic T cells were unable to control infection, which remained chronically patent (Figs. 8.3.8 & 9). Unprimed splenic T cells did provide some protection upon adoptive transfer, manifested by a significantly reduced parasitaemia with respect to non-reconstituted mice ($p < 0.01$) (Figs. 8.3.8 & 9). For both groups of depleted recipients, however, mice inoculated with naive splenic T cells at the time of challenge always exhibited a parasitaemia of not less than 1% throughout infection; thus, adoptively transferred lymphocytes not previously exposed to P. c. chabaudi AS did not have the capacity to replace the resistance to homologous infection normally provided by the host complement of mature Ly-4-bearing lymphocytes.

By comparison, repopulating inocula of malaria-primed Ly-4^+ lymphocyte lines, taken either from d 16 of a primary infection (WEP 775) or after recovery from a secondary infection (WEP 737), transferred the potential to control P. c. chabaudi AS challenge. Hence, MAb-treated recipients of either Ly-4^+ line showed similar disease characteristics as did those mice which had been adult-thymectomised without further manipulation (these already having been shown to be immunocompetent with regard to P. c. chabaudi AS infection; Fig. 8.3.7). Otherwise immunologically incompetent mice reconstituted with populations of primed Ly-4^+ lines, stable for phenotype and presumably also function, exhibited courses of infection typical of mice possessing a fully intact immune system. Thus, primary parasitaemia was resolved by d 18 p.i.,

followed by a subpatency of 4-5 d (WEP 737) or 7-8 d (WEP 775) and parasite clearance by d 50 p.i. (Figs. 8.3.8 & 9). That the manifestations of protection observed upon adoptive transfer of each of the Ly-4$^+$ lines were actually engendered by these lymphocytes was apparent by examining the kinetics of infection, which were characteristic of the Ly-4-bearing lymphocyte populations inoculated. Hence, in both Ly-4$^+$-depleted and double-depleted mice, recipients of WEP 775 recrudesced later than did recipients of WEP 737 (Figs. 8.3.8 & 9). This divergence in the onset of recrudescence in mice receiving these two Ly-4$^+$ lines was characteristic of their adoptive transfer and had been noted previously in both naive and immunosuppressed mice (Chapters 5-7).

For Ly-2$^+$-depleted animals, the protection conferred by adoptive transfer of Ly-4$^+$ lymphocytes was not so obvious; this was not because of any lesser activity of the reconstituted lymphocytes but rather due to the fact that mice lacking a peripheral circulatory Ly-2$^+$ population were still quite capable of controlling infection (Fig. 8.3.10). Nevertheless, the specific anti-plasmodial reactivity given upon transfer of the P. c. chabaudi AS-primed Ly-4$^+$ lines was distinguishable from the acquired resistance to primary infection of the background host immune response. For each group of mice, the pattern and level of primary parasitaemia was very similar (Fig. 8.3.10), showing that Ly-2$^+$-depletion or Ly-4$^+$ line reconstitution had no apparent effect on the outcome of the acute infection. Thereafter, the subsequent courses of infection in adult-thymectomised and in adult-thymectomised and Ly-2$^+$-depleted mice were alike, as illustrated by a similar onset, peak and clearance of recrudescent parasitaemia. This again served to emphasise the lack of acquired resistance resident within the Ly-2$^+$ T cell subset, for its removal had no adverse effect on the mediation of anti-plasmodial immunity. For mice receiving either WEP 775 or WEP 737 Ly-4$^+$ cells, a substantial degree of protection was conferred by the adoptive transfer of these populations; this was manifested as significantly longer subpatencies ($p < 0.01$), followed by lower ($p < 0.01$) and shorter lasting ($p < 0.01$) recrudescences (Fig. 8.3.10), compared to either splenic T cell recipients or unreconstituted, Ly-2$^+$-depleted mice. In fact, mice given an inoculum of naive splenic T lymphocytes showed the same level and length of recrudescence as did either unreconstituted control group and actually recrudesced much more quickly, subpatency lasting only 24 hr (c.f. 6-7 d in thymectomised mice or thymus-ablated, anti-Ly-2 MAb-treated mice) (Fig. 8.3.10). Therefore, as for their adoptive transfer to Ly-4$^+$-deficient recipients (Figs.

8.3.8 & 9), unprimed populations of spleen cells enriched for T lymphocytes conferred little specific protection upon P. c. chabaudi AS challenge.

Adoptive transfer of the WEP 775 line appeared to restore the Ly-4$^+$ activity to mice depleted of this T cell subset (Fig. 8.3.11). Such animals showed a course of infection not unlike that for mice that had been adult-thymectomised, but still retaining both subsets of circulatory T cells. The best protection conferred by the adoptive transfer of WEP 775 lymphocytes was to Ly-2$^+$-depleted mice. Compared to Ly-4$^+$- or double-depleted recipients, enhanced protection was detectable as an extended subpatency (16 d rather than 7-8 d), a significantly lower recrudescence ($p < 0.01$) and a shorter length of secondary patency (8 d rather than 13-14 d). It is not clear whether this greater immunity in Ly-2$^+$-depleted animals was due to the removal of Ly-2$^+$ cells, and therefore a loss of possible suppressor activity, or alternatively, the presence of a full, undepleted Ly-4$^+$ T cell compartment, or perhaps both. As for inoculation of WEP775 cells, transfer of WEP 737 lymphocytes conferred similar levels of protection to variously depleted recipients hosts of P. c. chabaudi AS infection (Fig. 8.3.12). In this instance, however, reconstituted Ly-4$^+$-depleted mice recrudesced at the same time as did the adult-thymectomised control, and not after, as for WEP 775-transferred mice (Fig. 8.3.11). This discrepancy in the appearance of recrudescence in recipients of the two Ly-4$^+$ lines did not detract from the protective immunity conferred to T cell-depleted mice upon reconstitution of either population. It did serve to stress the fact that although each Ly-4$^+$ line was fully effective at restoring immune reactivity to that of levels seen in unmanipulated animals, the underlying mechanisms by which this protection was mediated by lymphocytes belonging to either WEP 775 or WEP 737 populations are very probably different.

Figure 8.3.13 shows the courses of infection upon adoptive transfer of naive splenic T cells to mice deficient of either or both Ly-2$^+$ or Ly-4$^+$ T cell subsets. It can be seen that this mixed population of unprimed T lymphocytes of both phenotypes was unable to restore Ly-4$^+$ function to mice from which this T cell compartment had been removed, and hence they suffered chronic blood-borne infections. For mice that controlled infection, similar magnitudes of both primary and secondary parasitaemias were recorded for Ly-2$^+$-depleted, naive splenic T lymphocyte repopulated animals and for adult-thymectomised mice (Fig. 8.3.13). The additional presence of naive spleen cells in Ly-2$^+$-deficient mice therefore did not seem to enhance the level of protective immunity observed. The transient secondary recrudescence in such Ly-2$^+$-depleted

recipients of a splenic T cell preparation (Fig. 8.3.13) was presumably due to partial reconstitution of Ly-2^+ lymphocytes to these mice. It has already been shown that extension of P. c. chabaudi AS infection through the appearance of a tertiary patency occurred in intact (Fig. 8.3.7) but not in Ly-2^+-depleted (Fig. 8.3.6) mice. Also, WEP 775- and WEP 737-transferred recipients exhibited a secondary recrudescence in all situations except after Ly-2^+ T cell subset elimination (Figs. 8.3.11 & 12). It would thus appear that the presence of a Ly-2^+ population was a necessary prerequisite for the appearance of breakthrough variant P. c. chabaudi AS pRBC, presumably due to immunosuppression of an otherwise usually immunologically competent host immune system.

8.4 Discussion

The experiments described in this chapter were designed to evaluate the roles of the major T lymphocyte subsets of Ly-2 and Ly-4-bearing cells in the immune response of an unprimed host to primary infection with P. c. chabaudi AS. As well as studying the ability of the background immunity of the host immune system to provide sufficient acquired resistance to control challenge infection, the capacity of in vivo-primed, in vitro-propagated P. c. chabaudi AS-specific Ly-4^+ lines to transfer immunity adoptively was examined in a controlled system selectively manipulated to deplete either or both subsets of T lymphocytes.

Selective depletion was achieved readily with rat IgG_{2b} monoclonal anti-Ly-2 or -Ly-4 MAbs, which, when given i.v., were highly immunosuppressive. For example, mice treated with the anti-Ly-2 MAb showed a specific depletion of 97% compared to control mice (Table 8.2.1). This degree of elimination compares favourably with those recorded originally using the identical MAbs (Cobbold et al 1984). In each instance, animals depleted by serotherapy still retained some residual Ly-2^+ or Ly-4^+ cells, as also reported in other studies (Wofsy & Seaman 1985, Howard et al 1989). In this study, effective depletion was assayed by immunofluorescence of peripheral blood lymphocytes only, although it was presumed that all mature T lymphocytes would be exposed to circulating MAbs and therefore be susceptible to lysis. This assumption was not unreasonable since IgG_{2b} Abs have been found to be very effective in eliminating the appropriate T cells in the blood, spleen, lymph nodes and bone marrow after in vivo administration (Cobbold et al 1984, 1985, Nash et al 1987).

The first protocol followed for in vivo manipulation used weekly inoculations of MAbs to

achieve T cell depletion (after Süss et al 1988). It was shown subsequently that the dose of MAbs used was considerably in excess of that required for effective immunosuppression. Adult-thymectomised mice that received just two injections of IgG_{2b} Ab prior to challenge exhibited a level of depletion of peripheral blood T cells as great as that observed in mice receiving more regular treatments for the full time course of infection (60 d) (Tables 8.2.1 & 8.3.1). This finding is little surprising in view of the fact that Cobbold et al (1984, 1985) found that a profound immunodepletion of target T lymphocytes could be sustained for periods up to one year in adult-thymectomised animals. They showed that over a 12 month observation period, there was no detectable change in Ly-4 or Ly-2 expression in appropriately depleted mice. This demonstrated that any remaining Ly-2- or Ly-4-bearing T cells were unable to repopulate deficient animals and that, under these conditions, the mature $Ly\text{-}2^-$ or $Ly\text{-}4^-$ phenotypes were stable and irreversible and not subject to phenotypic switching. Previously, long term immunosuppression could only be achieved, albeit inefficiently, by using populations of T lymphocytes depleted of $Ly\text{-}1^+$ or $Ly\text{-}2^+$ subsets in vitro to reconstitute lethally irradiated, thymectomised mice (Huber et al 1976). This methodology was used, amongst others, by Jayawardena et al (1979) and by Leke et al (1981) to study experimental malaria infection. Such T cell-depleted animals may have a contribution from radio-resistant lymphocytes (LeFrancois & Bevan 1984), a complication which is avoided by in vivo MAb treatment. Moreover, the production of an Ab response to the injected MAbs that is a feature of in vivo depletion of primates and calves (Howard et al 1989) was not observed with murine recipients. Mice inoculated with rat IgG_{2b} MAbs directed against either Ly-2 or Ly-4 Ags become tolerant to those Abs (Benjamin & Waldmann 1986, Gutstein et al 1986). Furthermore, it has been shown for the murine system that the most effective immunosuppression, which leads to the induction of tolerance, is obtained with Abs that can both deplete T cells and exert functional inhibition (Cobbold et al 1985). These workers showed that the best suited Ig isotype to perform this task was the IgG_{2b} class used in this study.

The first depletion experiment using these rat MAbs to murine $Ly\text{-}2^+$ and $Ly\text{-}4^+$ T lymphocytes gave essentially the same results as those obtained by Süss et al (1988), also with P. c. chabaudi AS. The strain of mouse used was C57BL/6 ($H\text{-}2^b$) rather than NIH ($H\text{-}2^q$) used here, but for both, infection with this malaria parasite is usually non-lethal (Stevenson et al 1982). Both sets of data showed unequivocally that $Ly\text{-}4^+$ cells are an essential component of the protective immune response to P. c. chabaudi AS.

As described herein, Süss et al (1988) showed that mice lacking a peripheral Ly-4$^+$ lymphocyte compartment had significantly higher primary parasitaemias than did control groups, which they were unable to reduce below 20% for the duration of the experiment. Again, the chronic parasitaemia was very stable, a phenomenon also reported for P. c. chabaudi infection of adult-thymectomised and irradiated mice (Leke et al 1981). Süss et al attributed the partial limitation of parasitaemia to a persistent reticulocytosis (this parasite does not show a preference for younger RBC), and also the low titres of transient malaria-specific IgM Abs in the peripheral blood of such Ly-4$^+$-depleted mice challenged with P. c. chabaudi AS.

Neither this study nor the one by Süss et al (1988), however, allowed delineation of the functions of the Ly-4-bearing cells in vivo. It is possible that they mediate their effect as helper cells either for production of specific Ab (B cell-dependent mechanisms), or for the generation of specific cytotoxic T cells, or alternatively as initiators of macrophage activation and delayed-type hypersensitivity reactions (B cell-independent mechanisms). The anti-Ly-4 MAb used could not discriminate between T_H1- and T_H2-type cells and thus further dissection of the effector mechanisms of protection involving these lymphocytes necessitated the adoptive transfer of T cell lines of defined phenotypic and functional specificity.

The initial depletion experiment showed that mice deficient of mature Ly-2-bearing lymphocytes were quite capable of mounting a parasite-clearing protective response similar to that observed in intact animals, a finding which concurred with the studies of Süss et al (1988) and of Kumar et al (1989), with P. c. chabaudi AS and P. vinckei, respectively. Süss et al expressed the opinion that, under experimental conditions, a role for Ly-2$^+$ cells in host immunity to P. c. chabaudi AS infection could not be discounted since it is possible that in the absence of Ly-4$^+$ cells and their soluble mediators, Ly-2$^+$ effector cells could not be activated appropriately. Although it is not known whether Ly-2-bearing T lymphocytes in vivo require an exogenous IL-2 source (supplied, at least in vitro, by Ly-4$^+$ cells), in a second experiment, recombinant IL-2 was given to Ly-4$^+$-depleted mice. This did not affect parasitaemia significantly, challenged recipients still being incapable of clearing infection (Süss et al 1988). Recently, Podoba & Stevenson (1989) showed a role for Ly-2$^+$ T cells in immunity to the blood stages of P. c. chabaudi AS infection. In vivo depletion of Ly-2-bearing lymphocytes had no effect on the prepatent period or peak parasitaemia. However, compared to controls, Ly-2$^+$-depleted mice had two recrudescent patencies. These

workers consider that both T cell subsets contribute to acquired immunity to P. c. chabaudi AS pRBC, but in the absence of Ly-4$^+$ lymphocytes, those of the Ly-2$^+$ phenotype cannot function (Stevenson, M.M., personal communication). These data, together with adoptive transfer studies, such as those by Cavacini et al (1986) and by Vinetz et al (1990) in P. c. adami and P. yoelii infections, suggest that T lymphocytes belonging to the Ly-2$^+$ subset do not play a major protective role in host immunity to murine malarias. These findings are in direct contrast to the crucial role proposed for Ly-2$^+$ cells in the protective response against the exoerythrocytic stages of Plasmodium (Schofield et al 1987, Weiss et al 1988). Indeed, Ly-2-bearing cells may have a deleterious effect upon the host immune response to infection with P. c. chabaudi AS. The slight immunosuppressive effect in the presence of Ly-2$^+$ lymphocytes which is absent upon their removal agrees with the suggestion that activated T suppressor cells may contribute to the immunosuppression associated with P. falciparum malaria in humans (Troye-Blomberg et al 1984, Riley et al 1989 a).

In the parallel experiments described here and by Süss et al (1988), MAb treatment started shortly prior to P. c. chabaudi AS challenge and continued throughout the course of the ensuing infections. Most recently, this methodology has been refined by initiating Ly-4$^+$ and/or Ly-2$^+$ T cell removal at various times during infection, in order to examine whether or not the presence of Ly-4$^+$ lymphocytes is required throughout an infection to exert a protective response (Langhorne et al 1990). It was found that removal of the Ly-4$^+$ T cell subset at the beginning of infection or during the acute phase of infection rendered mice incapable of reducing their parasite load. In contrast, loss of this T cell phenotype after this time affected the parasitaemia only transiently, manifested as a slightly enhanced recrudescence, which was subsequently resolved. Later still, depletion of the Ly-4$^+$ compartment from mice recovered from a primary infection had no detectable effect on the protective secondary response to P. c. chabaudi AS upon rechallenge. Collectively, these data showed that Ly-4$^+$ cells are essential only to control the primary parasitaemia and that recrudescent parasitaemia can be cleared in their absence. Measurement of malaria-specific Abs during infection revealed that the ability to control parasitaemia correlated with the appearance of P. c. chabaudi AS-specific IgG. Langhorne et al suggested a mechanism of parasite control requiring the presence of Ly-4$^+$ lymphocytes during the acute pre-IgG period. These would presumably be of the T_H1 subset and limit acute parasitaemia through Ab-independent machanisms. In view of this, it is interesting to note that the Ly-4$^+$ lines derived from

d 16 and d 20 of a primary P. c. chabaudi AS infection were shown to have a B cell-independent reactivity in vivo (Chapter 7). Control of parasitaemia in the second phase of infection correlated both with the ability to make IgG Abs and with the prevalence of Ly-4$^+$ T_H2-type helper cells for Ab synthesis (Langhorne et al 1989 a). It is evident that Ab-mediated resistance can occur in the absence of the Ly-4$^+$ helper function, but the presence of these lymphocytes is desirable, if only to help B cells switch from IgM to IgG production. Again, it is relevant that those Ly-4$^+$ lines raised to P. c. chabaudi AS in vivo taken from rechallenged mice exhibited a B cell dependency to confer protection in vivo (Chapter 7).

The second series of depletions, for which mice were reconstituted with Ly-4$^+$ lines, necessitated the surgical removal of the thymus in advance of parasite challenge. Comparison of the courses of infection in these animals compared to those in sham-thymectomised or non-thymectomised control mice revealed that thymectomy at the age of five weeks had a negligible effect on the outcome of P. c. chabaudi AS infection in mice depleted by serotherapy. It has been reported that thymectomy in adult life results in little detectable effect on levels of recirculating lymphocytes, and, further, that the primary immune response is unimpaired for up to one year after surgery (Taylor 1965, Millar 1965). This suggests the longevity of peripheral blood lymphocytes and emphasises the importance of MAb depletion used here. With regard to malaria infection, Seitz (1972) showed that thymectomy at six weeks of age did not influence the course of P. berghei infection if mice were allowed two months recuperation after surgery. These findings together suggest that the thymus plays no direct part in host immunity to ongoing malaria infection, except for its providing T lymphocytes. These populations migrate from the thymus to peripheral lymphoid tissues mainly during the neonatal period, and are maintained throughout the life of the animal (Weissman 1967). In contrast to adult thymectomy, neonatal thymus removal results in severe depletion of all peripheral T lymphocytes and major defects in both humoral and cellular immune responses (Millar 1962, Good et al 1962). Hence, for malaria, a more severe disease in neonatally-thymectomised animals and a shortened life span compared to that of infected controls was found for P. berghei infection of rats (Brown et al 1968 a) and of hamsters (Chapman & Hanson 1971). Seitz (1976), however, reported that neonatal thymectomy had no influence on the outcome of P. berghei challenge; it may be that species differences in host resistance may account for this discrepancy, but this is not established. There is also the possibility that thymus ablation of newborn mice, which

leads to a lesion in the mature T cell component of the immune system, results in the development of alternative effector immune mechanisms. This possibility has not been reported for malaria, but has for infection of congenitally athymic mice with the facultative intracellular parasite Listeria monocytogenes (Nickol & Bonventre 1977). Nude mice showed heightened innate resistance to this bacterial pathogen, a phenomenon attributed to the activation of fixed tissue macrophages. Activation of macrophages with enhanced microbicidal activities appeared to arise as a direct result of T cell deficiency, since thymus implantation abrogated the high resistance of nude mice to L. monocytogenes challenge. In support of this possible mechanism of parasite control not requiring the presence of T lymphocytes are data showing products of activated macrophages to be toxic for a variety of plasmodia in vitro (Clark & Hunt 1983, Dockrell & Playfair 1983, Ockenhouse et al 1984). It has to be stressed, however, that the production of such toxic mediators normally occurs as a result of T cell-induced activation in vivo, when activated macrophages can be envisaged to play a role in Ab-independent mechanisms of immunity to murine malarias, but their activation in isolation of T cells is difficult to reconcile.

The importance of the Ly-4^+ T cell subset to immunity induced by infection with P. c. chabaudi AS in NIH mice was examined both by in vivo depletion of this population, and of Ly-2^+ cells (discussed previously), and by reconstitution of these depleted animals with homologously primed in vitro-propagated Ly-4^+ lines. Adoptive transfer of malaria-specific T cell lines to recipients selectively depleted of either one or both Ly-4^+ or Ly-2^+ T cell compartments had not previously been performed. Kumar et al (1989) did, however, perform a similar study by transferring immune spleen cells from freshly sacrificed mice recovered from a primary infection with P. vinckei. In the BALB/c mouse strain used, P. vinckei is lethal even in immunocompetent hosts, but solid immunity can be induced by two successive drug-cured challenges. It was demonstrated that Ly-4-bearing lymphocytes are absolutely necessary for immunity to P. vinckei in these experimentally manipulated animals. Ly-4^+-depleted mice that were previously immune to P. vinckei died after inoculation of homologous pRBC. Furthermore, the transfer of a population of Ly-4^+-enriched splenic T cells, taken from immune donor mice, to Ly-4^+-deficient recipients reconstituted immunity. These results very closely concur with those described in this chapter, albeit that Kumar et al used a more complex host-parasite system. The fact that P. vinckei is uniformly lethal upon primary infection necessitated the artificial induction of immunity through drug cure to produce

animals that could then be Ly-4$^+$-depleted and reconstituted. This, however, did not invalidate the findings which bear comparison with those in the non-lethal P. c. chabaudi AS/NIH mouse model.

For the study of immunity to P. vinckei (Kumar et al 1989), splenectomy of mice rendered immune by drug-cured infection caused complete loss of immunity. Repletion of immune splenectomised mice with their own separated spleen cells failed to reconstitute protection, showing that an architecturally intact spleen was necessary for the expression of immunity. From this it can be inferred that, although Ly-4$^+$ lymphocytes are important mediators of anti-malarial immunity, they are alone insufficient to provide protection. The spleen is required for destroying pRBC in rodent malarias and for the characteristic drop in parasitaemia, crisis, during acute infection. Crisis is associated with a shift from a closed to an open circulation through the red pulp of the spleen (Quinn & Wyler 1979 b), which results in the exposure of pRBC to a high concentration of cellular elements.

In the present study, P. c. chabaudi AS-specific Ly-4$^+$ lymphocytes were demonstrated to be quite sufficient to restore immunity to mice otherwise deficient of Ly-4$^+$ cells. This was not the case, however, for the corresponding P. vinckei situation, where, despite the requirement for lymphocytes of the Ly-4$^+$ T cell subset, these cells were not sufficient to confer immunity (Kumar et al 1989). Whereas adoptive transfer of immune Ly-4-bearing cells to Ly-4$^+$-depleted immune mice reconstituted immunity in the majority of mice, the inoculation of the same immune Ly-4$^+$ cells into naive or nude mice had no protective effect on P. vinckei infection, which was invariably fatal. This differed from observations using P. c. adami, in which transfer of immune spleen cells (Cavacini et al 1986), a Ly-4$^+$ line (Brake et al 1986) or a Ly-4$^+$ clone (Brake et al 1988) could transfer protection to prevent an otherwise fatal infection in nude mice. Although it is conceivable that the failure of T cell transfer to effect immunity against P. vinckei in naive mice may be due to a requirement for the activation or recruitment of another cell type, such as monocytes, in the spleen, Kumar et al (1989) proposed that the more virulent nature of P. vinckei (i.e. its rapid rate of multiplication) probably explains the failure of Ly-4$^+$ transfer to render unprimed animals immune. The successful transfer of protection to Ly-4$^+$-depleted but not to naive mice is evidence that the Ly-4$^+$ T lymphocyte itself is not the effector cell, but instead an intermediary mediator of anti-plasmodial immunity. The transfer of immunity to P. c. chabaudi AS and to P. c. adami with Ly-4-bearing T cell clones that secrete IFN-γ (Chapter 9 and

Brake et al 1988) suggests that this lymphokine may activate macrophages or monocytes to kill intracellular malaria parasites.

Depletion of the Ly-2$^+$ T lymphocyte complement of mice suffering from P. c. chabaudi AS (this chapter and Süss et al 1988) and P. vinckei (Kumar et al 1989) infections did not affect the mediation of host immunity. Likewise, Vinetz et al (1990) showed that elimination of Ly-2$^+$ cells during primary infection of P. yoelii in BALB/c mice did not diminish the capacity of these mice to resolve their infections. Furthermore, adoptive transfer of immune Ly-2$^+$ splenic lymphocytes from BALB/c and C57BL/10 donors to congenitally athymic BALB/c or C57BL/10 recipients, respectively, did not transfer immunity, and, upon P. yoelii challenge, recipient mice succumbed to infection. In addition, inoculation of Ly-4$^+$-depleted CBA/CaJ mice with P. yoelii-primed Ly-2$^+$ cells also did not give protection to homologous challenge, from which all mice died. This methodology was the corollary of the transfer of Ly-4$^+$ lines primed to P. c. chabaudi AS to Ly-4$^+$- and/or Ly-2$^+$-depleted recipients at homologous challenge that is described in this chapter. Vinetz et al (1990) failed to observe any direct effect of Ly-2-bearing T cells on immunity to the asexual stages of P. yoelii, as they were neither protective nor did they enhance immunity. This supported the earlier work of Jayawardena et al (1982) that Ly-1$^+$ but not Ly-2$^+$ cells could transfer protection to P. yoelii infection. Both these experiments were in disagreement with the claim by Mogil et al (1987) that adoptive transfer of either Ly-1$^+$ or Ly-2$^+$ T cells could give protection. These differences were reconciled by Vinetz et al (1990), who suggested a minor but significant contamination of Ly-4$^+$ cells in the in vitro purified Ly-2$^+$ T cell population upon in vitro depletion of spleen cells that preceded adoptive transfer to P. yoelii challenged recipients in the report of Mogil et al (1987). This anomalous report apart, data suggest that Ly-2$^+$ lymphocytes, in the absence of Ly-4$^+$ cells, do not play any role in the protective response of mice to P. yoelii. This notion is supported by experiments on other murine malarias, including those described herein.

For the P. yoelii model, the reciprocal adoptive transfers of Ly-4$^+$-enriched immune splenic T cells or unfractionated immune spleen cells to syngeneic nude recipients rapidly transferred protection against P. yoelii. Challenged mice resolved homologous infection and recovered (Vinetz et al 1990). The transfer of protection by Ly-4$^+$ cells in a T lymphocyte-deficient system for a different murine malaria, P. yoelii, confirmed that immunity to blood stage murine malaria parasites resided in the Ly-4$^+$ T cell compartment, as shown here for P. c. chabaudi AS (both by depletion and adoptive

transfer). Furthermore, these findings vindicated the generation and propagation of stable T cell lines of the Ly-4^+ phenotype specific for P. c. chabaudi AS to examine the protective immune response to homologous (Chapters 5, 7 & 8) and heterologous (Chapter 6) parasite challenge.

Table 8.2.1 Phenotypic analysis of *in vivo* T cell subset depletion by continuous MAb treatment.

Stain	Day 0	5	12	19	33	47	60
(a) Ly-4 depletion							
α-Ly-4	47	92	93	93	95	94	94
α-Ly-2	2.8	2.9	2.8	3.4	3.5	4.4	4.3
α-Thy-1	35	58	61	62	62	62	61
α-Rat Ig	2.7	2.8	2.5	2.2	2.3	1.9	2.0
(b) Ly-2 depletion							
α-Ly-4	1.5	2.4	2.4	3.2	2.9	3.2	3.5
α-Ly-2	42	95	97	97	96	97	97
α-Thy-1	16	28	29	30	28	30	31
α-Rat Ig	2.1	2.3	2.3	2.5	2.2	2.2	2.1
(c) Ly-2/Ly-4 depletion							
α-Ly-4	43	88	93	92	92	90	91
α-Ly-2	35	93	91	93	93	92	91
α-Thy-1	75	91	90	90	89	86	85
α-Rat Ig	2.1	1.9	1.9	2.0	2.2	2.3	2.1

% depletion compared to animals given no Ab treatment (0% depletion)

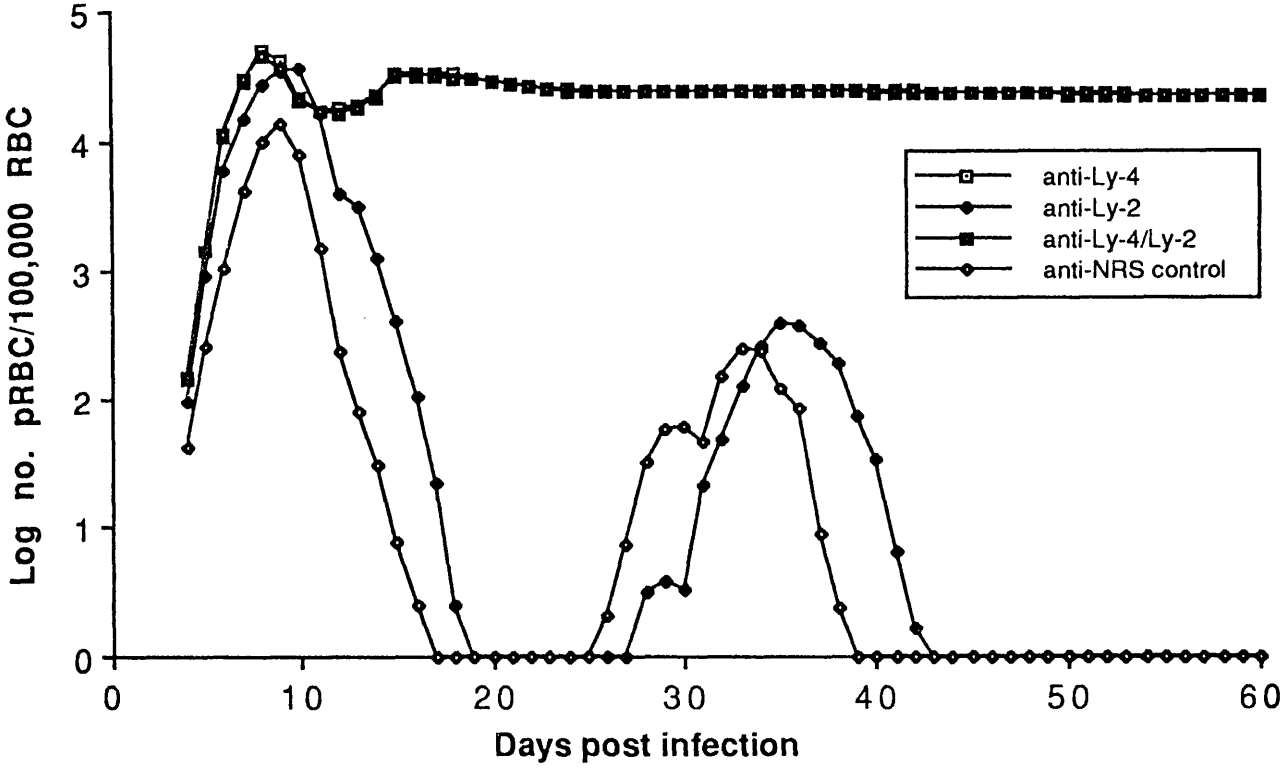

Fig. 8.2.1 Courses of infection in NIH naive mice treated <u>in vivo</u> with MAbs to Ly-4$^+$ and/or Ly-2$^+$ T lymphocyte subsets upon challenge with 1 x10^5 <u>P. c. chabaudi</u> AS pRBC i.v..

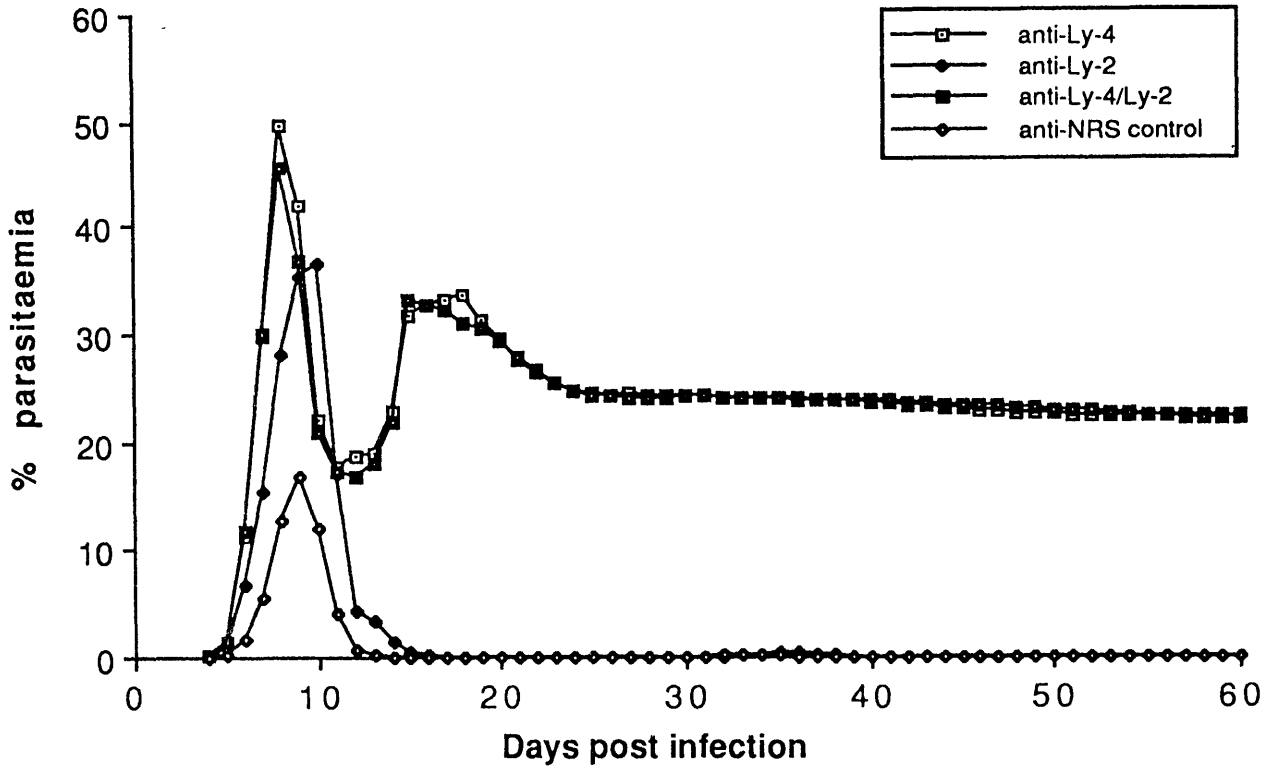

Fig. 8.2.2 Courses of infection in NIH naive mice treated <u>in vivo</u> with MAbs to Ly-4$^+$ and/or Ly-2$^+$ T lymphocyte subsets upon challenge with 1 x10^5 <u>P. c. chabaudi</u> AS pRBC i.v..

Table 8.3.1 Phenotypic analysis of *in vivo* T cell subset depletion by adult thymectomy and MAb pretreatment.

Stain	Day 0	5	12	19	33	47	60
(a) Ly-4 depletion							
α-Ly-4	89	88	90	92	91	92	92
α-Ly-2	2.6	2.8	2.8	3.1	3.3	3.6	3.8
α-Thy-1	57	56	59	62	61	63	62
α-Rat Ig	2.0	2.6	2.5	2.5	2.4	2.5	2.3
(b) Ly-2 depletion							
α-Ly-4	1.2	1.7	1.9	2.0	2.3	2.4	2.7
α-Ly-2	96	97	97	98	98	97	97
α-Thy-1	33	34	34	35	35	33	34
α-Rat Ig	2.1	1.9	1.9	2.2	2.4	1.9	2.0
(c) Ly-2/Ly-4 depletion							
α-Ly-4	86	87	86	88	88	87	87
α-Ly-2	94	95	94	97	97	95	96
α-Thy-1	91	92	89	93	93	91	90
α-Rat Ig	2.1	2.1	1.8	1.9	2.0	2.2	2.3

% depletion compared to non-thymectomised animals given no Ab treatment (0% depletion).

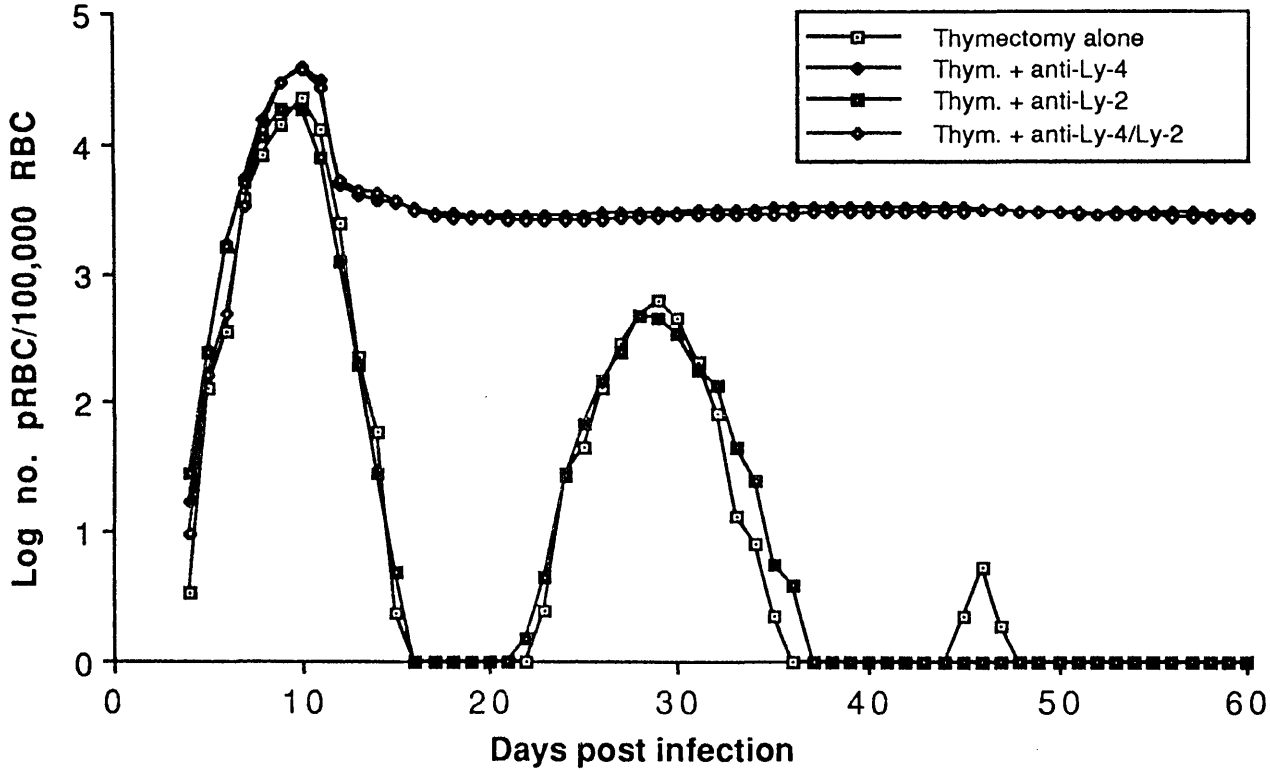

Fig. 8.3.1 Effect of adult thymectomy & MAb depletion treatments on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

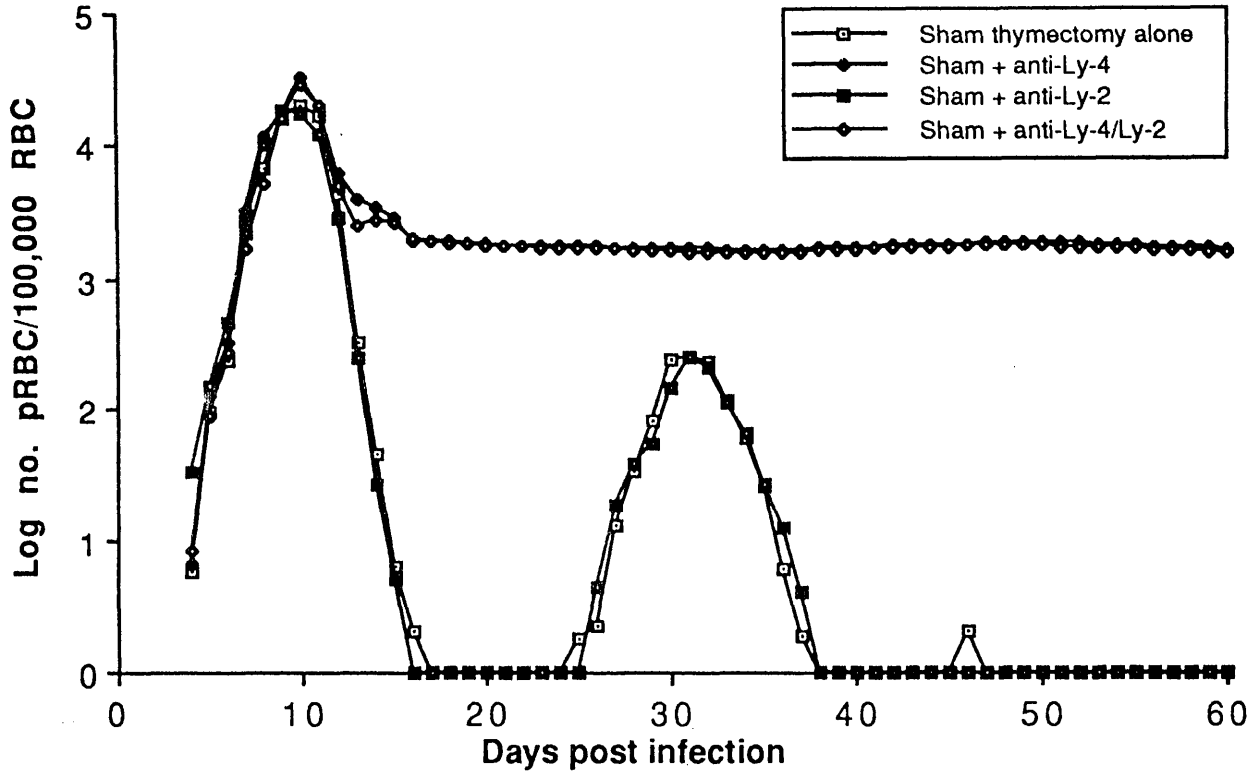

Fig. 8.3.2 Effect of sham thymectomy & MAb depletion treatments on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

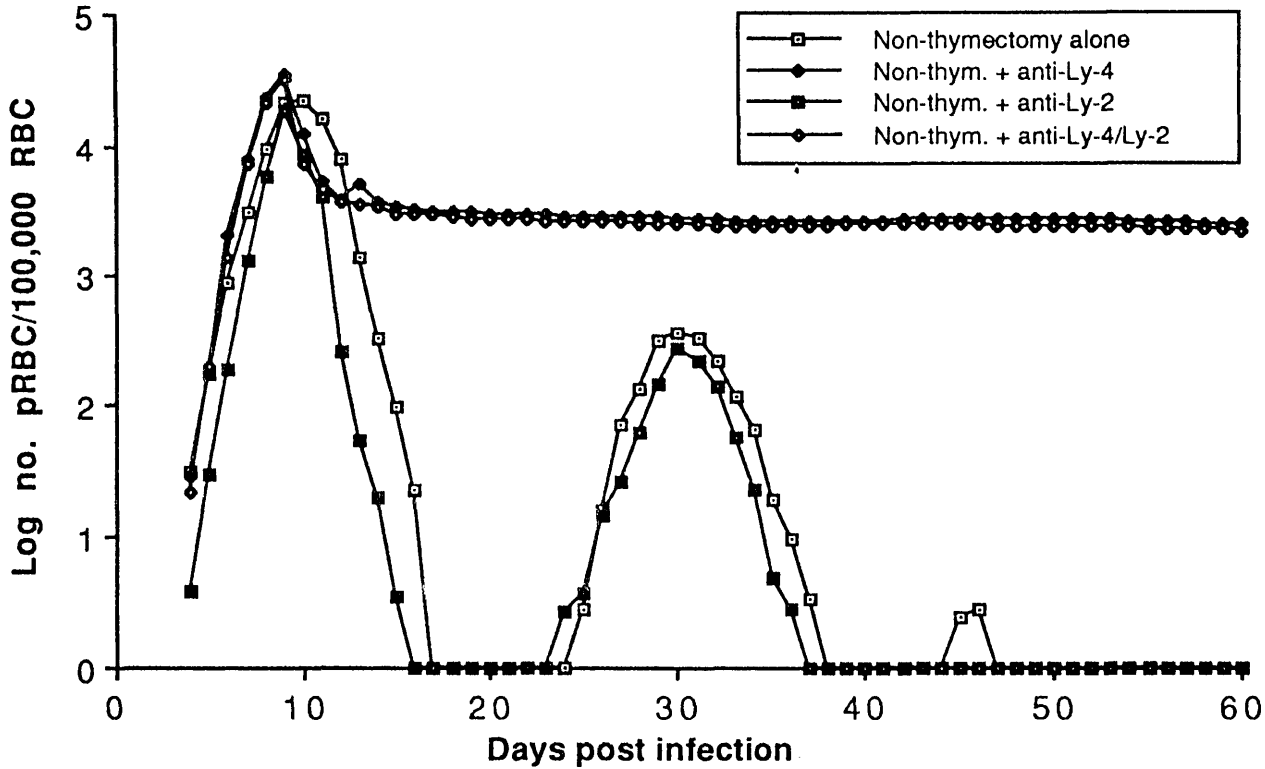

Fig. 8.3.3 Effect of MAb depletion pretreatments on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

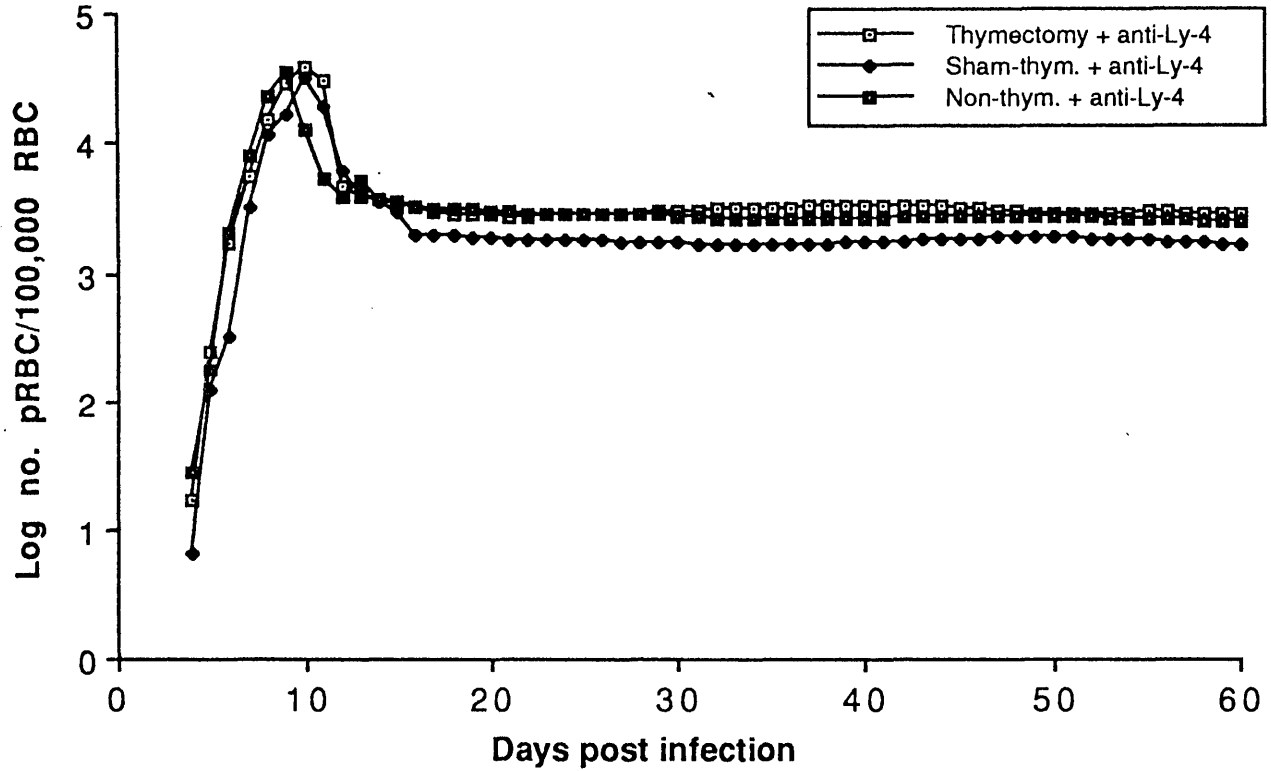

Fig. 8.3.4 Effect of anti-Ly-4 MAb pretreatment ± adult thymectomy on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

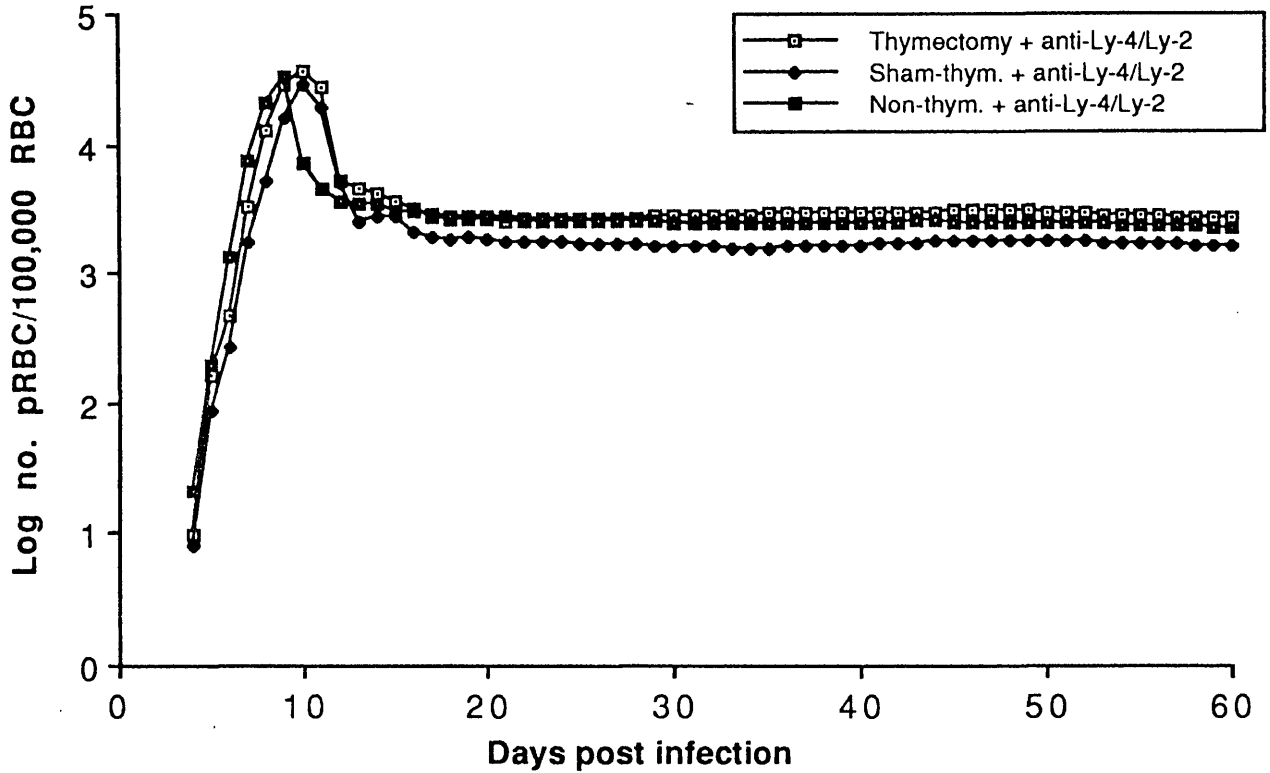

Fig. 8.3.5 Effect of anti-Ly-4 & anti-Ly-2 MAb pretreatments ± adult thymectomy on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

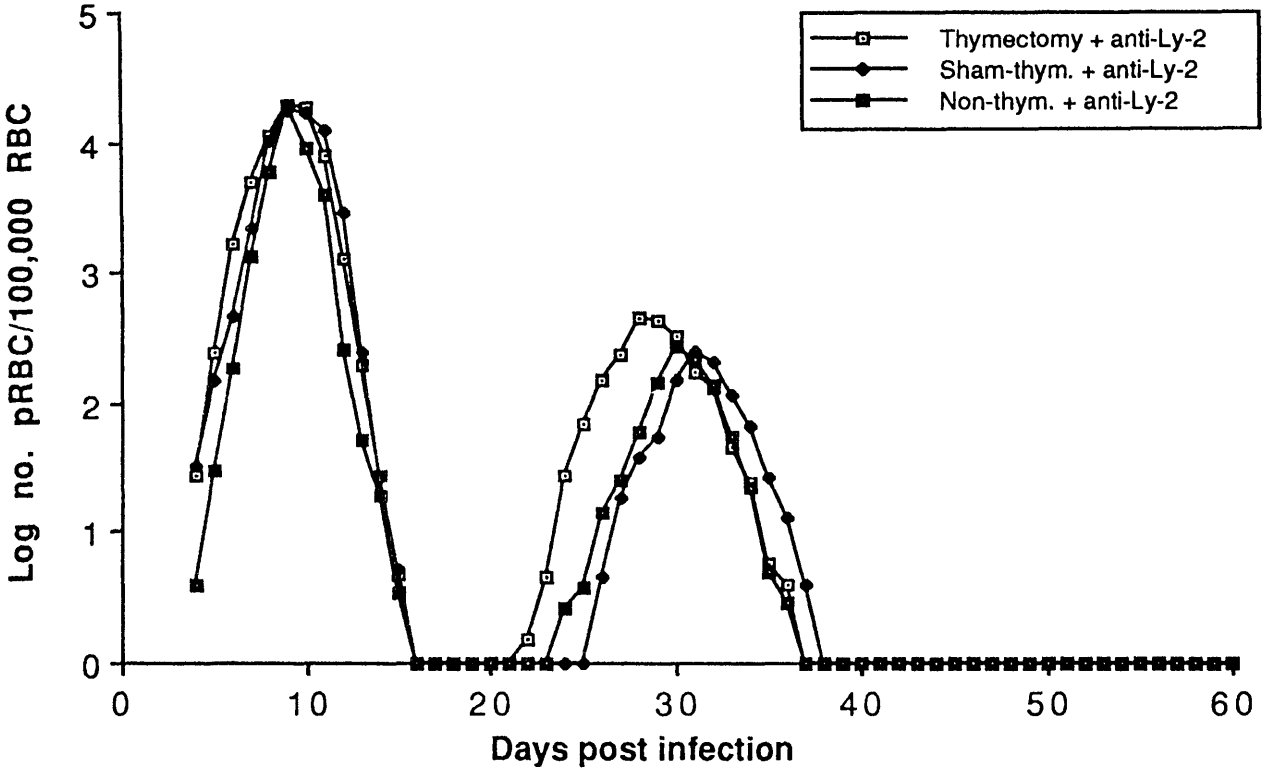

Fig. 8.3.6 Effect of anti-Ly-2 MAb pretreatment ± adult thymectomy on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

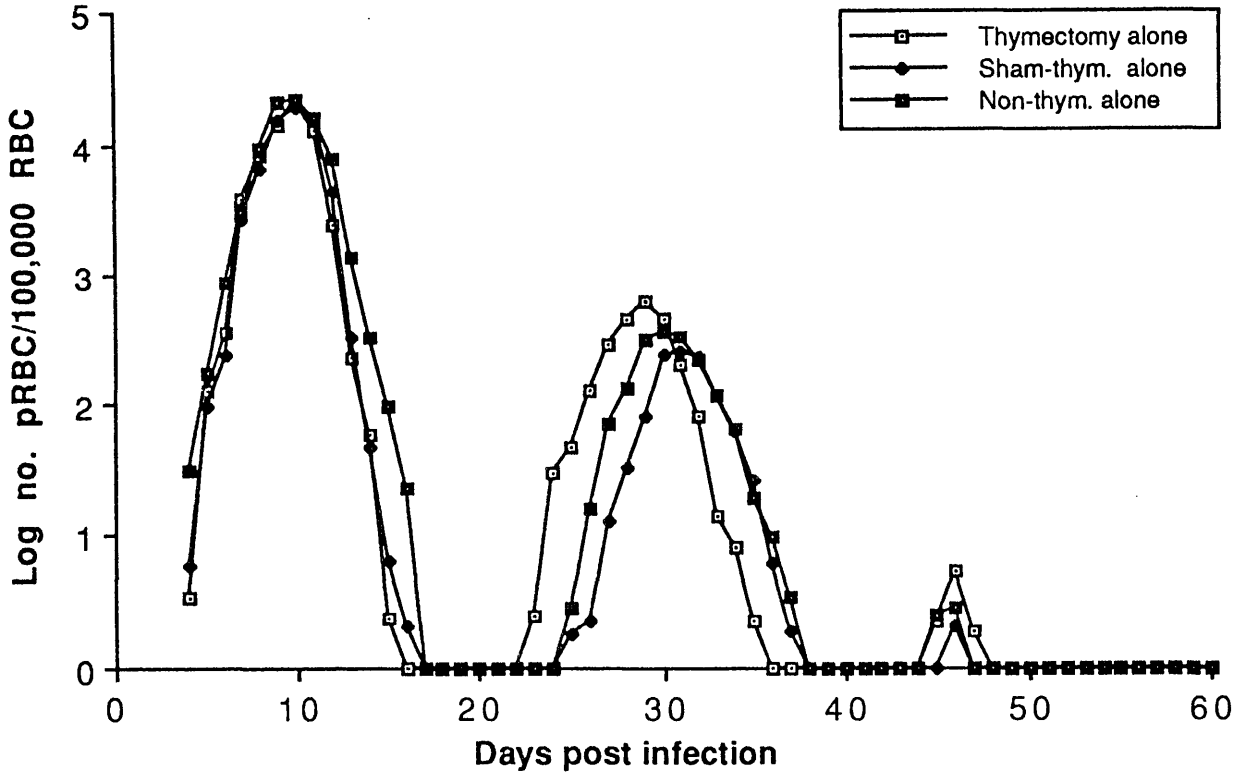

Fig. 8.3.7 Effect of adult or sham thymectomy alone on the course of infection in NIH naive mice challenged with 1 x10^5 P. c. chabaudi AS pRBC i.v..

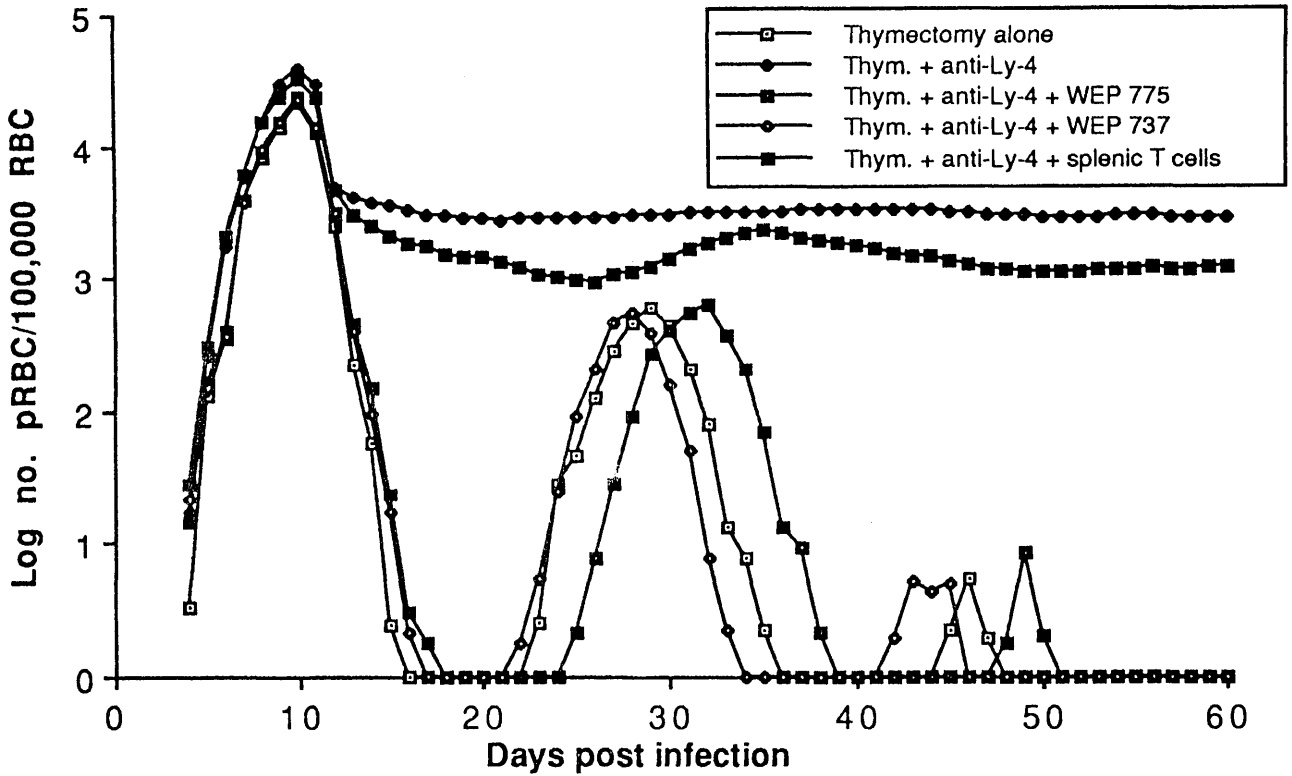

Fig. 8.3.8 Courses of infection in adult-thymectomised & Ly-4-depleted NIH naive recipients of WEP 775 and WEP 737 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v..

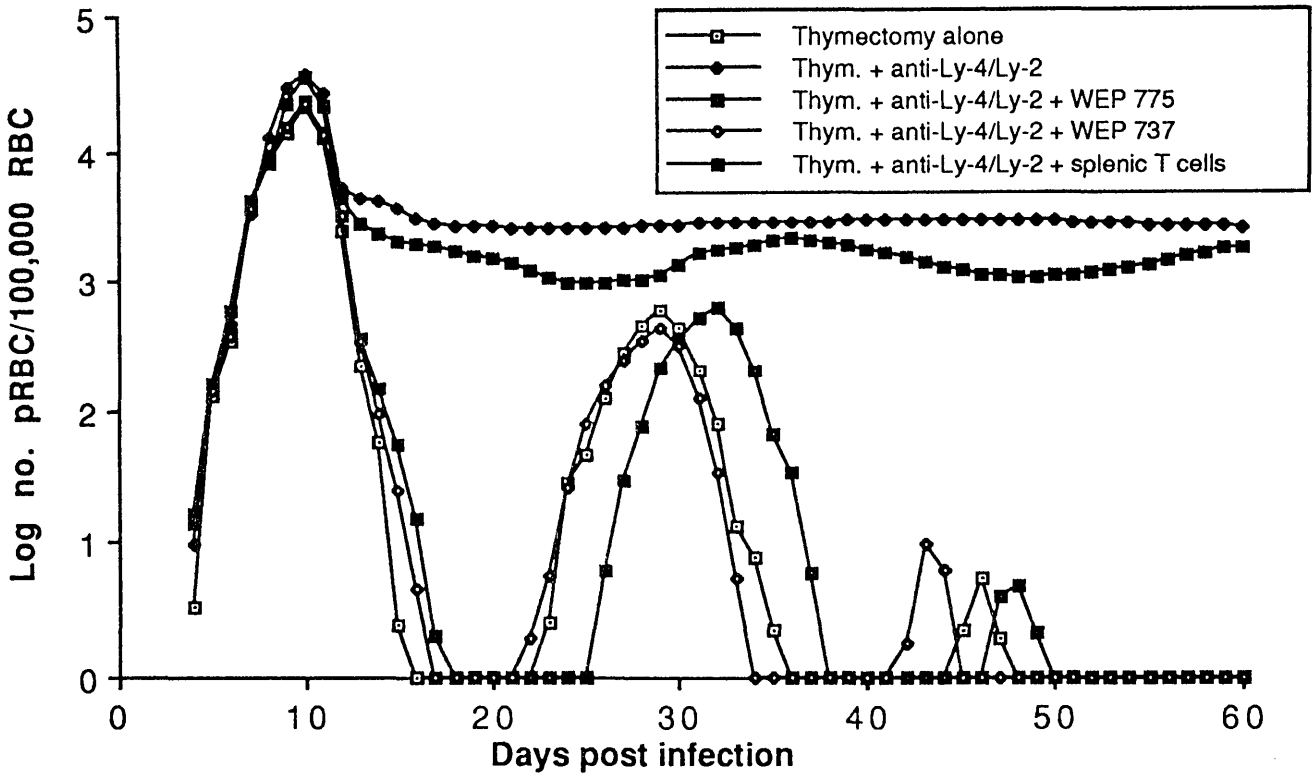

Fig. 8.3.9 Courses of infection in adult-thymectomised & Ly-4-/Ly-2-depleted NIH naive recipients of WEP 775 and of WEP 737 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v.

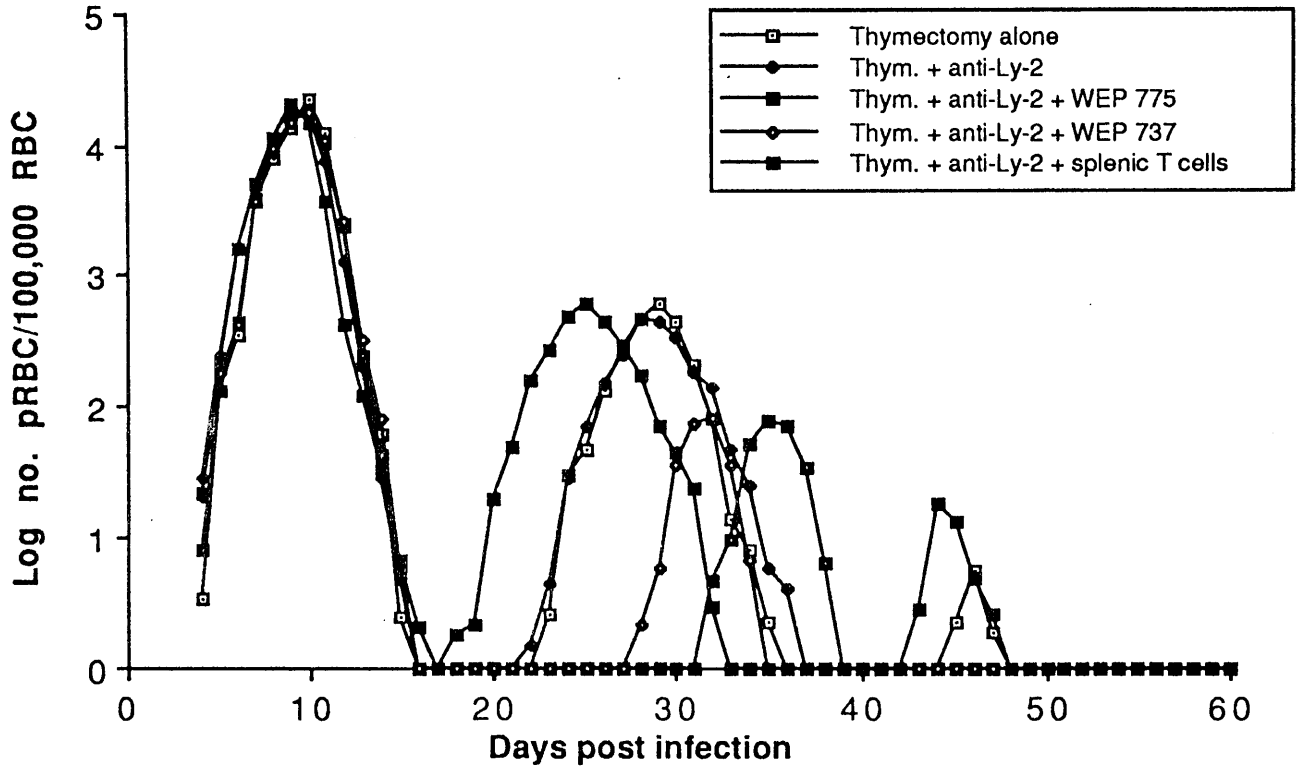

Fig. 8.3.10 Courses of infection in adult-thymectomised & Ly-2-depleted NIH naive recipients of WEP 775 and WEP 737 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v.

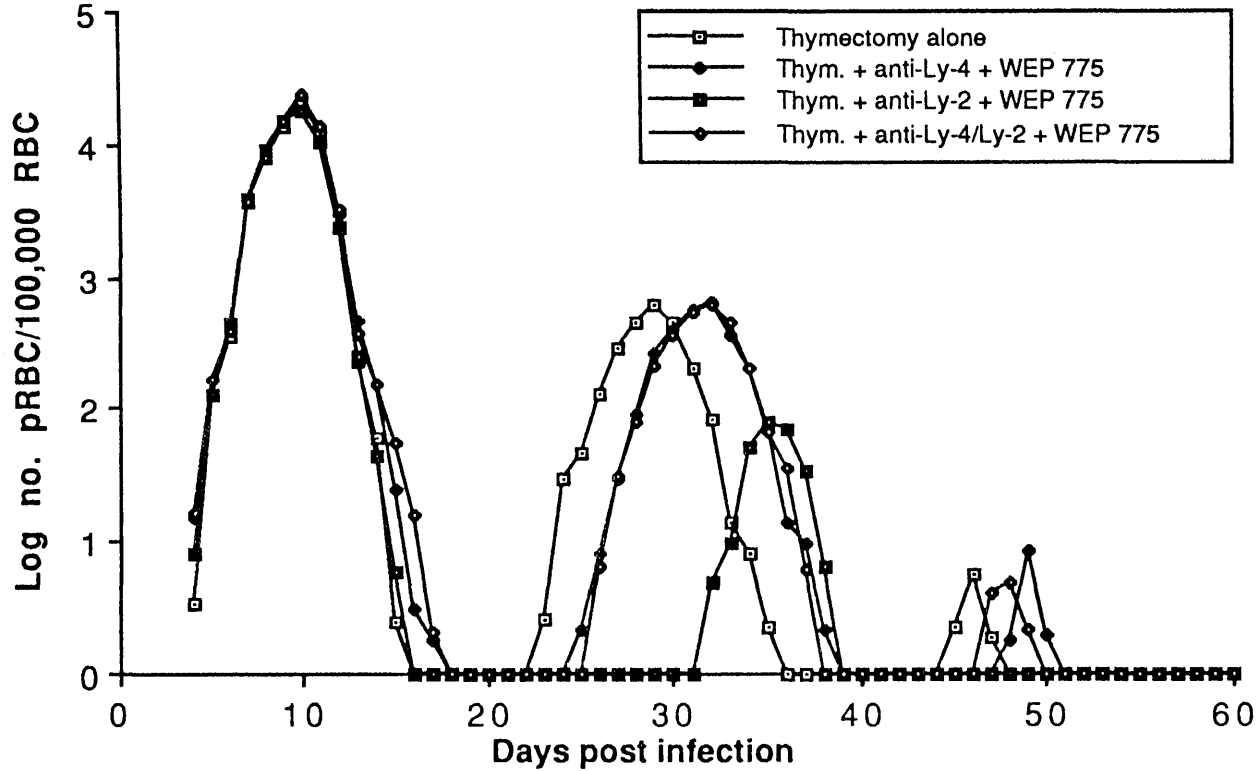

Fig. 8.3.11 Courses of infection in adult-thymectomised & Ly-4- and/or Ly-2-depleted NIH naive recipients of WEP 775 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v.

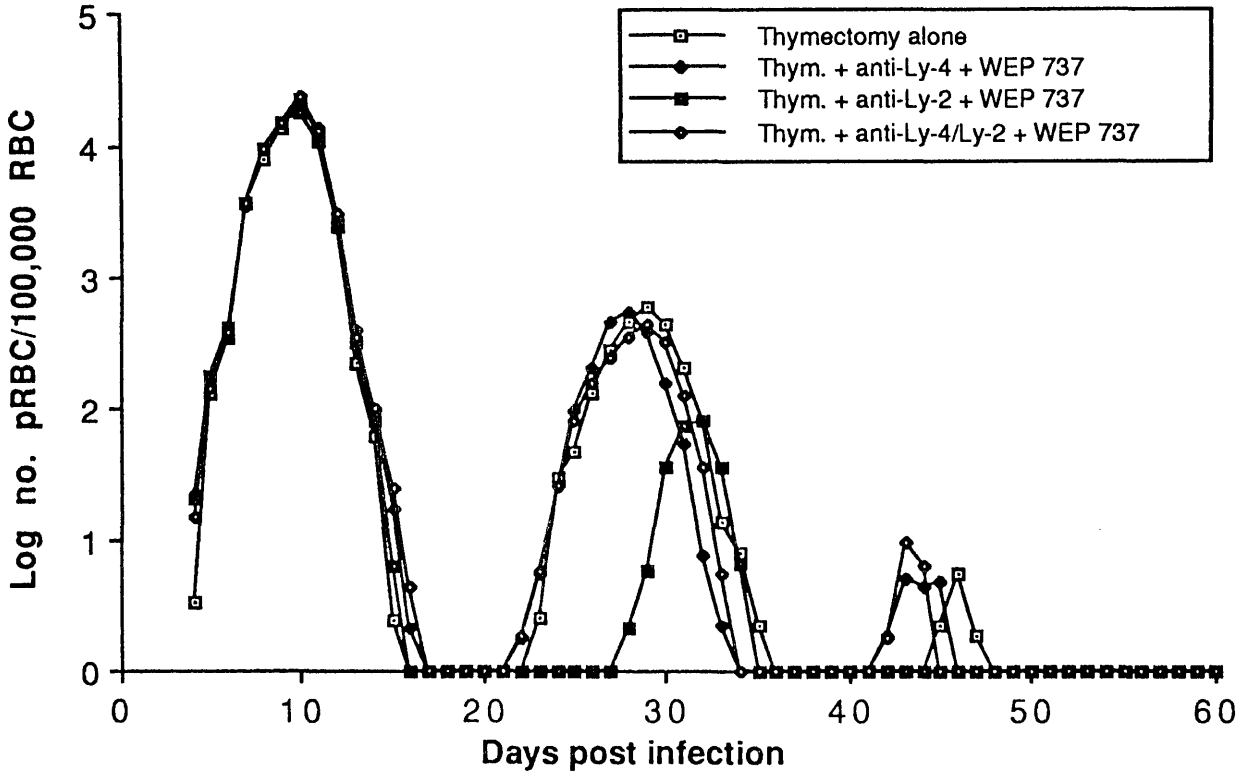

Fig. 8.3.12 Courses of infection in adult-thymectomised & Ly-4- and/or Ly-2-depleted NIH naive recipients of WEP 737 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v.

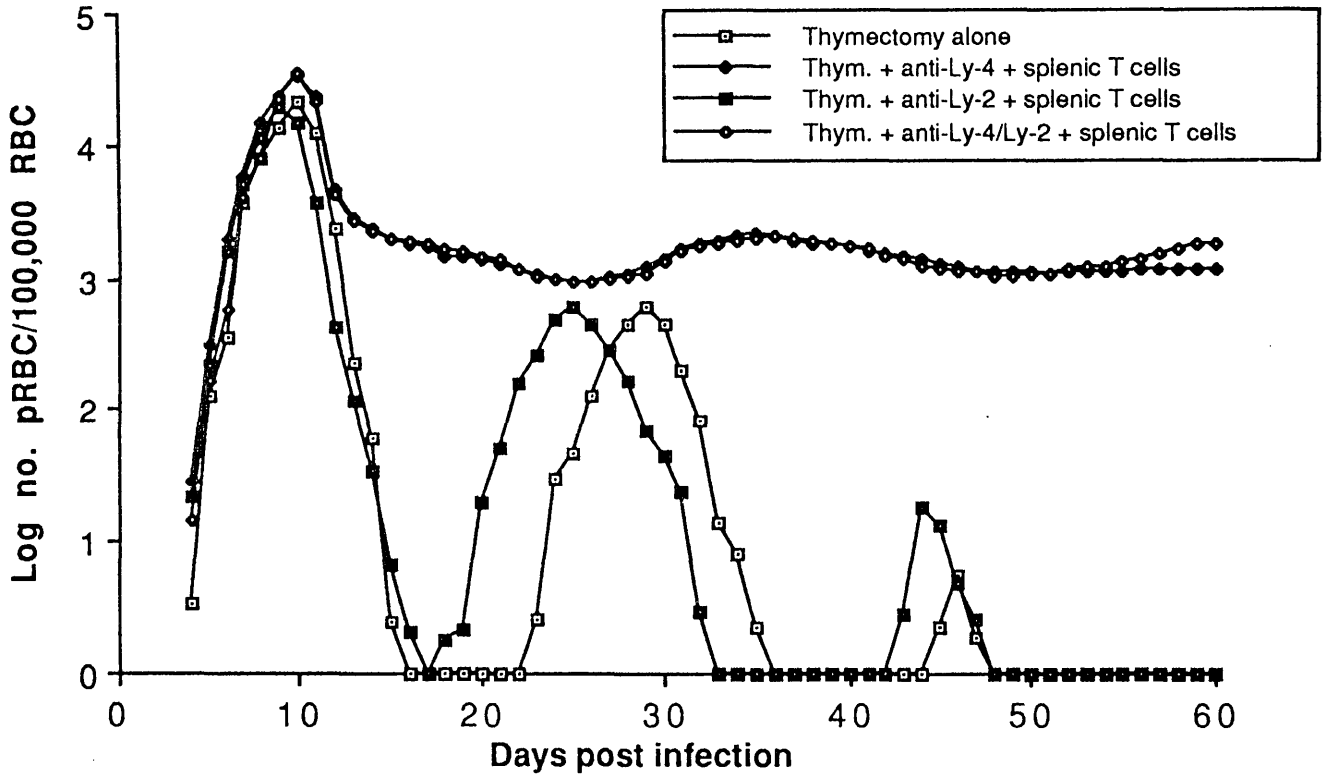

Fig. 8.3.13 Courses of infection in adult-thymectomised & Ly-4- and/or Ly-2-depleted NIH naive recipients of naive splenic T cells upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v.

CHAPTER 9

CHARACTERISATION OF T CELL LINES AND CLONES

9.1 Introduction

Three types of functionally distinct T lymphocytes have been described: helper, suppressor and cytotoxic T cells. Th cells mediate a variety of immunological functions and generally regulate the immune response. Cells of this lymphocyte class mediate Ab responses, and are also involved in the activation of Tc cells. Since the discovery that Th and Tc lymphocytes could be separated by differences in cell surface Ags (Cantor & Boyse 1975), several lines of evidence have suggested that further subdivisions exist within the Th cell population. Studies by Marrack & Kappler (1975) and by Janeway (1975 a) first proposed the existence of additional heterogeneity among Th cells, since when several reports have confirmed that Th cells were functionally heterogeneous (Janeway et al 1977, Tada et al 1978, Swierkosz et al 1979, Imperiale et al 1982) and that two main types could be distinguished based on the type of help provided to B cells (Tada et al 1978, Imperiale et al 1982).

In the last few years, with the development of techniques to clone and propagate functionally active and Ag-specific Th lymphocytes in vitro (Kimoto & Fathman 1980), their heterogeneity has become even more apparent. Mosmann and Coffman showed that long term clones of murine Ly-4$^+$ T cells could be divided into two non-overlapping subsets, based on patterns of lymphokine gene transcription and secretion (Mosmann et al 1986, Cherwinski et al 1987, Mosmann & Coffman 1987). One subset, designated T_H1, secretes uniquely IL-2, IFN-γ and lymphotoxin, whilst the other, designated T_H2, secretes uniquely IL-4, IL-5 and IL-6. Several other lymphokines, notably IL-3 and GM-CSF, appear to be produced by both cell types, although in some cases in different quantities (Mosmann et al 1986). These secretion patterns have been confirmed in several panels of Th clones, and this is currently the best defined criterion for delineation of mouse Ly-4-bearing T lymphocyte subsets. An alternative classification, proposed by Bottomly and colleagues (Kim et al 1985, Killar et al 1987, Rasmussen et al 1988) divides Th cells into 'helper' and 'inflammatory' subtypes. These two categories are, for the most part, equivalent to the T_H2 and T_H1 subsets, respectively. Subsequent studies in several laboratories have shown that cloned Ly-4$^+$ T cell lines can usually be divided into T_H1/inflammatory and T_H2/helper types by either function or lymphokine secretion. Recently, however, the finding of murine T cell clones capable of producing both IL-4 and IFN-γ (Kelso & Gough 1988) has raised the possibility that some or all T_H1 and T_H2 clones may represent differentiated cells derived from a common precursor. Furthermore, among human T cell clones, the frequency of cells

making both IL-4 and IFN-γ is high (Maggi et al 1988), although marked differences in the ratios of these two products have been observed and have been linked to varying functions of the cells.

The characterisation of the two types of Th cells has been carried out in vitro using established clones (Mosmann et al 1986, Cherwinski et al 1987, Cher & Mosmann 1987). It is therefore possible that variables in the techniques used for isolating, growing and cloning Ly-4$^+$ lymphocytes may have selected clones that were not representative of their in vivo counterparts. However, recent evidence has been provided that the functional differences and production of distinct lymphokines by T_H1 and T_H2 populations in vitro may also be true of normal Ly-4$^+$ T cells in the mouse (Tite et al 1985, Bottomly et al 1989, Carding et al 1989). These developments in the characterisation of Ly-4-bearing lymphocytes serve to stress the relevance of using in vitro-propagated, Ag-specific T cell clones to dissect specific mechanisms of cell-mediated immunity, as will be described in this chapter for P. c. chabaudi AS infection of NIH mice.

Attributable to the different lymphokines secreted by these lymphocytes, T_H1 and T_H2 clones perform fundamentally different functions. Most important with regard to malaria, T_H1 cells mediate delayed-type hypersensitivity, and T_H2 cells act as helper cells for specific Ab production (Mosmann & Coffman 1987, Abbas 1987, Bottomly 1988). T_H2 clones may specifically enhance polyclonal Ab synthesis of particular isotypes, namely IgG_1 and IgE, presumably through IL-4 (Roehm et al 1984, Vitetta et al 1985, Coffman & Carty 1986). Indeed, there is now very strong evidence that the in vivo synthesis of IgE depends upon production of IL-4 and is opposed by IFN-γ (Finkelman et al 1986, 1988 a & b). Similarly, IFN-γ synthesis in vivo is responsible uniquely for IgG_{2a} Ab responses (Finkelman et al 1988 a). These observations have suggested that the immune response to various antigenic stimuli may be regulated by the differential activation of Ly-4$^+$ T cell subsets (Snapper & Paul 1987). For example, selective activation of T_H1 clones producing IL-2 and IFN-γ may occur during viral infection, leading to IgG_{2a} being the dominant subclass in the IgG anti-viral response (Coutelier et al 1987, Taylor et al 1990). In contrast, infection with helminths such as Nippostrongylus brasiliensis favours strongly secretion of IgE (Jarrett & Stewart 1972), through selective activation of T_H2 clones (producing IL-4, which can regulate IgE synthesis and enhance growth of mast cells) (Finkelman et al 1986). Recent studies of Leishmania major infection of various mouse strains has

shown that Ag-specific T cell clones from mice that limit their infection produce IL-2 and IFN-γ, whereas such clones from mice that develop progressive leishmaniasis produce mainly IL-4 (Heinzel et al 1989). These results support the contention that for the immunoregulation of this disease, Ly-4$^+$ cells that transfer protective immunity or exacerbation belong to different Th subsets and respond to different parasite Ags (Scott et al 1988).

With regard to malarial infection, the determination of the functional characterisation of the Ly-4$^+$ T cells responding to malarial Ags is itself of interest, but may also have important implications for anti-malarial vaccine development, since this will necessarily select Ags which induce the appropriate functions of the Ly-4$^+$ lymphocyte in the host immune response to the asexual erythrocytic stages of Plasmodium. An investigation of the functional heterogeneity of Ly-4$^+$ cells in a P. c. chabaudi AS infection is particularly pertinent since it has been shown that Ly-4-bearing cells are necessary for a protective immune response to this parasite (Süss et al 1988, Langhorne et al 1990, also Chapter 8).

Previously in this thesis, it has been shown that in vitro-propagated, P. c. chabaudi AS-specific splenic T cell lines are capable of transferring protection adoptively to homologous challenge in immunocompetent and compromised hosts alike (Chapter 5, 7 & 8). Moreover, a dichotomy in the behaviour of the lines in vivo was revealed, two lines appearing to be Ab-dependent and two Ab-independent in their reactivity upon adoptive transfer (Chapters 7 & 8). This divergence in the requirement of Ly-4$^+$ lines to confer immunity is suggestive of an underlying functional heterogeneity between the different P. c. chabaudi AS-primed population. To determine if differential induction of T_H1 and T_H2 cells could explain the results of the documented immunisation studies, the properties of each of the cell lines were analysed. Their Ag specificity using conventional proliferation assays, and the lymphokine secretion profiles after stimulation were determined. It was hoped to elucidate at the cellular level the mechanisms responsible for the divergent patterns of protective immunity observed in vivo upon adoptive transfer of these Ly-4-bearing populations. To define these mechanisms more precisely, limiting dilution techniques were used to develop a series of Ly-4$^+$ lymphocyte clones, which were then expanded with plasmodial Ag and IL-2 (Chapter 4). As for the parent lines, the secretory products of the clones were analysed in an attempt to define protective factors. Furthermore, the ability of each monoclonal population to transfer protection adoptively to non-immune mice was examined.

Previously, Brake et al (1988) had shown a Ly-4$^+$ T cell clone specific for an unknown Ag of P. c. adami to be protective in vivo. As this clone produced IL-2 and IFN-γ upon antigenic stimulation, it was thought to belong to the T_H1 subset. This identification was not surprising in view of the Ab-independent nature of the host immune response to P. c. adami (Grun & Weidanz 1981, Cavacini et al 1986, Brake et al 1986). For the P. c. chabaudi AS/NIH mouse model, both humoral and cell-mediated components of immunity are thought to contribute, as exemplified by the homologously primed lines described herein. Thus, the release of lymphokines by each of the protective T cell clones in vivo may display a similar pattern to that observed for the P. c. adami-specific clone, or may display a distinct secretion profile, indicative of the exertion of a different anti-plasmodial effect. The findings detailed in this chapter extend the results of others who have reported T cell clones with protective activity against various intracellular pathogens, including influenza virus, Listeria monocytogenes, Mycobacterium bovis and Trypanosoma cruzi (Lukacher et al 1984, Kaufmann & Hahn 1982, Pedrazzini & Louis 1986, Nickell et al 1987).

9.2 Surface phenotyping of T cell lines & clones

Each in vitro-propagated P. c. chabaudi AS-specific splenic T lymphocyte line and clone used in this study was evaluated for the presence of different surface membrane markers by both indirect immunofluorescence (2.32) and by complement-mediated cytolysis (2.33). Figure 9.2.1 shows the results of the IFAT performed on each of the four in vivo-primed splenic T cell lines shortly after establishment of each as a stable population in bulk culture. This was the first phenotypic analysis of the cell lines following their generation and gave an indication of the efficacy of the procedures used to raise homogeneous Ly-4$^+$ T cell populations. For each line, > 91% of cells expressed the Thy-1 surface Ag, indicative of T lymphocytes. In addition, depending on the line, between 78-91% of the cells exhibited the Ly-4 Ag of the helper/inducer T cell subset, whereas only 4-8% displayed the Ly-2 marker characteristic of the suppressor/cytotoxic subset. The degree of B cell contamination was negligible, as was that from macrophages and monocytes (data not shown).

The small fraction of cells which failed to stain upon incubation with the pan-T cell marker did not do so for any of the other MAbs used. Although they may have represented non-T non-B lymphocytes, it is more probable that this residual non-fluorescent population contained T cells which could not be detected under the experimental

conditions used, highlighting an inherent error in this assay system. This body of cells, therefore, was not thought to represent a significant contamination of the malaria-specific populations raised, and thus did not invalidate the interpretation of the protective effect observed upon adoptive transfer of these lymphocytes (Chapters 5-8). In any case, it was later demonstrated unequivocally that the protective activity resided within the majority Ly-4$^+$ population of each lymphocyte line (Chapter 5).

These data indicate the relative homogeneity of each of the cell lines for expression of the Ly-4 surface Ag at the outset of in vitro cultivation. The spleen cells taken from mice for in vitro culture had all been exposed previously to P. c. chabaudi AS pRBC. This may or may not have preselected those cells for proliferation in vitro, but it is true that the proportion of Ly-4$^+$ cells increased with the time allowed for in vivo priming. Hence, spleens taken from animals recovered from a tertiary infection yielded 91% Ly-4$^+$ cells (WEP 723), whereas those taken from mice sacrificed on d 16 of primary infection gave only 78% Ly-4-bearing lymphocytes (WEP 775) (Fig. 9.2.1). Further experimentation revealed that upon continued subculture, not only did surface Ag expression for each line remain relatively constant, but that long term in vitro propagation actually enriched each population for lymphocytes of the Ly-4$^+$ T cell subset (data not shown). Hence, the Ly-4 marker was expressed by not less than 96% of all cultured lymphocytes at the time of cloning.

The extent of the homogeneity of the splenic T cell lines after initiation and propagation in vitro is apparent by comparing with a control IFAT which was performed on a population of naive spleen cells (Fig. 9.2.2). In this instance, all the MAbs used showed significant fluorescence, indicating the assortment of cell types that is usually present in this secondary lymphoid organ. Indeed, only 27% of freshly cultured naive splenocytes displayed the Ly-4$^+$ phenotype. The increase in the proportion of cells bearing the Ly-4 marker from 27% to > 90% after the initial stages of in vitro propagation was indicative of the positive selection pressure for Ly-4$^+$ lymphocytes imposed by the culture protocol followed (2.26).

The complement-mediated cytotoxicity assay was used with both anti-Ly-4 and anti-Ly-2 MAbs to confirm the Ly-4$^+$ characterisation of the T lymphocyte lines raised to P. c. chabaudi AS. The cytotoxicity of the anti-Ly-4 MAb towards each of the cell preparations tested is shown in Fig. 9.2.3. As can be seen, for both cell lines assayed (WEP 775 and WEP 737, those subsequently cloned), at the lowest two titres used, 1/10 and 1/50, the levels of lysis observed, and thus by implication, the proportions of

cells carrying the Ly-4 determinant, were similar to those recorded by indirect immunofluorescence (Fig. 9.2.1). In fact, the fraction of cells characterised as belonging to the Ly-4$^+$ subset was greater for the cytolysis assay than for IFAT. Thus, for instance, the proportion of Ly-4-bearing WEP 737 cells was as high as 91% here, but only 80% using immunofluorescence. Despite this slight discrepancy between the sensitivity of the two assays, it is clear that for both systems the phenotypic characterisation of the P. c. chabaudi AS-specific lines tested is that of predominantly Ly-4-bearing lymphocytes. The sensitivity of the anti-Ly-4 MAb used was evidenced by the lack of cytotoxicity towards a control preparation of naive splenic B cells, and lysis of only those 43% of naive splenic T cells exhibiting the Ly-4 surface Ag (Fig. 9.2.3). For the corresponding assay using the anti-Ly-2 MAb (Fig. 9.2.4), the highest level of lysis recorded was 39% for the naive splenic T lymphocyte population. For both WEP 775 and WEP 737 preparations, only 7-8% of cells lysed upon incubation with this MAb. This suggested a degree of contamination of the T cell lines with Ly-2$^+$ cells that correlated extremely well with that determined by IFAT (Fig. 9.2.1). Regardless of the origin of the splenic T cell lines assayed, the cytotoxicity recorded in the presence of either MAb used or the normal rat serum control was very similar. This was demonstrated by the similarity of the MAb titre profiles for WEP 775 (Fig. 9.2.5) and for WEP 737 (Fig. 9.2.6) lymphocyte lines. In each case, even at the highest MAb titre tested, 1/5000, the number of cells lysed by incubating with the anti-Ly-4 MAb was considerably in excess of those lysed by either of the other preparations used. Again, this indicated the degree of enrichment of the normal splenic T cell population (cytotoxicity profile shown in Fig. 9.2.7) for the Ly-4$^+$ subset that was achieved by the in vitro culture methodology followed.

The P. c. chabaudi AS-specific Ly-4-bearing lines previously documented to transfer protection adoptively to primary challenged mice of varying immunological status (Chapters 5-8) were used as the basis for the development of cloned cells (Chapter 4). Of 10 and 11 clones derived and expanded from each of the two parent lines, WEP 737 and WEP 775, respectively, used for limiting dilution cultivation, four of each were used for subsequent in vitro and in vivo experimentation. Table 9.2.1 shows the phenotypic characterisation of these cloned in vitro-propagated lines. For each clone, regardless of origin, surface immunofluorescence revealed a total homogeneity of the population for expression of the Thy-1 and Ly-4 cell surface Ags. Indeed, in each instance, the number of positively fluorescing cells upon incubation with MAbs to either

marker was identical. Furthermore, for all clonal preparations tested, not one cell was noted as being Ly-2$^+$, nor was a positive identification made using either anti-θ or anti-κ MAbs. Thus, allowing for the limitations of this detection system that will occassionally yield a non-fluorescing cell, all the populations were characterised as being monoclonal with regard to surface phenotype, belonging to the Ly-4$^+$ Ly-2$^-$ subset of T lymphocytes. This finding was confirmed by typing each of the clones by complement-mediated cytotoxicity (Table 9.2.2). At the lowest dilution of anti-Ly-4 MAb used, 1/10, the degree of lysis of the cloned populations was never less than 99%. Although the level of cytotoxicity titred out with MAb dilution (Table 9.2.2), it was noticeable that for the cloned preparations, the degree of lysis observed at higher titrations, such as 1/1000, was markedly greater than that observed for the particular parent cell line (Figs. 9.2.5 & 6). Whether or not this was a reflection of the increased homogeneity of the T lymphocyte populations after cloning by limiting dilution is open to question.

9.3 Assay of Ag-specific proliferation of T cell clones

The proliferation of Ly-4$^+$ T cell lines in response to stimulation with a soluble lysate of P. c. chabaudi AS pRBC has been described previously (Chapter 4). It was demonstrated that the proliferative response of each splenic T lymphocyte line, derived from mice semi- or fully-immune to P. c. chabaudi AS, was parasite-specific and exhibited dose-response kinetics. The lymphocyte proliferation assay (2.34) was performed on each of the Ly-4$^+$ clones used for further analysis in order to verify the retention of the Ag-specific responsiveness of the cloned populations derived from parent lines of known specificity.

Figure 9.3.1 shows the proliferative responses of all eight clones used in this study. To enable comparison of the respective growth rates, both with each other and with the parent cell lines, each lymphocyte preparation was assayed at the same time, 7 d after the fourth round of antigenic stimulation post cloning. All the cloned populations exhibited a significant proliferation when incubated with P. c. chabaudi AS pRBC Ag, the kinetics of which were dose-dependent. In this regard, the response of the clones to antigenic stimulation was essentially identical to that observed upon activation of the uncloned parent lines (Fig. 4.11.1). Again, as for WEP 775 and WEP 737, each daughter clone showed optimal growth when cultured with 200 μg/ml pRBC lysate Ag (Fig. 9.3.1). Although there was a variation in the level of tritium incorporation

attained in response to incubation with this concentration of pRBC lysate, the variation was as marked between clones of the same origin (Figs. 9.3.2 & 3) as between those derived from different parent lines (Fig. 9.3.1). At the higher range of concentrations assayed, however, there was a noticeable difference in the proliferative responsiveness of the clones; those derived from WEP 775 had a greater growth rate than those attained from cloning WEP 737 (Fig. 9.3.1). Although the difference was statistically significant ($p < 0.05$), the biological significance of this variance is debatable in view of the generally reduced growth response of the P. c. chabaudi AS-primed lymphocytes in vitro upon homologous antigenic stimulation at such an antigenically high concentration of lysate. At concentrations of Ag below 250 μg/ml, there was variation in the level of proliferation of individual clones, but this was not significant ($p > 0.05$), and did not show any obvious pattern (Fig. 9.3.1).

A measure of the heightened proliferative responsiveness of Ly-4^+ clonal populations primed by natural infection to P. c. chabaudi AS and restimulated in vitro with homologous plasmodial Ags was gained by examination of the proliferation of control preparations of splenic T cells taken from naive or immunised mice (Fig. 9.3.1). Although the response of a mixed population of in vivo-primed splenic T lymphocytes to specific antigenic stimulation was greater than that of unprimed splenic T cells, both its magnitude and distribution over a range of lysate concentrations was considerably less ($p < 0.01$) than that shown by a homogeneous population of Ly-4^+ P. c. chabaudi AS-specific lymphocytes that each clone represented.

The P. c. chabaudi AS-specific proliferative responsiveness of each of the daughter cloned populations derived from splenic T cell lines primed in vivo to the homologous parasite was shown to be dependent upon the availability of APC. When Ly-4^+ clones were cultured either alone or with lysate, but without APC, no proliferation occurred. Similarly, the presence of APC but without pRBC lysate Ag, was insufficient to induce clonal proliferation (Fig. 9.3.1). It therefore appeared that Ag processing and presentation by syngeneic APC was a necessary step in the process of T cell activation leading to Ag-specific proliferation. Moreover, APC of the same haplotype (H-2^q) as the NIH strain responder cells were required for correct Ag presentation to all clones tested (data not shown), showing that this is an MHC-restricted phenomenon.

Finally, it was demonstrated that this response was directed to P. c. chabaudi AS Ags (of unknown identity) since antigenic stimulation with a control lysate of uninfected RBC gave significantly less proliferation ($p < 0.01$) (data not shown). In all of these

regards, the Ly-4-bearing monoclonal populations behaved in an identical fashion to their parent P. c. chabaudi AS-specific T lymphocyte lines (4.11 & 12). It was thus demonstrated that the populations attained by limiting dilution cloning retained the specificity of the original uncloned Ly-4$^+$ lines towards P. c. chabaudi AS, as exemplified by pRBC lysate-specific proliferation in vitro.

Since the monoclonality of the cloned populations was ensured, and demonstrated by surface phenotyping (9.2), the identity of the parasite-specific lymphoproliferation of both Ly-4-bearing lines and clones emphasises that this reactivity was representative of the majority Ly-4$^+$ cells in each T lymphocyte line preparation from which the cloned daughter populations were taken.

9.4 Assay of helper T cell function

The ability of P. c. chabaudi AS-specific, in vitro-propagated lymphocyte lines and clones of the Ly-4$^+$ T cell subset to induce splenic B cells to produce specific anti-plasmodial Abs in vitro was assayed by IFAT (2.39).

Considering the Ly-4$^+$ lines, there was a pronounced variation in the capacity of these lymphocytes to stimulate B cell differentiation in vitro. Those lines taken from donor mice after resolution of multiple infections, WEP 737 and WEP 723, induced a large specific Ab response, as shown by very high IFAT end point titres (Fig. 9.4.1). These Ly-4-bearing preparations were those which were considered to confer protection against homologous challenge in vivo by Ab-mediated mechanisms (Chapter 7). Conversely, those lines which had been shown previously to have a B cell-independent reactivity in vivo (Chapter 7), WEP 775 and WEP 779 (taken from donor mice during a primary P. c. chabaudi AS infection) were unable to promote anti-plasmodial Ab synthesis (Fig. 9.4.1). In fact, the Ab titres achieved by culturing these lines with parasite-primed B cells and homologous Ag were as low as those recorded by naive splenic T cells, and significantly lower than the titre attained by coculturing post-infective B cells with similarly primed splenic T lymphocytes ($p < 0.05$). These latter two groups of splenic T cells of mixed phenotype were a necessary inclusion to control for the normal levels of T cell help usually provided in vitro-propagated splenic lymphocytes.

The segregation of the four Ly-4$^+$ lines used in this study into two groups based upon their ability to induce an Ab response to P. c. chabaudi AS in vitro was confirmed at the clonal level. Each of the four clones derived from either WEP 775 or WEP 737 was

incubated with plasmodial-primed splenic B cells and P. c. chabaudi AS pRBC lysate and the culture S/N assayed by IFAT. Figure 9.4.2 shows that there was a very significant difference in the secondary Ab response induced by these two sets of phenotypically identical clones ($p < 0.01$). The reciprocal Ab titre of each clone was usually similar to that of its parent cell line, those clones derived from WEP 737 consistently inducing a greater Ab response from cocultured B cells than did WEP 775-derived clones. Although this was the general trend, there was some variation. For instance, the Ab titres for the daughter clones of WEP 775 ranged from 1/128 to 1/2048 (WEP 997 and WEP 996, respectively). Also, for the WEP 737-derived clones, the end point titre for WEP 986, 1/16384, was twice that for WEP 985, 1/8192 (Fig. 9.4.2). Obviously, for both WEP 996 and for WEP 986, the level of indirect immunofluorescence recorded was significantly greater than that of the respective parent lines, WEP 775 and WEP 737 ($p < 0.05$). In each case, however, the variation was not so great either to obscure the identity of the parent line or to invalidate the groupings of P. c. chabaudi AS-specific populations into B cell helper and non-helper categories. The difference between the lowest Ab titre of the proposed Ab helper clones (WEP 985; 1/8192) and the highest Ab titre of the non-helper clones (WEP 996; 1/2028) was not only statistically significant ($p < 0.01$), it suggested strongly a difference of biological significance, namely a dichotomy in the mechanisms by which WEP 775 and WEP 737 and their respective daughter clones mediate protection in vitro, and presumably therefore in vivo.

Further experimentation revealed that for each Ly-4$^+$ lymphocyte line, regardless of its ability to induce specific parasiticidal B cell responses (Fig. 9.4.1), the greatest Ab response was achieved for Ly-4-bearing cells cultured with pRBC Ag and post-infective B cells (Fig. 9.4.3). These Ab-producing lymphocytes were taken from the spleens of mice recently recovered from a primary P. c. chabaudi AS infection. The substantial response of these B cells upon restimulation with homologous Ag in vitro was therefore an anamnestic response, presumably characteristic of the large proportion of memory B cells thought to be present in the spleen following recovery from natural infection. Of the two variables in the culture system, the responder B cells of varying immunocompetence and the source of antigenic stimulation, it is clear that the latter made a greater contribution towards the detectable Ab response in vitro. This was suggested by the fact that the degree of B cell responsiveness induced by culturing any of the Ly-4$^+$ lines with pRBC lysate Ag and naive B cells was, in each case, significantly

greater than that induced by the reciprocal cultures containing uninfected RBC lysate and post-infective B cells ($p < 0.01$) (Fig. 9.4.3). The failure of P. c. chabaudi AS-primed B lymphocytes to synthesise specific Abs in vitro upon incubation with nRBC lysate demonstrated the Ag-specific nature of this response; like lymphoproliferation (4.11 and 9.3), cells were stimulated in vitro specifically by the plasmodial Ags to which they had been primed previously by natural infection. The lesser response of naive B cells compared to the corresponding cells previously exposed to P. c. chabaudi AS when both were cultured with pRBC lysate ($p< 0.05$ for both WEP 737 and WEP 723) (Fig. 9.4.3) reflected the fact that the naive lymphocytes were making a primary Ab response whereas for the post-infective cells, the kinetics and magnitude of Ab production were characteristic of a secondary response. For both situations, however, the maximal induction of B cell activation occurred in the presence of those T cells, WEP 737 and WEP 723, considered to be good T helpers for Ab production.

9.5 Assay of IL-2 and IL-4 production

An investigation was undertaken to examine whether or not antigenic stimulation of the Ly-4$^+$ P. c. chabaudi AS-specific lymphocytes in vitro induced the secretion of lymphokines that may play a role in the activation of protective immune effector mechanisms. The production of three different lymphokines was monitored, IL-2 and IL-4 described here, and also IFN-γ (9.6). The presence of IL-2 and/or IL-4 was assessed by testing the effect of different S/N dilutions on the growth of the CTLL-2 cell line, which responds to both these lymphokines. Monospecificity for IL-2 or IL-4 was achieved by incubation with or without an anti-IL-4 MAb, respectively (2.37).

Firstly, considering the ability of culture S/N taken from incubating each of the four Ly-4$^+$ parent lymphocyte lines to support the proliferation of the lymphokine-dependent cell line (Fig. 9.5.1), it can be seen that in the absence of blocking MAb, there was a striking divergency in the *tritium incorporation* levels attained. For the recombinant IL-2 control included to gauge the growth of the target cell line, cellular proliferation titred out directly with the dilution of IL-2 used. This formed the control against which all other c.p.m. values could be compared, for it was known that the responsiveness of CTLL-2 cells to IL-2 was much greater than that to IL-4 (2.37). Figure 9.5.1 shows that the degree of support given to the target cell line by S/N of WEP 775 and of WEP 779 cultured lymphocytes was considerable. At lower S/N dilutions, CTLL-2 cell growth, and thus, by inference, IL-2 and/or IL-4 concentration, was

comparable with that of similarly diluted pure IL-2. Thus, these S/N contained a potent activator of proliferation of the IL-2-/IL-4-dependent cell line, presumably IL-2 and/or IL-4. Indeed, at higher S/N dilutions, the ability of WEP 775- and of WEP 779-derived S/N to support CTLL-2 growth was actually greater than that of the recombinant IL-2 control.

In contrast, in the presence of S/N taken from culturing either WEP 737 or WEP 723 Ly-4^+ lines in vitro, only low level CTLL-2 proliferation took place (Fig. 9.5.1). These S/N did not lack an ability to support target cell proliferation, since the levels of tritium uptake at all but the highest dilutions tested were significantly raised ($p < 0.05$) compared to c.p.m. values for the negative control of CTLL-2 cells incubated in the absence of lymphokine stimulation. However, the support given to the target cell line by S/N of WEP 737 and of WEP 723 was significantly less than that afforded by S/N from either cultured naive or post-infective splenic T cells ($p < 0.01$). Indeed, for undiluted S/N, the c.p.m. values recorded for WEP 737 (2301) and for WEP 723 (1942) were an order of magnitude less than those recorded for WEP 775 (20723) and for WEP 779 (17403). This disparity between the abilities of S/N taken from each of the four Ly-4^+ lines to enable the IL-2-/IL-4-dependent CTLL-2 cell line to grow in vitro was due either to a dichotomy in the types of lymphokine elaborated, or alternatively, to a quantitative difference in the levels of IL-2 and/or IL-4 secreted; which was the case could be determined only by repeating this analysis in the presence of 11B11 cell line S/N (anti-IL-4 MAb).

Figure 9.5.2 shows the lymphokine secretion profiles for S/N from each of the four P. c. chabaudi AS-specific Ly-4-bearing lymphocyte lines, but this time incubating the target cells with an anti-IL-4 MAb to achieve monospecificity for the detection of IL-2. Again, there was a clear and significant difference in the capacities of the lymphocyte-derived S/N to promote the proliferation of the target line. Under these experimental conditions, the growth of the CTLL-2 cells was due entirely to the presence of IL-2 in the culture S/N. For WEP 775 and WEP 779, each gave a very similar proliferation profile, as measured by tritium incorporation, to that of the corresponding assay in the absence of anti-IL-4 MAb (Fig. 9.5.1). Comparison of the c.p.m. values attained both in the absence and presence of the anti-IL-4 MAb (Fig. 9.5.3) showed that the culture S/N from WEP 775 and WEP 779 supported an extremely similar level of target cell growth in both instances. Indeed, there was no significant difference between the proliferation of CTLL-2 cells with or without the presence of 11B11 S/N in the culture system for

both lymphocyte lines tested ($p > 0.05$). Thus, identical levels of support of target cell growth in the monospecific IL-2 assay (Fig. 9.5.2) and in the original IL-2/IL-4 assay (Fig. 9.5.1) not only showed that the WEP 775 and WEP 779 Ly-4^+ lines secreted high levels of IL-2 upon antigenic stimulation in vitro, they also implicated that these lymphocyte populations did not produce detectable quantities of IL-4, at least not under the experimental conditions employed. Although a note of caution is advisable concerning the interpretation of these data, it is highly likely that these P. c. chabaudi AS-specific Ly-4^+ lines were capable of secreting IL-2, but not IL-4, upon activation with pRBC lysate Ag in vitro. It would have been desirable to repeat this assay using an anti-IL-2 MAb, but unfortunately the anti-IL-2 Mab-secreting S4B6 cell line was not available. This would have demonstrated unequivocally the IL-2-secreting property of both WEP 775 and WEP 779 Ly-4^+ lines; however, that this reciprocal assay was not performed does not invalidate the findings (Cushley, W., Grencis, R.G., Wood, P.R., personal communication), and many reports have been published using either one or the other MAb to achieve monospecificity, but not both (e.g. Spinella et al 1990).

Examination of Fig. 9.5.1 would show that at the higher S/N dilutions assayed, the level of CTLL-2 proliferation supported by each of WEP 775 and WEP 779 culture S/N was significantly greater than that supported by the recombinant IL-2 control ($p < 0.05$). A similar disparity upon repetition of this assay with anti-IL-4 MAb (Fig. 9.5.2) ruled out the possibility of IL-4 contributing towards the enhanced growth support of these S/N. It is presumed this phenomenon was due to the presence in solution of one or more non-specific growth factors of undetermined identity in the culture S/N which acted to promote further the proliferation of the incubated CTLL-2 cells.

In view of the identical lymphokine secretion of WEP 775 and WEP 779 lymphocyte lines in the presence or absence of the anti-IL-4 MAb, initial inspection of Fig. 9.5.2 may lead one to summise that it is no different from Fig. 9.5.1. Detailed scrutiny would show this not to be the case, however, for there was a graphically small but biologically significant difference in the degrees of target cell proliferation attained by culturing S/N taken from WEP 737 or WEP 723 cultures without or with the addition of 11B11 cell culture S/N (Figs. 9.5.1 & 2, respectively). Direct comparison of these two assays (Fig. 9.5.4) reveals that for both Ly-4^+ lines at all S/N dilutions tested, the level of tritium incorporation recorded by culturing the target cells in the absence of the anti-IL-4 MAb was significantly greater than that recorded upon the addition of this MAb ($p < 0.01$). Moreover, for the monospecific IL-2 assay, the proliferation of the IL-2-/IL-

4-dependent cell line upon addition of either WEP 737 or WEP 723 culture S/N was reduced to the background level of the negative control (Fig. 9.5.2). The observation of a low grade stimulation of CTLL-2 growth in the original assay (Fig. 9.5.1) which was lost in the monospecific IL-2 assay (Fig. 9.5.2) indicates the presence of IL-4 in the WEP 737 and WEP 723 culture S/N. It would appear that these two Ly-4-bearing T cell populations produced IL-4 upon antigenic stimulation in vitro, and it was this which was responsible for the low level maintenance of the lymphokine-dependent target cell line used. Unlike the other two P. c. chabaudi AS-specific Ly-4$^+$ lines, WEP 775 and WEP 779, used throughout this study, the IL-4-secreting lines were unable to produce IL-2 upon activation in vitro. This was concluded from the fact that upon blocking the IL-4 activity present in either WEP 737 or WEP 723 culture S/N by incubation with the anti-IL-4 MAb, the CTLL-2 growth-supporting property of these S/N was abrogated completely (Fig. 9.5.4). It thus transpired that the dichotomy in the lymphokine secretion profiles of the four Ly-4$^+$ lymphocyte lines showed a qualitative rather than a quantitative effect. Hence, two lines (WEP 775 and WEP 779) secreted IL-2 but not IL-4 in vitro, whilst two others (WEP 737 and WEP 723) showed the reverse pattern of synthesis and secretion.

Comparison of the ability of S/N taken from either of the two control cultures used to enable the proliferation of the target cell line gave an indication of the proportions of lymphocytes of mixed heterogeneity capable of secreting different lymphokines. Figure 9.5.4 showed that, both in the absence and presence of 11B11 S/N, post-infective splenic T cell culture S/N was capable of supporting CTLL-2 cellular proliferation. In the absence of the anti-IL-4 MAb, the c.p.m. values attained were significantly greater than those when this MAb was added to the culture system. Unlike the WEP 737 or WEP 723 culture S/N, however, conversion to an IL-2 monospecific assay did not result in the abrogation of all lymphokine activity (Fig. 9.5.4). Thus, unlike any of the S/N of cultured homogeneous populations of in vitro-propagated Ly-4$^+$ lymphocyte lines, which contained either IL-2 (WEP 775 and WEP 779) or IL-4 (WEP 737 and WEP 723), but not both, S/N collected from similarly incubated splenic T cells isolated from mice recently recovered from a primary challenge with P. c. chabaudi possessed detectable quantities of both IL-2 and IL-4. This mixed lymphokine response was attributable to the heterogeneity of the T lymphocyte population resident within the spleen. To a lesser extent, naive splenic T cells also elaborated both IL-2 and IL-4 upon in vitro cultivation, but in this instance the degree of IL-4 production was relatively

slight compared to IL-2 synthesis (Fig. 9.5.4). The assays performed for IL-2 and for IL-4 secretion showed that there was a difference in the lymphokine secretion patterns amongst the four Ly-4$^+$ lines tested. Considerable IL-2 activities could be detected in culture S/N taken from the primary infection-derived lymphocyte lines (WEP 775 and WEP 779). These were the preparations thought to confer protection through B cell-independent effector functions in vivo (Chapter 7), and shown to lack a helper activity for Ab production in vitro (9.4). Conversely, those Ly-4-bearing lines shown to have a B cell dependence to engender protection against P. c. chabaudi AS challenge (WEP 737 and WEP 723) (Chapter 7) were those for which a T cell helper function for specific anti-malarial Ab production in vitro was demonstrated (9.4), and were those now shown to synthesise and secrete IL-4, but not IL-2, upon antigenic stimulation in vitro.

When this work was extended to assay for lymphokine activity of culture S/N taken from each of the daughter cloned lines of WEP 775 and WEP 737, the results obtained confirmed the division of the parent Ly-4$^+$ populations on the basis of differential lymphokine production. It was found that in all cases, the monoclonal populations possessed the same lymphokine secretion profiles as did their parent lines from which they were isolated. Although there was some variation between clones of the same origin in the magnitude of the support given to CTLL-2 test cells cocultured with S/N, and thus, by implication, the levels of IL-2 or IL-4 produced, these differences were only quantitative. Thus, the cloning procedure did not select for all cell types exhibiting a qualitatively distinct secretion of lymphokines to that of the uncloned parent population. Hence, as for helper T cell function, the daughter Ly-4$^+$ clones appeared to show a range of activities representative of the majority Ly-4-bearing lymphocytes from which they were derived.

Since the clones behaved like their parent lines with regard to lymphokine synthesis, Fig. 9.5.5 for the proliferation of the target cell line when incubated with clone culture S/N in the absence of anti-IL-4 MAb is essentially identical to Fig. 9.5.1 previously described. Likewise, the corresponding graphs for monospecific IL-2 secretion by the Ly-4$^+$ lines (Fig. 9.5.2) and clones (Fig. 9.5.6) are very similar.

For the WEP 775-derived T cell clones, comparison of bi- and mono-specific CTLL-2 proliferation assay data (Fig. 9.5.7) showed no significant difference ($p > 0.05$) between the two for each clone tested. Hence, there was no detectable loss of activity upon addition of the anti-IL-4 MAb to the culture system and the support of target cell growth was therefore attributable to the presence of IL-2 in each culture S/N. There

was no significant difference between the c.p.m. values recorded for three of the clones, WEP 997-999 ($p > 0.05$). However, in the case of WEP 996, culture S/N supported a significantly reduced level of CTLL-2 growth than did S/N taken from culturing in vitro any of the three other daughter clones of WEP 775 studied ($p < 0.05$) (Fig. 9.5.7). Although this difference in the levels of tritium incorporation is only slight compared to the large difference between the c.p.m. values recorded by incubating the target cell line with culture S/N of daughter clones of either WEP 775 or WEP 737, it may be of profound significance. This is because it is this clone, WEP 996, which, of the WEP 775-derived cloned lines, induced the highest anti-plasmodial Ab response (Fig. 9.4.2). Taken together, these findings suggest that there is a natural variation in the ability of P. c. chabaudi AS-specific Ly-4^+ clones to perform protective immune functions in vitro. Whether of not this qualitative difference in immune capacity of the Ly-4^+ clones has a bearing on either the mechanism of protective immunity followed in vivo or the efficacy of that effector function remains to be established.

As for the parent lymphocyte line, WEP 737, the capacity of culture S/N taken from any of the four daughter clones, WEP 985-988, to maintain the growth of the IL-2-/IL-4-dependent cell line CTLL-2 was abrogated completely by the addition of the anti-IL-4 MAb. Figure 9.5.8 compares the levels of tritium incorporation with those for the control groups tested, whereas the differential lymhokine secretion by these clones in the absence and presence of the anti-IL-4 MAb can be appreciated more readily by studying the same data in more detail (Fig. 9.5.9). The lack of growth of the target cells after blocking IL-4 activity showed conclusively that these clones did not secrete IL-2, but rather IL-4. The actual c.p.m. values attained for CTLL-2 growth were not necessarily a reflection of the absolute quantities of IL-4 produced by each cloned population in vitro, since this target line is known to respond more efficiently to stimulation with IL-2 than with IL-4. Despite this, the relative levels of tritium incorporation, and therefore IL-4 synthesis, could be compared amongst clones of the same WEP 737 origin. For each clone, the c.p.m. values recorded in the bi- and mono-specific assays were significantly different ($p < 0.01$) (Fig. 9.5.9). Comparison of the tritium incorporation levels induced by S/N from different clones under the same assay conditions, however, indicated that there was no significant difference in the IL-4 activity present in each of the four batches of culture S/N ($p > 0.05$). It is true that at each S/N dilution, WEP 986 S/N gave the most support to CTLL-2 cellular proliferation (Fig. 9.5.9), the same clone which induced the largest anti-P. c. chabaudi AS secondary

Ab response (Fig. 9.4.2). However, although this observation hints at a direct link between IL-4 secretion by Ly-4$^+$ lymphocyte clones and their ability to act as helper cells for specific Ab production, the standard deviations between both the levels of IL-4 synthesis and anti-malarial Ab titres amongst the four WEP 737 daughter clones were too small to support such a proposal.

9.6 Assay of IFN-γ production

In order to define further the nature of the specific protective mechanisms mediated by each of the Ly-4$^+$ T cell lines and clones that have been analysed, each was assayed for the production in vitro of the lymphokine IFN-γ. The concentration of IFN-γ in each S/N sample was measured by a plaque inhibition assay using Semliki Forest virus and L-929 cells (2.38). In the first instance, viral proliferation was recorded (tritium incorporation), this being indirectly proportional to IFN-γ concentration. The dilution of S/N that conferred a 50% level of protection to the target cell monolayer was used to calculate the IFN-γ titre of each sample (BRMP U/ml).

Figure 9.6.1 shows that there was a most noticeable difference in the levels of IFN-γ detected in S/N of the cultured Ly-4$^+$ lymphocyte lines. The P. c. chabaudi AS-specific lines WEP 775 and WEP 779 were those populations prepared by sacrificing semi-immune mice 16 and 20 d, respectively, after primary challenge. Upon in vitro culture under optimal conditions (incubation with pRBC lysate Ag and parasite-primed B cells), the collected S/N showed quite respectable IFN-γ titres of 92 U/ml (WEP 775) and 81 U/ml (WEP 779). The demonstration of an IFN-γ activity for these two Ly-4-bearing populations was not surprising in light of existing knowledge of the character of these lymphocytes. Previously, it had been reported that WEP 775 and WEP 779 were B cell-independent in their protective reactivity upon adoptive transfer (Chapter 7), which correlated well with their inability to promote a specific anti-plasmodial B cell response in vitro (9.4). More importantly with respect to this assay, it was shown that these lines produced considerable quantities of IL-2 upon appropriate antigenic stimulation in vitro (9.5). As will be discussed later, elaboration of both IL-2 and IFN-γ by malaria-specific murine and human T cell clones is a phenomenon which has been documented previously.

The remaining two Ly-4$^+$ populations studied, WEP 737 and WEP 723, isolated from the spleens of multiply infected donor mice, failed to prevent viral RNA synthesis and therefore recorded only base line IFN-γ titres for most culturing conditions employed

(Fig. 9.6.1) (it should be noted that the negative controls of conditioned medium alone were included, but not shown). WEP 737 and WEP 723 were the lines shown to confer protection through B cell-dependent mechanisms in vivo (Chapter 7), and in vitro that secreted IL-4 (9.5) and promoted an Ab response (9.4).

For each Ly-4$^+$ cell line, four different culture S/N were assayed for IFN-γ content. It was found that regardless of the capacity of each line to secrete this lymphokine in vitro (Fig. 9.6.1), the highest IFN-γ titres were attained upon antigenic stimulation of the P. c. chabaudi AS-specific lymphocytes with homologous parasite Ag (Fig. 9.6.2). As described before for both lymphoproliferation (4.11 & 9.3) and for helper T cell function (9.4), the response of the pRBC-primed Ly-4$^+$ cells upon incubation with nRBC lysate as a source of Ag was very poor, stressing the requirement of all lymphocyte functions in vitro for activation by specific plasmodial Ags.

The culture S/N tested for the presence of IFN-γ were the same as those assayed for helper T cell function (9.4). The cultures therefore contained B cells of varying immunological competence which were considered not to affect the lymphokine-secreting potential of the Ly-4$^+$ lymphocytes present. This was found to be essentially true, for although the IFN-γ titres for WEP 775 and WEP 779 cells cocultured with post-infective B cells were greater than the corresponding titres for S/N taken from cultures containing naive B cells, in each case, the difference was not significant ($p > 0.05$) (Fig. 9.6.1).

A further finding from the preliminary IFN-γ assays was the constancy of the differential levels of secretion attained with each of the four distinct culture conditions. Thus, for each different set of cultures, WEP 775 culture S/N had the highest IFN-γ activity, followed in order by S/N from WEP 779, WEP 737 and WEP 723 cultures (Fig. 9.6.2). It is interesting to note that this represents an ordered sequence of the Ly-4$^+$ lines based upon the length of priming to P. c. chabaudi AS achieved through natural immunisation. Thus, WEP 775 cells, which were exposed to plasmodial erythrocytic stages in vivo for a maximum of 16 d, produced the greatest concentrations of IFN-γ in vitro. Conversely, WEP 723 cells, isolated from donor mice after recovery from a tertiary infection, and therefore presumably fully immunologically primed to a range of antigenically variant pRBC, produced bearably detectable levels of IFN-γ in vitro (Figs. 9.6.1 & 2). It may be, therefore, that this inverse correlation between the exposure time to P. c. chabaudi AS in vivo prior to initiating each of the Ly-4$^+$ lines in vitro and their ability to secrete IFN-γ upon homologous antigenic stimulation in vitro

reflects an underlying heterogeneity with which each of these populations of identical phenotype exert their anti-plasmodial effects in vivo. Moreover, this suggests an apparent sequential appearance in vivo, first, of IFN-γ-secreting Ly-4-bearing lymphocytes, represented here by WEP 775 and WEP 779, and second, some time later, of IL-4-secreting Ly-4$^+$ cells that act as helpers for Ab synthesis, represented here by WEP 737 and WEP 723. Assuming that the effector functions displayed by each of the characterised lines of Ly-4$^+$ phenotype were representative of the predominant cell type present in vivo at the times when the lines were developed, this temporal regulation of the type of Ly-4$^+$ cell present in the peripheral blood, and thus of the prevailing protective reactivity, may reflect the natural course of the host immune response to a P. c. chabaudi AS challenge.

After it had been determined that culturing lymphocytes of the Ly-4$^+$ T cell subset in vitro with P. c. chabaudi AS pRBC lysate and post-infective B cells optimised the secretion of IFN-γ (Fig. 9.6.2), these cultural conditions were used to examine the IFN-γ secretion profiles of both Ly-4-bearing lines (Fig. 9.6.3) and clones (Fig. 9.6.4). As can be seen from Fig. 9.6.3, for each of the lymphocyte lines, the concentration of IFN-γ in the culture S/N titred out directly with the dilutions of the respective S/N. This pattern of IFN-γ distribution was observed for each line, irrespective of the actual absolute levels of lymphokine detected at each dilution. Indeed, the uniformity with which IFN-γ concentration fell with increasing S/N dilution for all four lines assayed meant that at the highest dilution tested, 1/32, WEP 775 and WEP 723 remained the highest and lowest IFN-γ producers, respectively (Fig. 9.6.3).

The cloned populations derived from WEP 775 or WEP 737 showed similar IFN-γ secretion profiles as did the respective parent lines (Fig. 9.6.4). Thus, the disparity between the levels of IFN-γ produced by WEP 775 and WEP 737 (Figs. 9.6.1 & 3) was also shown by their daughter clones. There was a significant difference between the concentrations of this lymphokine detected for the clones of different origin at all but the highest S/N dilution tested ($p < 0.01$) (Fig. 9.6.4). Within each group of four clones, there was little variation between the IFN-γ titres attained at each S/N dilution. It was again noticeable, however, that for WEP 996, culture S/N contained less biological activity than did S/N collected from other WEP 775-derived clones; on this occasion, however, this discrepancy was not of statistical significance ($p > 0.05$). Thus, for IFN-γ synthesis, as for all other assays performed to characterise functionally the Ly-4$^+$ preparations raised against P. c. chabaudi AS, the monoclonal populations expanded from

limiting dilution cultures of WEP 775 or WEP 737 showed a similar behaviour in vitro, both to each other and to the parent line from which they were derived.

9.7 Adoptive transfer of T cell clones to naive syngeneic recipients

In this chapter thus far, it has been shown that the cloned Ly-4$^+$ lymphocyte populations studied had the same activity in vitro as the uncloned parent lines. Such cells have been characterised for their surface phenotype (9.2), Ag-reactivity (9.3), B cell helper activity (9.4) and their secretion of various lymphokines (9.5 & 6). These assays had shown clearly that the heterogeneity between WEP 775 and WEP 737 of the anti-malarial immune effector functions displayed by these two Ly-4$^+$ lines was maintained after cloning. Having observed this divergence of behaviour of the clones in vitro, it was desirable to examine, firstly, whether these P. c. chabaudi AS-specific monoclonal Ly-4$^+$ populations could transfer protection in vivo, and secondly, if so, whether the heterogeneity of response in vitro could be detected at the level of the immune response to natural infection. In order to address these possibilities, the ability of each of the clones to transfer immunity adoptively to non-immune mice was examined. The experiments performed were essentially a repeat of those previously described for the adoptive transfer of the Ly-4$^+$ parent lines to naive NIH recipients (5.2 & 6.4), save inoculating cloned lymphocyte preparations. For each clone, 3.0 x10^7 cells were inoculated i.v. into each of five age-matched NIH female mice. To enable comparison, similar groups of mice receiving WEP 775, WEP 737 or naive splenic T cells were prepared. All animals were infected with 1 x10^5 pRBC P. c. chabaudi AS at the time of adoptive transfer, and the level of peripheral blood infection monitored by examination of blood smears.

Each of the Ly-4$^+$ clones conferred considerable protection against challenge infection with P. c. chabaudi AS upon adoptive transfer into naive syngeneic recipient mice. This was not unexpected considering the previously described protective capacity of the parent lymphocyte lines (Chapter 5). The adoptive transfer of WEP 775 and of WEP 737 cells into non-immune mice was repeated here (Fig. 9.7.1) to enable comparison with the transfer of daughter clones. As was noted before (5.2 & 6.4), recipients of either parent population showed a significantly suppressed recrudescent parasitaemia of shortened duration, compared to control mice inoculated with naive splenic T cells or with mice not receiving any additional lymphocytes ($p < 0.01$) (Fig. 9.7.1). However, unlike the two previous occasions when WEP 775 or WEP 737 Ly-4$^+$ lines have been

transferred to intact recipients (Figs. 5.2.5 & 6.4.1), the differences in the levels of protection conferred by these P. c. chabaudi AS-specific lines and by unprimed splenic T cells at homologous challenge were not as great. This time, the primary parasitaemias attained were similar both in their level and duration (Fig. 9.7.1). This anomaly notwithstanding, the transfers of WEP 775 and WEP 737 to naive NIH mice served as adequate controls for the analysis of the protective capacity of the derived Ly-4-bearing monoclonal populations. This was because comparisons were being made between the courses of infection of mice receiving each of the clones, and not between these and other cell transfers. Since the patterns of parasitaemia exhibited by mice given either WEP 775 or WEP 737 differed only in the onset of recrudescence (Figs. 5.2.5, 6.4.1 & 9.7.1), it was only this characteristic which was of direct interest as a gauge of protective immunity in this experiment. As it transpired, even for this parameter of protection, the variation observed here was not as pronounced as that described earlier; recipients of WEP 737 did recrudesce prior to those of WEP 775 (Fig. 9.7.1), but this difference was not statistically significant ($p > 0.05$).

Comparing the courses of infection of mice adoptively transferred each of the clones with the patterns of parasitaemia attained for the respective parent uncloned lines (Figs. 9.7.2 & 3), it is noticeable that, in general terms, the kinetics of blood stream P. c. chabaudi AS infection were very similar. Indeed, for both WEP 775- and WEP 737-derived clones, the dynamics of primary parasitaemia were identical to those of the respective parent lines upon transfer to naive mice (Figs. 9.7.2 & 3). For each daughter clone of WEP 737, upon adoptive transfer, there was no significant difference ($p > 0.05$) between the onset, magnitude or duration of recrudescence, compared with either that parasitaemia observed in recipients of the parent Ly-4^+ cell line or of other clones of similar parentage (Fig. 9.7.3). Likewise, for WEP 775-derived clones, recipient mice showed patterns of recrudescent parasitaemia that were insignificantly different from those observed for WEP 775-inoculated animals ($p > 0.05$) (Fig. 9.7.2). However, although the manifestation of protection conferred by each of the clones was similar to that of the parent cell line, there were variations between the recrudescent parasitaemias seen in recipients of different clones, which, compared to each other, were of significance. For instance, mice given WEP 996 recrudesced on d 26 p.i., 3 d earlier than did mice transferred WEP 997 ($p < 0.05$).

As the daughter clones of WEP 775 and of WEP 737 essentially conferred similar protection to naive mice upon P. c. chabaudi AS challenge as did the parent malaria-

specific lines (Figs. 9.7.2 & 3), in general terms, recipients of WEP 775-derived clones recrudesced earlier than did their counterparts given clones of WEP 737 origin. Comparison of Figs. 9.7.2 & 3 shows this to be so, for the onset of recrudescence for recipients of clones WEP 996-999 was between 26-29 d p.i. (Fig. 9.7.2), compared to 25-26 d p.i. for mice given one of clones WEP 985-988 (Fig. 9.7.3). From these data, it could be argued that the mediation of different effector functions in vitro (previously described in this chapter) correlates with a divergence in the protective capacity of these clones upon adoptive transfer. However, such an interpretation would be misleading because the differences detailed above were, in most cases, not statistically significant ($p > 0.05$). The variation in the behaviour of different Ly-4$^+$ clones of the same derivation meant that, for some, recipient mice did indeed show distinctly different kinetics of recrudescent parasitaemia compared to recipients of clones of alternative origin. This is exemplified by Fig. 9.7.4 which shows the extremes of variation with regard to recrudescence. WEP 986 and WEP 988 were those WEP 737-isolated Ly-4$^+$ clones that recrudesced the earliest (d 25 p.i.), whilst WEP 997 and WEP 999 were those clones derived from WEP 775 that recrudesced the latest (d 29 and d 28 p.i., respectively). The difference in onset of recrudescence seen here was significant ($p < 0.05$), which supports the contention that this reflects the dichotomy of the reactivity of the clones at the cellular level. However, this finding was not universal, and the differences in origin of some clones conferring a similar protection in vivo with regard to recrudescent parasitaemia (Fig. 9.7.5) was not at all obvious. Figure 9.7.5 depicts the courses of infection in P. c. chabaudi AS-infected recipient mice upon adoptive transfer of either WEP 996 and WEP 998 (of WEP 775 origin) or WEP 985 and WEP 987 (of WEP 737 origin). For each, breakthrough recrudescence was observed either on d 26 or d 27 p.i., so there was no significant difference in this parameter of protection between these particular clones ($p > 0.05$).

For all the monoclonal Ly-4$^+$ populations examined for the adoptive transfer of immunity to homologous challenge, a measurably greater degree of protection was engendered compared to the transfer of unprimed naive T cells; this was manifested as a significant lowering of the peak recrudecence ($p < 0.01$) (Figs. 9.7.4 & 5), and occurred irrespective of the variation in the timing of the recrudescence between recipients of different clones. Thus, the relatively subtle differences in the kinetics of parasite clearance between mice given Ly-4$^+$ clones of the same or different origin should not detract from the enhanced capacity of each of these P. c. chabaudi AS-specific

populations to control homologous primary infection compared to similar numbers of non-immune T cells.

Comparison of the courses of infection in mice receiving different cloned populations with those of the parent cell lines, WEP 775 and WEP 737, revealed that the distribution of recrudescent parasitaemias observed upon adoptive transfer of different clones ranged about a mean that included the parent lines. Thus, for Fig. 9.7.6, which shows the extremes of recrudescence attained in this study, the time over which secondary parasitaemia was observed was 25-41 d p.i., compared to 27-39 d p.i. and 26-38 d p.i. for recipients of WEP 775 or WEP 737, respectively. The fact that the parasitaemia for recipients of either uncloned line formed the mid point of the range of recrudescences (Figs. 9.7.6 & 7) indicated that there was a normal distribution (non-skewed) of variation in this parameter of protection exhibited by clones of the same origin, from which it could be inferred that the deviation of the behaviour of each clone from the norm was not of likely biological significance. Further, detailed examination of the patterns of parasitaemia attained upon adoptive transfer of each of the Ly-4$^+$ preparations showed that for all clones and both lines studied, the length of the patent recrudescence was between 12-13 d (14 d for controls), regardless of the onset of secondary parasitaemia (Figs. 9.7.6 & 7). Thus, the variation in the course of infection in recipients of different P. c. chabaudi AS-specific clones did not relate at all to the magnitude or duration of either acute or recrudescent patencies (Figs. 9.7.6 & 7). Rather, the difference in onset of recrudescence that was sometimes observed (Fig. 9.7.6) was a result of the slight variation in the length of the prerecrudescent subpatent period. Hence, the latest breakthrough recrudescence, on d 29 p.i., for recipients of WEP 997, was preceded by the longest recorded subpatency (10 d). Likewise, for the similarly WEP 775-derived WEP 996, the subpatent period was only 6 d, resulting in detection of recrudescent pRBC in the peripheral blood by d 26 p.i. (Fig. 9.7.2). In this regard, it should be noted that WEP 996 and WEP 997 showed slight but appreciable differences in levels of activity in vitro with respect to lymphokine secretion (9.5 & 6) and helper function for Ab production (9.4). However, these clones represented the extremes of responsiveness in vitro, and whether or not such minor differences at the cellular level could be converted into significant changes in the control of challenge infection of competent host animals is debatable. The preliminary investigation described revealed that the protective activity of the P. c. chabaudi AS-specific clones of Ly-4$^+$ phenotype did show some heterogeneity in vivo. Whether or not this was of

significance, and, if so, whether it could be attributed to differences in the effector function of monoclonal populations of identical phenotype could be answered only by examining the transfer of protection to immunologically deprived mice.

9.8 Discussion

An obligatory requirement for lymphocytes of the Ly-4$^+$ T cell subset in the protective immune response to infection with P. c. chabaudi AS is now undisputed (Langhorne 1989). Langhorne and colleagues have investigated the type of T cells involved in immunity to P. c. chabaudi AS by application of a limiting dilution assay system on splenic lymphocytes isolated from mice during the course of a primary infection (Langhorne & Simon 1989, Langhorne et al 1989 a) and by rat MAbs against murine Ly-2 and Ly-4 determinants to deplete mice of specific T cells prior to or during infection (Süss et al 1988, Langhorne et al 1989 b). This latter technique was also used herein (Chapter 8), both studies finding a major role for Ly-4$^+$ but not for Ly-2$^+$ T cells in controlling peripheral blood infection. Throughout this thesis, however, a different approach has been documented: the establishment in vitro of P. c. chabaudi AS-specific T cell lines from repeatedly stimulated bulk cultures (Chapter 4) and their adoptive transfer to both immunocompetent (Chapters 5 & 6) and compromised (Chapters 7 & 8) non-immune mice. Upon transfer of the four lymphocyte lines studied to immunologically suppressed recipients, it was demonstrated that those lines taken from reinfected mice (WEP 737 and WEP 723) were B cell-dependent in vivo, whereas those lines taken on d 16 (WEP 775) and d 20 (WEP 779) of primary infection were largely B cell-independent in vivo. To examine whether this heterogeneity of the T cell response elicited during a P. c. chabaudi AS infection in NIH mice was indicative of a dichotomy in the mechanisms by which these in vivo-primed lines mediate protection, each was subjected to functional analysis in vitro, as described in this chapter. In addition, since heterogeneous populations of T cells were used, it was considered that it may be both difficult to dissect the specific mechanisms of T lymphocyte-mediated immunity involved, and to justify the conclusions drawn from the results. Thus, limiting dilution techniques were used to develop a series of clones which were then expanded with plasmodial Ag and anti-CD3 MAb (Chapter 4). These too were assayed for functional activity in vitro and also for their ability to confer protection during a primary infection in this parasite-host model of malaria.

The phenotypic characterisation detailed in this chapter confirmed the predicted identity

of the in vitro-propagated T lymphocyte lines primed to P. c. chabaudi AS as belonging to the Ly-4$^+$ T cell subset. This was not surprising as, although the characterisation of the uncloned lines is included in this chapter to enable direct comparison with that of the respective daughter clones, these assays were actually performed prior to the adoptive transfer experiments described in Chapters 5-8. Thus, although it was not stated, these reconstitution studies were performed in the knowledge of the identity of the P. c. chabaudi AS-specific lines being of the Ly-4$^+$ phenotype, which therefore validified the conclusions reached. At the initiation of in vitro culture, expression of the Ly-4 Ag amongst the four propagated lines, as determined by IFAT, was between 78-91%. This compared favourably with the 75% positive fluorescence detected with the Ly-1 marker at the same stage of culture of a P. c. adami-specific T cell line (Brake et al 1986). This is the only comparable study which has been made, for which spleens were removed from BALB/c ByJ mice recently recovered from a secondary infection and cultured in vitro with specific P. c. adami Ag and syngeneic APC. The methodology followed to generate this line was therefore essentially similar to that followed for the initiation of the WEP 737 line (Chapter 4). For the P. c. adami-primed line, expression of the Ly-1 cell surface determinant increased from 75% to 80% over a two month culture period, with a concomitant drop in the proportion of contaminating Ly-2$^+$ cells from 8% to 6% (Brake et al 1986). For WEP 737, a similar enrichment of the lymphocyte population for cells of the majority Ly-4$^+$ characterisation was also observed; thus, exhibition of the Ly-4 Ag rose from 88% to 95% after several subcultures, whilst the contamination through Ly-2$^+$ lymphocytes fell from 6% to 3% over the same period of time (data not shown). These findings suggested that both in vitro culture techniques used not only selected for lymphocytes of the Ly-4-bearing T cell subset at the outset of propagation but continued to do so upon prolonged in vitro cultivation, so giving rise to Plasmodium-specific lines which were close to homogeneity for expression of the Ly-4$^+$ phenotype. For WEP 775 and WEP 737, such a state of homogeneity was achieved by cloning each population by limiting dilution, whereupon each of the clones tested expressed the Ly-4$^+$Ly-2$^-$ surface phenotype, as judged by both indirect immunofluorescence and complement-mediated cytotoxicity. Similarly, Brake et al (1988) typed nine cloned lines of the original P. c. adami-raised population as having an identical phenotypic characterisation.

For the eight monoclonal populations analysed, each was shown to proliferate in vitro specifically in response to P. c. chabaudi AS Ag, in the form of a pRBC lysate, presented

by syngeneic APC. In this respect, the clones behaved similarly to their parent uncloned lines, WEP 775 or WEP 737 (Chapter 4). These results concurred with those of Brake et al (1988) with P. c. adami-specific Ly-4$^+$ clones, which were shown to maintain plasmodial Ag specificity and were H-2-restricted when cultured in vitro. In addition, in a similar experimental system, Baldwin et al (1987) reported the MHC-restricted Ag-specific proliferation in vitro of bovine helper T cell clones raised against lymphocytes infected with Theileria parva (Mugaga). In this study, clones proliferated in the absence of exogenous IL-2 in a dose-dependent manner to specific Ag, an observation repeated here with both lines (4.11) and clones (9.3) specific for P. c. chabaudi AS. The proliferation profiles of lines and clones alike showed that an optimal rate of growth was achieved at a pRBC lysate concentration of 200 µg/ml, above which Ag-induced suppression of the proliferative response occurred. This inhibition of Ag-specific proliferation of T cell clones at high Ag concentrations has been attributed to the blocking of cell division following repeated exposure of T lymphocytes to Ag-APC complexes (Suzuki et al 1988).

Since the delineation of Ly-4-bearing T lymphocytes into two subsets based on the secretion of different lymphokines (Mosmann et al 1986), evidence has accumulated that clones representing T_H1- and T_H2-types are functionally different. It has been demonstrated that T_H1 and T_H2 clones use IL-2 or IL-4, respectively, as autocrine growth factors in vitro (Fernandez-Botran et al 1986, Lichtman et al 1987, Kupper et al 1987). In addition, T_H2 cells can also use IL-2 as a paracrine growth factor, and their responsiveness to IL-2 and IL-4 increases concomitantly after antigenic stimulation (Vitetta et al 1987). These findings suggested that the proliferation of normal T_H2 cells could be regulated, at least in part, by T_H1 cells. Indeed, Fernandez-Botran et al (1988) demonstrated that for a mixed population of T_H lymphocytes, the proliferative response in vitro was predominantly one of T_H1 cells. In the present study, monoclonal and not heterogeneous populations were studied, so no direct comparison can be made. However, at the optimal concentration of Ag shown to induce Ly-4$^+$ T lymphocyte proliferation (200 µg/ml), there was no significant difference between the detected growth of WEP 996-999 and of WEP 985-988, considered to be T_H1 and T_H2 cells on the basis of differential lymphokine production (discussed later). Enhanced growth of the WEP 996-999 clones was observed, however, at the higher end of the range of Ag concentrations assayed. These results concur with current opinion in that the growth in isolation of WEP 996-999 clones and of WEP 985-988 clones was

presumably after autocrine synthesis of IL-2 and IL-4, respectively. The fact that cloned populations of P. c. chabaudi AS-specific Ly-4$^+$ lymphocytes were able to grow in response to appropriate antigenic stimulation but in the absence of exogenous T cell growth factors showed that this proliferative response was under autocrine regulation. The growth of the cloned T_H2 populations (WEP 985-988) in vitro also showed that this was, at least, a secondary response to plasmodial Ag stimulation. This is because IL-4 production by splenic T cells taken from naive donor mice was shown to be completely dependent upon the presence of IL-2, either intentially added to cultures or produced in situ (Ben-Sasson et al 1990). None of the WEP 737-derived clones required exogenous IL-2 to proliferate in vitro, thus demonstrating that these lymphocytes had been activated previously by IL-2 during the process of priming by natural infection. The reduced growth of clones WEP 985-988 (T_H2) compared to that of clones WEP 996-999 (T_H1) at superoptimal concentrations of pRBC lysate was thought not to be of significance to the mediation of different effector functions by these two classes of Ly-4-bearing cells. This phenomenon was most probably attributable to the greater sensitivity of the T_H2 clones compared to the T_H1 clones with regard to suppression of proliferation at high Ag concentrations (Suzuki et al 1988). It was considered unlikely that this depression of the T_H2 response was due to a regulatory effect of T_H1-derived IFN-γ. This was because any contamination of the WEP 737 parent line with cells of the T_H1 Ly-4$^+$ phenotype was eliminated by cloning this population, thereby giving rise to the WEP 985-988 monoclonal populations. Thus, any IFN-γ possibly present in the culture S/N of in vitro-propagated WEP 737 cells would have been removed at the cloning stage. In any case, if the decreased proliferation was caused by the inhibitory effect of IFN-γ, this would have occurred, to a greater or lesser degree, at all pRBC lysate concentrations assayed. Hence, the down-regulation of IL-2- and IL-4-mediated proliferation of T_H2 but not T_H1 cells in mixed cultures in vitro (Fernandez-Botran et al 1988, Gajewski & Fitch 1988), and probably in vivo, could not account for the differential Ag-induced suppression of cloned Ly-4$^+$ lymphocytes detailed herein.

With regard to malaria-induced proliferation of T lymphocytes taken from donors previously exposed to plasmodia, this is thought to provide a measure both of the degree of priming to parasite-derived Ags and of the existing immunological memory (Troye-Blomberg & Perlmann 1988). By this rationale, it could be reasoned that those T_H2 clones (WEP 985-988) derived from WEP 737, which itself was initiated using splenic T lymphocytes from mice recovered from a secondary P. c. chabaudi AS infection,

would be expected to proliferate more under identical conditions of stimulation with homologous Ag and syngeneic APC than those T_H1 clones (WEP 996-999), derived from WEP 775, itself established in vitro from mice splenectomised on d 16 of a primary infection. However, the proliferation assays described in this chapter do nothing to support any proposed difference in the growth rates of P. c. chabaudi AS-specific Ly-4^+ clones primed in vivo for different lengths of time. This does not mean that these findings show a negative correlation between proliferation and the extent of priming either, since there was no significant difference in the growth of clones of either WEP 775 or WEP 737 origin over most of the pRBC lysate concentration range assayed. It is interesting to note that, as a consequence of those clones primed in vivo to P. c. chabaudi AS for two infections (WEP 985-988) belonging to the T_H2 T cell subset, the predicted results of the in vitro proliferative responsiveness of these populations would appear to be contradictory. Current immunological literature would favour an enhanced proliferation of T_H1 over T_H2 lymphocytes, whilst malaria studies indicate a level of growth in vitro commensurate with the length of priming in vivo. As it transpired, neither of these two claims could be supported by the available data, and it is of interest to speculate that these potentially antagonistic factors led to the proliferative responses of all the Ly-4-bearing cloned populations being insignicantly different from eachother. Alternatively, and more probably, the detected lack of variation for the proliferation rates amongst the clones tested would indicate an insufficient sensitivity of this assay system to detect possibly marginal differences between the growth rates of lymphocytes of varying origin or helper T cell subset.

In contrast to the studies of proliferation in vitro, the measurement of T helper cell activation, by determination of T-dependent Ig production in an in vitro T-B cooperation system, did show a heterogeneity of response of both lines and clones primed to P. c. chabaudi AS in vivo to varying degrees. It was found that the lines primed by multiple natural infections (WEP 737 and WEP 723) gave substantial Ab titres when assayed by IFAT, whereas those lines exposed to P. c. chabaudi AS only transiently during either the first 16 or 20 d of a primary infection (WEP 775 or WEP 779, respectively) gave only modest titres. Thus, WEP 737 and WEP 723, which had been shown previously to confer protection in vivo by B cell-dependent mechanisms (Chapter 7), were demonstrated to be good helper cells for specific Ab production, whilst this was not so for WEP 775 and WEP 779, known to exert anti-parasiticidal effects in vivo in the relative absence of B cells. These results were repeated at the clonal level, using WEP

775- and WEP 737-derived populations. Together, these findings concur with those of Langhorne et al (1989 a), who, also with P. c. chabaudi AS, demonstrated that the majority of Ly-4^+ lymphocytes elicited during the acute phase of primary infection were relatively poor helpers for Ab synthesis whilst the frequency of good helpers increased by the later stages of a patent peripheral blood infection.

It had been shown previously that the optimal concentration of P. c. chabaudi AS pRBC lysate to induce Ag-specific reactivity in vitro was 200 µg/ml (Chapter 4). This was hence used thereafter as the standard Ag concentration for all in vitro culture procedures, including assaying for helper T cell function. This proved to be very satisfactory, for at this pRBC lysate concentration, a clear delineation could be made between those Ly-4^+ cells offering effective help for anti-P. c. chabaudi AS Ig production and those not doing so. The method used to assess helper T cell activity was a modification of that described by Pearson et al (1983), who cultured 5 $x10^6$ spleen cells with 2 $x10^6$ whole infected RBC as a source of Ag. At this relatively high P. c. chabaudi AS Ag concentration, splenic lymphocytes were stimulated to produce specific Ab in vitro. Contradictory results have been shown for anti-P. falciparum Ab secretion in vitro, which was induced by low Ag concentrations (10-100 ng/ml soluble protein) (Kabilan et al 1987). At these low doses, either crude or Pf155-enriched P. falciparum Ag preparations induced IgG secretion in syngeneic B cells cocultured with T cells extracted from a P. falciparum-infected donor, whereas the control RBC ghost Ag did not. That this was indeed a T helper cell-dependent function was demonstrated by the lack of IgG secretion in the absence of T cells. High Ag doses (> 10 µg/ml) induced an extensive polyclonal IgG secretion, also seen with the RBC ghost Ag, and in lymphocyte cultures of both P. falciparum and control P. vivax patients. It may be thought that this variance represents a difference between the two malaria parasites studied, P. falciparum and P. c. chabaudi AS. It is clear that there was a difference in the concentration of plasmodial Ags required to induce parasite-specific Ig responses. However, the available data do not suggest that malaria-specific Ab is the only Ig secreted in the culture system used in the assay described herein, since in this study, the level of P. c. chabaudi AS-specific Abs was measured, but not the level of all Ig secretion. Thus, either at the 200 µg/ml pRBC lysate Ag concentration used, or at a higher Ag dosage, it is likely that polyclonal Ab responses would have been detected, if this had been examined. Polyclonal lymphocyte activation by P. falciparum Ag, as reflected by an increased Ig secretion, has been reported previously (Greenwood et al

1979, Ballet et al 1981, Kataaha et al 1984). Polyclonal Ab responses, however, are not restricted to T and B lymphocytes cocultured in vitro with P. falciparum pRBC. Goldring et al (1989) established that a Ly-4$^+$ T cell clone, shown to be protective upon adoptive transfer to nude mice at homologous P. c. adami challenge (Brake et al 1988), recognised a relatively large number of Ag specificities upon immunoprecipitation with sera collected from nude mice reconstituted with the identical T cell clone. From these findings, Goldring et al suggested that it would not be possible to determine the Ag specificity of cloned Ly-4$^+$ cells in malaria infections by their capacity to provide help to restricted populations of B lymphocytes. As P. c. adami has a very close phylogenetic relationship to P. c. chabaudi AS, it is possible that they share common cell surface Ags, which would imply a polyclonality of Ig secretion by B cells in response to specific stimulation by the latter parasite. This polyclonal response is quite distinct from the exquisitely-defined monoclonal Ab production that has been detected both in this study and by Kabilan et al (1987), which may occur either at different concentrations of Ag to the polyclonal response, or alternatively as a component of the polyclonal activation over a range of Ag concentrations, which may or may not be of physiological significance to the analogous in vivo situation. The detection of specific anti-plasmodial Ig at widely divergent concentrations of Ag in vitro in this study and that of Kabilan et al (1987) should not detract from the fact that a specific T helper cell requirement was needed in order to activate B cell differentiation and secretion of specific Ab in both instances.

The requirement for a helper T cell function was fulfilled in the assay detailed in this chapter by those lymphocytes categorised as T_H2, i.e. WEP 737, WEP 723 and the WEP 737-derived clones WEP 985-988. These data agree with the currently accepted view that T_H2 lymphocytes provide efficient help for B cell growth and differentiation (Kim et al 1985, Killar et al 1987, Boom et al 1988, De Kruyff et al 1989). T_H2 clones can stimulate significant clonal expansion and Ig secretion by 50-80% of splenic B cells in limiting dilution cultures (Lebman & Coffman 1988), and can stimulate populations of either small resting B cells (Boom et al 1988) or large B cells (Rasmussen et al 1988), through the mediation of either IL-4 and IL-5, or IL-5 alone, respectively.

In contrast, the ability of the T_H1 subset to function in a helper role for B cells is considerably more controversial. T_H1 clones have been reported to provide help for Ag-specific responses from primed B cells (Giedlin et al 1986, De Kruyff et al 1989) and for primary responses from hapten-purified unprimed B cells (Stevens et al 1988). However, other workers have accounted the failure of T_H1 clones to provide help for

primary responses either to specific Ags (Kim et al 1985, Killar et al 1987) or in the rabbit Ig polyclonal system (Boom et al 1988). These apparent discrepancies have now been reconciled by the fact that IFN-γ, present in some culture S/N but not in others, has a regulatory effect on B cell activity. It has been shown that IFN-γ at low concentrations acts as a specific antagonist of IL-4-mediated effects on B cells (Coffman & Carty 1986, Coffman et al 1986, Snapper & Paul 1987), and, at higher concentrations, acts as an inhibitor of B cell growth and differentiation (Coffman & Carty 1986, Mosmann & Coffman 1989 a). T_H1 clones have even been shown to be inhibitory for responses stimulated by T_H2 clones (Bottomly et al 1983, Friedman et al 1985), and much of this inhibition can be blocked with anti-IFN-γ Abs (Mosmann & Coffman 1989 b). In the assays performed, those clones designated to be T_H1 lymphocytes, WEP 997-999, as well as the Ly-4$^+$ lines, WEP 775 and WEP 779, induced a B cell response, as determined by IFAT of specific anti-P. c. chabaudi AS Ig, not significantly greater than that of the background controls. Thus, under the cultural conditions employed, these malaria-primed Ly-4-bearing cells did not appear to provide any appreciable help to B cells for Ig synthesis. These findings concur with those of other studies, since at the relative high dosage of pRBC lysate Ag used, activated T_H1 lymphocytes would be expected to be directly suppressive in cultures optimally stimulated by T_H2 cells (Bottomly et al 1983, Friedman et al 1985), as indeed was the case. The lack of helper activity of these particular P. c. chabaudi AS-specific Ly-4$^+$ lymphocytes in the T-B cell cooperation system used correlates well with the IFN-γ activity found in S/N taken from similar cultures. Thus, the secretion of IFN-γ by these T_H1 cells, but not by the T_H2 cells that mediate an Ig response, may account for the distinct inability of these populations to stimulate Ab production in vitro. One notable exception to the general failure of those Ly-4$^+$ populations primed to P. c. chabaudi AS during a primary infection to induce a significant B cell response in vitro was WEP 996, the incubation of which with post-infective B lymphocytes did enable the latter to mount an appreciable Ab response upon antigenic stimulation. Although the reciprocal IFAT titre attained was significantly below that of the lowest titre for T_H2-promoted Ab production, WEP 996 induced a degree of Ig synthesis significantly in excess of those recorded for other T_H1 cultures. This is consistent with the fact that WEP 996 culture S/N contained a reduced level of IFN-γ compared to those S/N of other WEP 775-derived Ly-4$^+$ clones. This intriguing finding was similar to that of De Kruyff et al (1989), who demonstrated a heterogeneity amongst T_H1 clones with regard to helper T cell

function. This study showed that although most monoclonal T_H1 populations induced little or no Ab synthesis with TNP-primed B cells, others were very effective at inducing anti-TNP IgG responses in similarly primed B cells. It was supposed that despite helper and non-helper T_H1 clones producing similar amounts of IL-2, non-helper T_H1 cells may secrete higher levels of inhibitory factors, including IFN-γ. De Kruyff et al concluded that although T cell clones can be classified as T_H1- or T_H2- types according to patterns of IL-2, IFN-γ or IL-4 synthesis, the functional capacity of Ly-4^+ lymphocytes to induce Ab secretion cannot be predicted solely by their ability to produce these lymphokines. In the instance of WEP 996, there was a considerable quantitative difference between both the levels of anti-P. c. chabaudi AS Ig induced, and the levels of IFN-γ secreted, by WEP 996 compared to the T_H2 clones, WEP 985-988. Therefore, this Ly-4-bearing clone, designated T_H1 because of its ability to secrete IL-2 and IFN-γ but not IL-4, could not be mistaken for a T_H2 clone. However, the pronounced divergence of the reactivity in vitro of this clone with respect to helper T cell function, and, to a lesser extent, IFN-γ secretion, compared to the other T_H1 clones, WEP 997-999, and to the parent Ly-4^+ lymphocyte line, WEP 775, did suggest a functional heterogeneity in vitro amongst the four P. c. chabaudi AS-specific Ly-4^+ clones. Whether or not this slight heterogeneity in vitro amongst the T_H1 clones compared to the clearly defined differences between the two groups of T_H1 and T_H2 lymphocytes is of significance in vivo is not known.

In the helper T cell assay described herein, for each of the P. c. chabaudi AS-specific Ly-4^+ lines, the highest Ab titres were realised upon incubation with pRBC lysate and post-infective B cells. Since these B cells were parasite-primed in vivo, the reactivity observed in vitro was an anamnestic response. By comparison, the level of specific Ab synthesis that took place when naive B cells were used was significantly lower, suggesting that this was a primary Ab response. In this study, the isotype of Ig secreted upon specific stimulation with P. c. chabaudi AS Ag was not examined. However, the available data do support the findings of Kabilan et al (1987) who did subject the Ig produced to isotypic analysis. They showed that a prerequisite for the induction of anti-P. falciparum Abs in vitro was that the donors had detectable amounts of homologous Abs in their sera. The induced Abs were primarily of IgG isotype, very little IgM being found in P. falciparum-exposed cultures. It was concluded that what was measured in these experiments was a secondary response in vitro of in vivo-primed lymphocytes. As an identical T-B cooperation assay was performed herein, it is very probable that the

specific anti-P. c. chabaudi AS Abs detected were predominantly of IgG isotype. These conclusions were also supported by the results of assaying cultures for secretion of Abs to Pf155 in a modified IFAT (Perlmann et al 1984). Specific Abs to this Ag were found only among donors with elevated serum titres to Pf155. The importance to the detection of Abs in vitro of priming in vivo has also been reported for other systems using various antigenic sources, such as tetanus toxoid (Volkman et al 1982) or polio virus (Emini et al 1983).

It should be noted that the secretion of IgG by P. c. chabaudi AS-primed B cells when cultured in vitro with homologous Ag and either WEP 737 or WEP 723 Ly-4$^+$ lymphocyte lines could have been predicted on the basis of the reported strong IgG_1-enhancing activity of T_H2 clones (Vitetta et al 1985). This induction occurred in the absence of IL-4, suggesting that T_H2 cells can activate substantial IgG_1 responses, even in the presence of the IL-4 antagonist, IFN-γ (Coffman et al 1988). This is in contrast to the unique dependence of IgE responses on IL-4 and the ability of relatively low concentrations of IFN-γ to inhibit this activity of IL-4 (Coffman & Carty 1986). For the uncloned WEP 737 and WEP 723 Ly-4$^+$ populations, therefore, any traces of IFN-γ secreted into culture by T_H1 cells possibly present would suppress the synthesis of IgE but not of IgG_1. Hence, the IFAT titres under optimal conditions for Ab secretion were essentially similar for WEP 737 and its clones, WEP 985-988, reflecting IgG_1 secretion by the in vivo-primed B cells. The low level of Ab production induced by WEP 775 and WEP 779, and clones WEP 996-999 derived from the former, was most likely of IgG_{2a}. This is because not only have T_H1 clones been shown to induce substantially more IgG_{2a} than T_H2 clones (Stevens et al 1988, Coffman et al 1988), but also that this response requires IFN-γ (Snapper & Paul 1987, Finkelman et al 1988 a). As detailed in this chapter, all of the proposed T_H1 populations, WEP 775, WEP 779 and WEP 996-999, were demonstrated to secrete IFN-γ in vitro, from which it could be inferred that what little Ig was detected for these Ly-4$^+$ populations in the helper T cell assay was IgG_{2a}. Although the proposed induction of secretion of IgG_1 and of IgG_{2a} by T_H2 and T_H1 P. c. chabaudi AS-specific Ly-4-bearing clones, respectively, agrees with current T cell dogma, these claims have to be substantiated.

The same assay system as used here and by Kabilan et al (1987) has also been used to investigate the helper function of P. falciparum-specific T cell clones which were shown to induce malaria-specific Ab production in syngeneic B cells in vitro (Sinigaglia et al 1987). The similarity of the findings emphasises the relevance to the human condition

of data collected from rodent models, such as the P. c. chabaudi AS-NIH model described throughout this thesis. One such study revealed a direct correlation between the ability of lymphocytes of either splenic or peripheral blood origin from P. c. chabaudi AS-infected mice to produce specific Abs after antigenic stimulation in vitro and the ability of such mice to control homologous challenge infection (Pearson et al 1983). Although Ab detected by IFAT is not necessarily protective, these authors showed that the level of immunological memory in primed lymphocytes from mice recovered from primary P. c. chabaudi AS infection, as measured by an Ab response in vitro, did reflect the capacity of the mice, similar in all respects to the immune cell donors, to mount an effective secondary response to a further challenge. In the present study, the induction of secondary Ab responses in vitro by those populations thought to be of the T_H2 Ly-4^+ subset, WEP 737 and WEP 985-988, correlated well with their ability to confer protection upon adoptive transfer to naive syngeneic mice challenged with P. c. chabaudi AS. However, those Ly-4^+ lymphocytes belonging to the T_H1 subset, WEP 775 and its derived clones WEP 996-999, were able to control primary challenge equally well after adoptive transfer without the ability to induce substantial secondary Ab responses in vitro. From these contradictory results, it is clear that these two distinct types of Ly-4-bearing P. c. chabaudi AS-specific lymphocytes mediate protection against homologous challenge by different immune responses, only one of which involves a major Ab component.

Thus far in this discussion, the malaria-primed Ly-4^+ T cell lines and clones described have been shown to fall into two discrete subsets on the basis of their relative ability to provide a helper function for B cell activation and induction of Ab synthesis. This is consistent with the previous findings of Kim et al (1985) and of Cher & Mosmann (1987), showing that two types of Ly-4-bearing lymphocyte clones can be distinguished which differ in some effector functions, including B cell help. New interest has been focussed on the possibility of helper T cell subsets by the findings that the pattern of secretion of non-overlapping groups of lymphokines can delineate two types of functionally distinct Ly-4^+ cell (Mosmann et al 1986, Cherwinski et al 1987). T_H1 lines secrete unique lymphokines IL-2, IFN-γ (Mosmann et al 1986) and lymphotoxin (Cherwinski et al 1987) when stimulated by either Ag or Con A. In contrast, cells of the T_H2 subset produce IL-4 and IL-5 in response to Ag or Con A stimulation (Mosmann et al 1986). Such differences in lymphokine secretion would be expected to lead to dramatic differences in the function of such subtypes of Ly-4^+ T cells.

This is pertinent to the study of the development of protective immunity to P. c. chabaudi AS since a heterogeneity in reactivity of the homologously primed Ly-4$^+$ lines and clones to Ags of P. c. chabaudi AS pRBC in vitro may explain the divergent responses of the lines in vivo (Chapter 5-8) and their different requirement to confer protective immunity upon adoptive transfer (Chapter 8).

Three monospecific bioassays were performed to detect the presence of IL-2, IL-4 or IFN-γ. For the first two, due to the multiplicity of lymphokine action, the basic CTLL-2 proliferation assay was modified by using the anti-IL-4 MAb (Ohara & Paul 1985) to achieve monospecificity for IL-2. It was found that the patterns of lymphokine secretion separated the Ly-4-bearing populations analysed into the same two groupings made with respect to activation of B cells and their differentiation into Ab-producing cells. Moreover, this distinction was made between lymphocyte preparations that produced both IL-2 and IFN-γ, but not IL-4, or IL-4 but not IL-2 or IFN-γ, i.e. the T_H1 and T_H2 categories introduced by Mosmann et al (1986) to classify long term helper T cell lines according to their lymphokine secretion profiles. Hence, those lines and clones that had no Ab helper activity in vitro, WEP 775, WEP 779 and WEP 996-999, secreted both IL-2 and IFN-γ upon antigenic stimulation in vitro. Each of these populations was considered to be of the T_H1 subset. Conversely, those lines and clones that did induce a specific anti-plasmodial Ab response in vitro, WEP 737, WEP 723 and WEP 985-988, produced IL-4 uniquely amongst the three lymphokines tested. Hence, these Ly-4$^+$ preparations appeared to belong to the T_H2 subset. There was, therefore, a correlation between the lymphokines secreted by a given P. c. chabaudi AS-specific Ly-4$^+$ lymphocyte population and its capacity to participate in Ab-dependent mechanisms of immunity. IL-4 produced by T_H2 cells stimulated the production of IgG_1 by cocultured activated B cells, an activity which was inhibited by the presence of IFN-γ, secreted by T_H1 cells. This confirmed the previous work of Coffman & Carty (1986) and of Snapper & Paul (1987), working with LPS-stimulated B cells. With specific regard to malaria, Brake et al (1988) had previously reported the characterisation of a Ly-4$^+$ cell clone which elaborated IL-2 and IFN-γ when stimulated by P. c. adami pRBC Ag in vitro. Likewise, in similar systems, Ly-4-bearing clones raised to Leishmania major (Scott et al 1990) and to Listeria monocytogenes (Magee & Wing 1988) produced exclusively IL-2 and IFN-γ upon stimulation in vitro. All of these clones had a lymphokine secretion pattern indicative of cells of the T_H1 Ly-4$^+$ subset. The data presented herein extend the work of Brake et al (1988) in that not only were four cloned populations

raised to P. c. chabaudi AS shown to produce IL-2 and IFN-γ in vitro (WEP 996-999; T_H1 typing), but four further clones were found to secrete not IL-2 and IFN-γ, but IL-4 upon appropriate stimulation in vitro. By these parameters, these clones (WEP 985-988) were classified as being T_H2 lymphocytes, this being the first time monoclonal populations of malaria-specific Ly-4$^+$ T cells of the T_H2 subset have been propagated successfully in vitro with plasmodial Ag.

Using the same P. c. chabaudi AS parasite as used here, Langhorne and colleagues (Langhorne & Simon 1989 , Langhorne et al 1989 a) described the precursor frequencies of malaria-reactive T cells in a limiting dilution assay. It was claimed by these authors that this system had the advantage over bulk cultures of providing information on T cell responses at the clonal level, without the bias of in vitro selection. However, such a view has been disputed by Street et al (1990), who reported that it was equally possible to show a heterogeneity of mouse helper T cells from bulk cultures and limiting dilution cloning. Despite this discrepancy in determination of the results of different assays, essentially similar findings were revealed by Langhorne et al as in the experiments presented in this chapter. The initial studies of this group indicated that the response of primed Ly-4$^+$ T lymphocytes to P. c. chabaudi AS pRBC Ag was heterogeneous (Langhorne & Simon 1989). The precursor frequency of proliferating Ly-4-bearing T cells was greater than that of specific helper T cells, implying that there was a proportion of activated Ly-4$^+$ lymphocytes which did not function in a helper capacity. Similarly, for the four Ly-4$^+$ lines primed by natural infection to P. c. chabaudi AS and propagated in vitro, only two appeared to function as helper cells for Ab production, whilst two others not only failed to induce B cell activation but also exhibited the lymphokine secretion pattern of T_H1 cells. Despite these differences between the reactivity of the various P. c. chabaudi AS-specific Ly-4-bearing lines, each has been shown to proliferate directly in response to homologous Ags presented in an MHC-restricted manner. There was, therefore, no direct link between proliferation and IFN-γ production, indicating that these two phenomena, which both reflect T cell activation, may be partially independent processes. Likewise, for P. falciparum-exposed individuals, no obvious correlation between lymphoproliferation and IFN-γ secretion in response to antigenic stimulation has been found, both for malaria-immune individuals and acute malaria patients (Troye-Blomberg et al 1985, 1987, Riley et al 1988 a). Furthermore, Troye-Blomberg et al (1984, 1985) had earlier performed experiments the results of which indicated that specific T cell-dependent B lymphocyte

activation could be induced in patients with acute P. falciparum infection in whom IL-2 production may be abortive or suppressed. It was concluded that the T cell fraction involved in B cell help in this particular instance was an IL-4-producing population distinct from the major IL-2-producing fractions (Troye-Blomberg & Perlmann 1988). Whilst these studies did not use cloned human $CD4^+$ lymphocyte populations, it is clear that those cultures producing IFN-γ or IL-4 corresponded to what would now be termed T_H1 or T_H2 cells, respectively. Thus, the finding that IL-2 synthesis and helper activity for Ab production were independent properties of $CD4^+$ lymphocytes was an early indication of the segregation of malaria-primed T cells into T_H1 and T_H2 compartments, which itself was a reflection of the different effector functions displayed by the host immune system that can be activated in response to malarial infection. More recently, Langhorne et al (1989 a) showed that under limiting dilution conditions, there was an obvious lack of correlation between IFN-γ production and T helper cell function, suggesting that in clonal cultures these functions may be carried out by distinct cells. These findings concur with those described herein, using both long term lines and monoclonal populations of P. c. chabaudi AS-specific Ly-4^+ lymphocytes. In contrast, both studies demonstrated a strong correlation between the secretion of IL-2 and IFN-γ, the production of both these lymphokines being a parameter of T_H1-like function. Since the measurement of IL-4 proved difficult in microculture, Langhorne et al (1989 a) did not make a direct measurement of T_H2-like function. However, it appeared that T_H2-type Ly-4^+ clones were the more efficient at activating resting B cells into malaria-specific Ab production, presumably due to the activities of IL-4 and IL-5 (Swain et al 1988 a). Using the bulk culture technique, it was possible to perform an IL-4 monospecific bioassay, from which T_H2 characterisation could be extrapolated. It was found that the preliminary typing of those populations giving B cell help as T_H2 cells was vindicated, since these lymphocytes alone of those assayed, produced detectable quantities of IL-4 in vitro.

Early experiments by Langhorne & Simon (1989) were carried out using splenic T cells isolated from mice recovered from a primary infection; these lymphocytes had been exposed to P. c. chabaudi AS pRBC through immunisation of the donor animals and therefore had reached a state of immunological competence similar to that attained by some of the Ly-4-bearing lymphocyte populations used in this study. Here, WEP 737 and WEP 985-988 cells were primed to P. c. chabaudi AS by two successive challenges, whilst WEP 723 lymphocytes were taken from mice recovered from a tertiary infection.

These long term in vitro-propagated Ly-4$^+$ populations were classified as fitting within the T_H2 subset as culture S/N collected from each contained substantial levels of IL-4. This is consistent with the finding of Langhorne & Simon (1989) that the precursor frequencies of T helper cells from animals convalescing from primary infection were always greater than those for IL-2 or IFN-γ production. Further studies were undertaken by Langhorne et al (1989 a & b) to determine the type of T lymphocytes involved in immunity to P. c. chabaudi AS during the course of a primary infection. Assays were performed at two time points after challenge: 7 d p.i., at the onset of patent parasitaemia, and 28 d p.i., when the parasitaemia was subpatent. Early in infection, 7 d p.i., there were more microcultures which produced IFN-γ than contained helper T cells for specific anti-plasmodial Ab production, and the precursor frequency tended to decrease as the infection progressed. Thus, the majority of Ly-4-bearing T cells specific for P. c. chabaudi AS early in infection were of the T_H1 subset. In contrast, after 28 d p.i., the majority of parasite-specific Ly-4$^+$ cells provided help for Ab synthesis, thus resembling T_H2 lymphocytes. Since the proportion of T_H2-type cells increased as the level of total responder lymphocytes decreased, the relative frequency of T_H2 cells within all T cells responding to P. c. chabaudi AS, therefore, tended to rise throughout infection. In the experiments detailed in this chapter, those Ly-4$^+$ populations characterised as T_H1, WEP 775 and its cloned derivatives WEP 996-999, and also WEP 779, were recovered from challenged mice either on d 16 or d 20 of primary infection. These lymphocytes were collected at times between those time points used by Langhorne et al (1989 a & b), so no direct comparison can be made. However, it would appear that the consistent characterisation of Ly-4$^+$ lymphocytes isolated on either d 16 or d 20 p.i. as being cells that secreted IL-2 and IFN-γ, but not IL-4, i.e. of the T_H1-type, would fit broadly with the data presented by Langhorne et al (1989 a & b). At 16 or 20 d p.i., the parasitaemia was either in remission or subpatent, at which time Langhorne et al suggested that the proportions of T_H1 and T_H2 precursors were approximately equal (Langhorne 1989). Thus, it may have been chance that both lines derived from spleens of mice infected for this length of time exhibited the T_H1 Ly-4$^+$ T cell response. However, the findings presented do stress the importance of the Ab-independent cell-mediated immune response until such a time following challenge when an effective humoral immunity can be mounted. The advantage of applying a limiting dilution assay to an investigation of the effector mechanisms controlling a primary infection of P. c. chabaudi AS is that it provides information on the relative frequencies

of the different T cells participating in a protective immune response. This is not the case with lines raised by bulk culture in vitro propagation unless many different lines are initiated and then maintained together, which is a practical implausibility. For the two lines used herein, WEP 775 and WEP 779, their reactivity in vitro suggested that they belonged to the T_H1 subset, a finding that is not unreasonable in view of the fact that they were taken from donor animals at a time when serum Ab levels had not peaked.

It has been demonstrated here that the T_H1 and T_H2 clones studied produced different T cell growth factors upon incubation in vitro, IL-2 and IL-4, respectively. These two lymphokines are each capable of mediating proliferation of activated Ly-4^+ clones (Kurt-Jones et al 1987, Greenbaum et al 1988), and thus have not been reported to have conflicting effects on the responsiveness of either T cell subset. Indeed, Fernandez-Botran et al (1988) showed that both T_H1 and T_H2 cells respond to IL-4 and IL-2 shortly after antigenic stimulation and that responsiveness to both lymphokines decreases with time after activation. Moreover, when both IL-2 and IL-4 were added to cultures of T_H1 or T_H2 clones, the proliferative response of both cell types was synergistic, suggesting that the presence of both lymphokines may be required for an optimal response. There is, therefore, no evidence, both from this study and from those of others, that IL-4, secreted by T_H2 lymphocytes, is a specific inhibitor of IL-2-mediated lymphoproliferation during malarial infection. Previous reports had indicated that infection of mice with P. berghei or P. yoelii leads to depressed splenic proliferative responses to mitogens and this depression has been linked to defective IL-2 production (Lelchuk et al 1984). Inhibitors of IL-2 present in normal mouse serum (Hardt et al 1981) and in increased amounts during malarial infection (Lelchuk & Playfair 1985, Male et al 1985) were thought to play a part in maintaining immunosuppression. This has been confirmed recently by Natarajan et al (1988), who showed that sera of mice undergoing lethal P. berghei infection contained a factor which inhibited the production of and/or the response to IL-2. The inhibitor appeared to function by binding to its putative target cell, suggested by the observation that excess IL-2 could not rescue CTLL cells from the effects of the inhibitor. On this basis, this factor could not be IL-4 since it is known from the work detailed in this chapter that the CTLL-2 cell line is responsive to both IL-2 and IL-4. Moreover, if the inhibition is competitive, as available data would suggest, IL-2 and the unknown factor would presumably have the same target molecule, the IL-2 receptor, on the T cell surface, which is not the case for IL-2 and IL-4, which have distinct membrane-bound receptors

(Vitetta et al 1988). Therefore, although IL-4 and IL-2 are secreted by subsets of Ly-4$^+$ T lymphocytes having different roles in host immunity to asexual stage plasmodia, their effects are not directly antagonistic, partly perhaps because they tend to mediate protection at different times following challenge of the host animal (Langhorne et al 1989 a & b, also this chapter). It is possible that a pluripotent lymphokine such as IFN-γ, which has a large number of biological activities on various cell types, may, under certain conditions, cause the malaria-induced immunosuppression reported both for human (Troye-Blomberg et al 1985) and murine (Lelchuk et al 1984, Natarajan et al 1988) malaria. Thus, it has been reported that T_H2 but not T_H1 proliferation is inhibited by IFN-γ (Fernandez-Botran et al 1988, Gajewski & Fitch 1988). This would concur with data showing that a strong candidate for the production of an IL-2 inhibitor is a T cell population (T_H1?) (Hardt et al 1981, Lelchuk & Playfair 1985, Honda et al 1985, Emara & Battisto 1987). This possibility has yet to be substantiated; indeed, the similar levels of proliferation upon specific antigenic stimulation in vitro shown by all the Ly-4$^+$ lines and clones assayed suggested that, under the experimental conditions prevailing, IFN-γ synthesis had no bearing on the relative proliferative responses of primed T_H1 and T_H2 lymphocytes upon activation with homologous Ag. These data add to the apparently conflicting information available regarding the nature of the presently undefined factor involved in or causing immunosuppression during malarial infection. Another possibility for the identity of this factor is the much mooted cytokine synthesis inhibition factor (Fiorentino et al 1989), but this would be envisaged to have a far wider ranging effect on the immune system than the exquisitely specific blocking of IL-2 synthesis. Investigations into the activity of this molecule are in their infancy and to date no reports of its effects on the immune system of a malarious individual, human or otherwise, have been published.

If it is not established whether or not IFN-γ plays a part in suppression of Ly-4$^+$ T cell proliferation during an immune response to malaria, it is known that IFN-γ acts as an antagonist of all the activities of IL-4 on B cells (Mond et al 1985, 1986, Coffman & Carty 1986, Rabin et al 1986). As these two lymphokines are secreted by different T_H subsets, it infers that the mediation of varying effector functions by cells of either T_H1 or T_H2 subset is achieved principally by elaboration of either IFN-γ or IL-4, respectively. It was first appreciated that IFN-γ plays a role in immunity to malaria when it was shown to be demonstrable in the sera of mice infected with P. berghei (Hwang et al 1968). Shortly after, Jahiel et al (1968 a & b) reported that various IFN

inducers exerted a protective effect in similarly infected animals. More recently, several workers have examined the kinetics of IFN-γ production by spleen cells in vitro in response to malarial Ag during the course of infection. Stevenson et al (1990) found that following P. c. chabaudi AS challenge of C57BL/6 mice, the peak of IFN-γ secretion occurred just before peak parasitaemia, followed by a decrease to negligible IFN-γ production by 25 d p.i.. This agreed with the earlier report of Sauvager et al (1979) that P. berghei was a good inducer of circulating IFN at the beginning of patent infection soon after challenge, when IFN could be detected in the sera of infected mice. Furthermore, Ojo-Amaize et al (1981) found comparatively high levels of anti-viral activity in sera of P. falciparum-infected children, the titres of which correlated with the degree of parasitaemia. With regard to P. c. chabaudi AS, Langhorne and colleagues extended their previous findings (Langhorne & Simon 1989, Langhorne et al 1989 a) by showing that the early response of lymphocytes of the T_H1 subset to pRBC Ag in vitro correlated well with the transient appearance of IFN-γ in the serum of infected mice (Slade & Langhorne 1989). This occurred 2-3 d before peak primary parasitaemia, and thus concurred with the finding of Stevenson et al (1990) using an identical parasite-mouse model. Of significance is the fact that the results of the present study also agree with those of Slade & Langhorne (1989) and of Stevenson et al (1990). Since long term Ly-4-bearing lines and clones primed to P. c. chabaudi AS were isolated on a limited number of occasions instead of preparing limiting dilution cultures at frequent intervals following infection, direct comparisons between experiments could not be made. However, it was demonstrated unequivocally that those lines and clones derived relatively early after challenge of donor mice, WEP 775, WEP 779 and WEP 996-999, secreted comparatively high levels of IFN-γ upon in vitro culture. By comparison, S/N collected from cultures of those populations primed to malaria through multiple infections, WEP 737, WEP 723 and WEP 985-988, recorded substantially lower but significant IFN-γ titres. The fact that circulating IFN-γ could actually be measured after rechallenge suggests that a significant number of T_H1 Ly-4^+ lymphocytes are still able to mount an IFN-γ-mediated response to reinfection, despite the predominance of Ab helper cells of the T_H2 subset previously observed in immune mice (Langhorne et al 1989 a & b). This finding has been confirmed by Meding et al (in press), who were able to detect IFN-γ at extremely low titres in the sera of rechallenged mice, using a sensitive ELISA assay. The weight of evidence points to IFN-γ playing an important part in the initial clearance of acute malarial infection, since the presence of this lymphokine

has been reported consistently as a predominant feature of the Ly-4$^+$ T cell response to asexual stage pRBC soon after challenge, at a time when there is relatively little help for Ab production and only a low titre of Plasmodium-specific Ig in the peripheral blood (Slade & Langhorne 1989, Stevenson et al 1990, also this chapter).

For the human malaria P. falciparum, CD4$^+$ lymphocytes collected from infected individuals have been shown to produce IFN-γ in vitro in response to stimulation with the asexual erythrocytic stages (Rhodes-Feuillette et al 1985 a & b, Sinigaglia & Pink 1985, Troye-Blomberg et al 1985, 1987). Although cells secreting IFN-γ would be expected to be of the T_H1-type, the lymphokine expression patterns of human CD4-bearing T cell clones do not fall clearly into different subsets. Large panels of cloned populations have been tested for expression of each of IL-2, IL-4 and IFN-γ and clones synthesising all of the possible combinations of these lymphokines have been observed (Del Prete et al 1988, Maggi et al 1988, Paliard et al 1988, Pène et al 1988, Rotteveel et al 1988, Umetsu et al 1988).

The characterisation of the two types of murine helper T cells has been carried out in vitro using established cloned populations (Mosmann et al 1986, Cherwinski et al 1987, Killar et al 1987, Cher & Mosmann 1987, also this chapter). It is therefore possible that variable in the techniques used for isolating, propagating and cloning Ly-4$^+$ lines may select clones that are not representative of their in vivo counterparts. Direct evidence of T_H1 or T_H2 subsets prior to Ag-driven activation in vivo is lacking. However, since the phenotype and lymphokine secretion profiles of each of the clones used in this study and those of others are stable, it would appear that most clones fit into one of the two categories described by Mosmann et al (1986), and, furthermore, are representative of the type and distribution of lymphocytes to be found in vivo in uninfected naive mice (Bottomly 1988, Swain et al 1988 a, Mosmann & Coffman 1989 a & b).

Although characteristic patterns of lymphokine expression can be used as functional markers to distinguish murine T_H1 and T_H2 cells (Mosmann et al 1986, Cherwinski et al 1987), no cell surface marker has been described that is present exclusively on either type of Ly-4-bearing lymphocyte. However, Birkeland et al (1988) have used two MAbs recognising different epitopes on the common leucocyte Ag, CD45R, to distiguish between functionally different helper T cells. The different subtypes of clones stain to varying degrees: 'CD45R-low' and 'CD45-high' T cells produce IL-4 or IL-2 and IFN-γ and correspond to T_H2 and T_H1 lymphocytes, respectively (Bottomly 1988).

Subsequent studies using these MAbs in an IFAT assay have correlated the phenotype of normal Ly-4^+ cells (CD45-low and -high) with their pattern of lymphokine secretion and other functional properties to provide strong evidence that analogues of T_H1 and T_H2 lymphocytes exist in vivo (Birkeland et al 1988, Bottomly 1988).

The effector mechanisms of the host immune response which are operative during a P. c. chabaudi AS infection will depend largely upon the nature of the Ly-4^+ cells present at different times following challenge. The experiments detailed herein show that the Ly-4^+ T cell response to P. c. chabaudi AS pRBC is heterogeneous, in that distinct functions can be performed by different responding lymphocytes. The patterns of reactivity of malaria-specific Ly-4-bearing populations in vitro generally support the idea of two functionally distinct Ly-4^+ subsets. Those cell lines (WEP 775 and WEP 779) and clones (WEP 996-999), shown to be B cell-independent in vivo (Chapter 7) secreted high levels of both IL-2 and IFN-γ upon antigenic stimulation in vitro, whilst the B cell-dependent populations (WEP 737, WEP 723 and WEP 985-988) acted as effective helper cells for specific Ab synthesis in vitro. Not only do these results suggest that the Ly-4-bearing cells fall into the two proposed helper T cell subsets, T_H1 and T_H2, respectively, but the fact that those populations that performed a B cell helper function in vitro were the same ones that had a B cell requirement to confer protection in vivo stresses the relevance of the analysis of effector functions of Ly-4^+ cells at the clonal level to the in vivo situation. It should be noted that it was those lines and clones derived at an early stage (d 16 and d 20 p.i.) of primary infection that had the effector repertoire of the inflammatory-type T_H1 Ly-4^+ subset. Moreover, those populations taken for in vitro culture after resolution of further infections displayed the helper activity for Ab production characteristic of T_H2 Ly-4^+ cells. These data, gained using isolated, long term cultured lines and clones raised to P. c. chabaudi AS, correlate strongly with those of Langhorne et al (1989 a), applying a limiting dilution assay to the same parasite. These workers showed that for P. c. chabaudi AS-challenged C57BL/6 mice (compared to NIH mice used here), the acute infection corresponding to the primary parasitaemia was characterised by those T cells of the Ly-4^+ subset which secreted predominantly IL-2 and IFN-γ. During the later stages of infection, once peripheral blood parasitaemia has been controlled, the Ly-4^+ T lymphocytes responding specifically to malarial Ag in vitro were mainly helper T cells for the production of specific anti-plasmodial Abs. Both these different approaches aimed at dissecting the cell-mediated immune response elicited during a blood stage infection of P. c. chabaudi

AS suggest that distinct protective effector mechanisms are operative in early and late stages of primary infection. It appears that there may be a sequential appearance during the course of infection of T_H1, followed by T_H2 cells specific for P. c. chabaudi AS-infected RBC. This flux in the relative frequencies of parasite-specific Ly-4^+ lymphocytes in infected animals may be due to the temporally-regulated activation of Ly-4^+ subsets by the same or different pRBC Ags. Alternatively, it could reflect the predetermined pathway of differentiation of every Ly-4-bearing lymphocyte undergoing an immune response in vivo. As yet, neither possibility has been substantiated. Recently, however, the picture has been clarified partially by experiments, the results of which have suggested the existence of precursors of T_H1 and T_H2 subsets (Kelso & Gough 1988, Mosmann & Coffman 1989 a). It has not been proven yet whether the precursors are committed to a particular cell type before expressing the mature lymphokine secretion pattern, or whether a common precursor lymphocyte can be induced to differentiate into either T_H1 or T_H2 cells.

The balance between Ly-4^+ lymphocyte subsets producing different lymphokines that has been described both here and elsewhere (Langhorne et al 1989 a & b, Langhorne 1989) for the development of immunity to the asexual blood stages of P. c. chabaudi AS may be unique to this malaria parasite. In similar studies examining the role of Ly-4-bearing T cells in protective immunity to other murine plasmodia, it was found that the magnitude and kinetics of the lymphokine response varied markedly to that described for P. c. chabaudi AS. Shear et al (1990) have shown that both T_H1 and T_H2 subsets of Ly-4^+ cells are activated during P. c. adami infection. As for P. c. chabaudi AS infection, the peak IFN-γ response occurred 2-3 d before peak parasitaemia, whereafter the level of IFN-γ declined to control levels at which it remained. In contrast to the situation reported herein, however, maximal IL-4 levels were reached not later in infection but on d 6 p.i., several days prior to peak parasitaemia. The modest IL-4 titres then decreased and remained low for the rest of the study period. Hence, for P. c. adami challenge of naive mice, the T_H1 response was very similar to that observable upon P. c. chabaudi AS infection, but the T_H2 response was not nearly as great and occurred considerably earlier than that in P. c. chabaudi AS-infected animals. Likewise, for P. vinckei-challenged mice, T_H1 cells were activated immediately following infection, giving rise to peak IFN-γ release on d 4 p.i., a few days before maximum parasitaemia was attained (Perlmann et al 1990). In this model, however, the number of cells secreting IL-4 was indistinguishable from that found in control mice, and the amounts of

IL-4 produced were low even when the mice were rechallenged with P. vinckei pRBC shortly before testing. Therefore, no discernable T_H2 activity was involved in regulating immunity to this malaria parasite. These apparently contradictory findings in different murine models of malaria can be reconciled by the fact that acquired immunity to P. vinckei and to P. c. adami infections is Ly-4^+ lymphocyte-dependent and can develop in the absence of functional B cells (Weidanz & Long 1988, Long 1988). Studies performed by Grun & Weidanz (1981) demonstrated that B cell-deficient mice resolved acute primary infections of P. c. adami with the same kinetics as normal mice and were immune to subsequent challenge with homologous pRBC. Although P. vinckei infection is uniformly lethal in mice, both B cell-deficient and immunologically intact animals develop immunity after two drug-cured infections with this parasite (Kumar et al 1989). Thus, Ab-independent mechanisms of immunity have been shown to be primarily responsible for resolution of acute infection caused by these plasmodial species. This is not the case for the host immune response to P. c. chabaudi AS, which is known to have a substantial humoral component (McDonald & Phillips 1978). The role of specific Abs in immunity to P. c. chabaudi AS, and the cooperation of T and B cells in this response, have been confirmed by experiments described in this thesis showing the B cell-dependent behaviour of certain malaria-primed Ly-4^+ lymphocyte lines upon adoptive transfer to immunocompromised recipients (Chapter 7). Hence, the work presented here and by Langhorne et al (1989 a & b) showing a distinct temporal pattern of lymphokine production during the course of infection with P. c. chabaudi AS, reflects the fact that both Ab-dependent and Ab-independent effector mechanisms play a role in the protective immune response to this parasite in vivo. It has been shown both here (Chapter 8) and by Süss et al (1988) that Ly-4^+ T lymphocytes are an essential component of the acquired immune response to P. c. chabaudi AS, and presumably these cells contribute both in a helper capacity for Ig production and by activating macrophages through the mediation of IFN-γ. In this context, the sequential release of the lymphokines IFN-γ and IL-4 by different subsets of Ly-4^+ cells is entirely explicable. Moreover, Langhorne et al (1990) have reported recently that for effective pRBC clearance, the presence of Ly-4^+ lymphocytes is required only in the acute phase of P. c. chabaudi AS infection, at which time IFN-γ-secreting T_H1 cells have been shown to predominate (Langhorne et al 1989 a, also this chapter). Later, after IgG has been produced, parasitaemia can be controlled in the absence of Ly-4-bearing cells, although the presence of the majority T_H2 cells at this time does act to promote B cell

differentiation and induction of Ab synthesis.

Apart from conducting extensive studies in vitro on each of the P. c. chabaudi AS-specific Ly-4$^+$ clones generated by cloning established cultured cell lines by limiting dilution, the effector function of each clone was shown in vivo by its adoptive transfer to non-immune recipients. It was demonstrated that all eight Ly-4-bearing monoclonal populations tested were able to confer protection against challenge with blood stage P. c. chabaudi AS. Of these clones, four had been typed as belonging to the T_H1 Ly-4$^+$ subset, and another four to the T_H2 subset, based upon both functional characteristics and lymphokine expression. The protection engendered by these parasite-specific clones appeared to correlate with their respective reactivity in vitro. Thus, although it was not proven, the data indicated that the protective capacity of the T_H1 clones (WEP 996-999) in vivo resulted from their ability to produce IFN-γ. Likewise, the property of the T_H2 clones (WEP 985-988) to induce B cell activation in vitro presumably enabled them to adoptively transfer protection. There was no obvious difference in the way in which recipients of clones of either type controlled infection, as observed by similar patterns of parasitaemia. This was a reflection of the fact that immunity to the asexual erythrocytic stages of P. c. chabaudi AS is mediated by both Ab-dependent and Ab-independent mechanisms (Weidanz & Long 1988). This, in turn, implies that both T_H1 and T_H2 clones have the capacity to transfer resistance in this experimental model. This is in contrast, for example, to host immunity to leishmaniasis, which is associated with the stimulation of the T_H1 subset of Ly-4-bearing lymphocytes, whereas susceptibility and disease promotion is associated with T_H2 cells (Scott et al 1988, 1990, Heinzel et al 1989).

With regard to malarial infection, only one other group had previously investigated the capacity of T cell clones to mediate specific protection against blood stage plasmodia in vivo. Working with P. c. adami, Brake et al (1986) first reported the successful transfer of immunity to immunologically deficient mice with an Ag-specific, IL-2-expanded Ly-4$^+$ line, a finding which concurred with experiments detailed previously (Chapter 7 & 8) using the AS strain of the other subspecies of this malaria parasite, P. c. chabaudi. In both cases, the protective in vitro-propagated lines were cloned to provide malaria-specific populations of homogeneous phenotype, which were subjected to analysis both in vitro and in vivo. Brake et al (1988) presented data showing that a clonal population of Ly-4-bearing T cells could adoptively protect susceptible nude mice against challenge infection with P. c. adami. This clone secreted both IFN-γ and IL-2,

and therefore was of the T_H1 subset. However, this was only one of 10 clones tested, the rest of which failed to transfer immunity and were thus not protective. For the adoptive transfers described in this chapter, all eight clones were able to confer protective activity upon inoculation into naive recipients, although unlike the study of Brake et al (1988), these animals were immunologically fully competent. As the clones gave a similar degree of protection to non-immune recipients as did their respective parent cell lines, there is no reason to believe that the resistance transferred by each of the parent populations to immunologically compromised animals (Chapters 7 & 8) would not be repeated in the case of the cloned lines. This is particularly so because, as has been shown here, each of the clones retained the reactivity in vitro of the Ly-4^+ line from which it was derived.

The data detailed herein therefore demonstrates the ability of clones of either T_H1 or T_H2 subsets of Ly-4^+ phenotype to engender protection in vivo against challenge with the same P. c. chabaudi AS parasite to which the clones had been raised. These findings extend the results of Brake et al (1988) in confirming that T_H1 cells are capable of conferring protection upon reconstitution of malaria-infected animals. These results also agree with the studies of Magee & Wing (1988), who showed that cloned Ly-4^+ lymphocytes protected mice against another intracellular pathogen, Listeria monocytogenes, by secreting IFN-γ. Elaboration of this lymphokine by human $CD4^+$ clones, specific for the asexual erythrocytic stages of P. falciparum has been reported (Sinigaglia & Pink 1985, Chizzolini & Perrin 1986), a finding which promises well for the development of an anti-malaria vaccine, since it has been reasoned that if Ab-independent mechanisms of immunity are operative in murine malarias, they should also be effective in primate malarias (Good & Miller 1989). The corollary to the human condition also stresses the importance of such work on murine models of malaria that has indicated the necessity for T cell epitopes in a putative subunit vaccine.

This study has demonstrated for the first time the capability of Ly-4^+ T cell clones of the T_H2 subset to mediate effective immunity in vivo against blood stage malarial infection. In the only other similar study, Brake et al (1988) did not attain an IL-4-secreting clone upon limiting dilution culture of a P. c. adami-specific Ly-4^+ line, presumably because immunity to this malaria parasite is largely Ab-independent (Weidanz & Long 1988, Long 1988). The demonstration of protective T_H2 cloned populations against P. c. chabaudi AS is in contrast to previous reports of adoptively transferred T_H2 Ly-4-bearing clones actually exacerbating infections of Leishmania

tropica (Liew 1983), L. major (Scott et al 1988, 1990) and Trypanosoma cruzi (Spinella et al 1990) in recipient mice. In the first two instances, disease progression was characterised by the non-healing of visceral and cutaneous lesions, whilst for chronic Chagas' disease, exacerbation was typified by polyclonal B cell activation, mainly of cells secreting IgG_2 isotypes. The reason for this disparity in the behaviour of T_H2 lymphocytes in vivo in mice challenged with different intracellular parasites is not known. Certainly, for malaria, there is no equivalent of the immunopathological consequences seen upon adoptive transfer of T_H2 clones to animals suffering from either leishmaniasis or trypanosomiasis. Even for the murine malarias P. vinckei and P. c. adami, immunity to which is considered to be B cell-independent (Weidanz & Long 1988), there is no evidence to suggest that the T_H2 subset of the Ly-4^+ T lymphocyte phenotype is involved in the immunopathology of malaria. Indeed, there is evidence implicating not the T_H2, but the T_H1 subset in the cerebral complication of P. berghei ANKA infection of CBA/Ca mice (Grau et al 1989 c). This concurs with the recent finding of Mendis et al (1990), who suggested that individuals acquire natural immunity to P. vivax by avoiding the induction of high levels of IFN-γ and TNF. Evidence showed that whilst these cytokines mediated killing of pRBC during clinical paroxysms of P. vivax infection when present in sera at low titres, they also appeared to be critical intermediates in mechanisms of clinical disease in malaria.

For both the adoptive transfers of P. c. chabaudi AS-Ly-4^+ clones described here, and for the transfer of a P. c. adami-primed Ly-4-bearing clone (Brake et al 1988), recipient mice displayed significant patent parasitaemia before resolution of acute infection. This suggested that in each model, the transferred Ly-4^+ cells were not directly parasiticidal, but that lymphokine-mediated activation of other host effector functions may have led to eventual clearance of peripheral blood infection. It is assumed that the lymphokine secretion pattern in vitro of either T_H1 or T_H2 subset of Ly-4^+ lymphocyte clones raised against P. c. chabaudi AS correlates with the mechanism of protective immunity followed in vivo. Thus, it could be envisaged that IL-2 may be secreted by T_H1 clones into a localised environment in vivo in response to stimulation by plasmodial Ag upon adoptive transfer to a challenged animal. This, in turn, may lead to the clonal expansion of WEP 996-999 cells in vivo, thereby resulting in increased levels of IFN-γ and subsequent activation of macrophages or other effector cells. Similarly, after inoculation of T_H2 clones into recipient mice, these may then proliferate in response to autocrine IL-4 or to endogenous IL-2, whereupon WEP 985-

988 could induce specific humoral immunity by helping B cells to produce anti-P. c. chabaudi AS Abs.

The significance of the successful adoptive transfer of malaria-primed cultured Ly-4$^+$ lymphocytes lies in the possibility of dissecting the mechanisms by which these cells mediate protection as well as identifying plasmodial Ags capable of inducing protective immune responses. However, neither the P. c. chabaudi AS Ags nor the P. c. adami Ags recognised by either the protective Ly-4-bearing lines and clone(s) employed in this thesis or by Brake et al (1986, 1988) have yet been identified. This could be achieved by constructing cDNA libraries and identifying the relevant antigenic epitopes expressed by screening with the respective T cell populations. In the present study, such an undertaking was not attempted, but it is an objective of future work to determine to which P. c. chabaudi AS Ags the Ly-4$^+$ clones are responsive. Although in this study, all eight monoclonal T cell populations conferred protective activity in vivo, Brake et al (1988) demonstrated that only a single clone of 10 tested was protective. This begs the question of whether a variety of plasmodial Ags and T cell epitopes on parasite-expressed peptides can elicit a Ly-4$^+$ T lymphocyte response or whether the number of protective Ags is more limited. The identification and distribution of putative protective blood stage Ags is of direct relevance to the prospects of developing anti-malaria vaccine candidates incorporating T cell epitopes of either recombinant or synthetic origin.

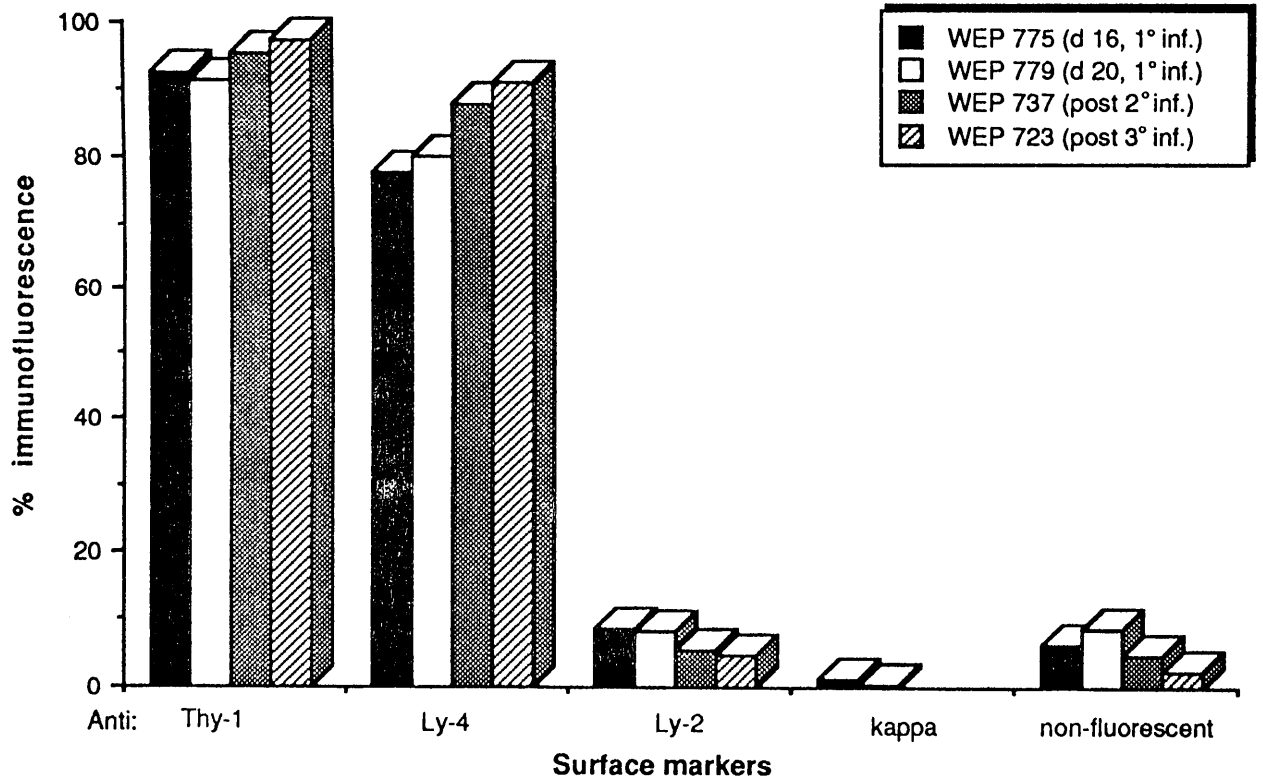

Fig. 9.2.1 Similar phenotyping of P. c. chabaudi AS-specific T lymphocyte lines by indirect immunofluorescence

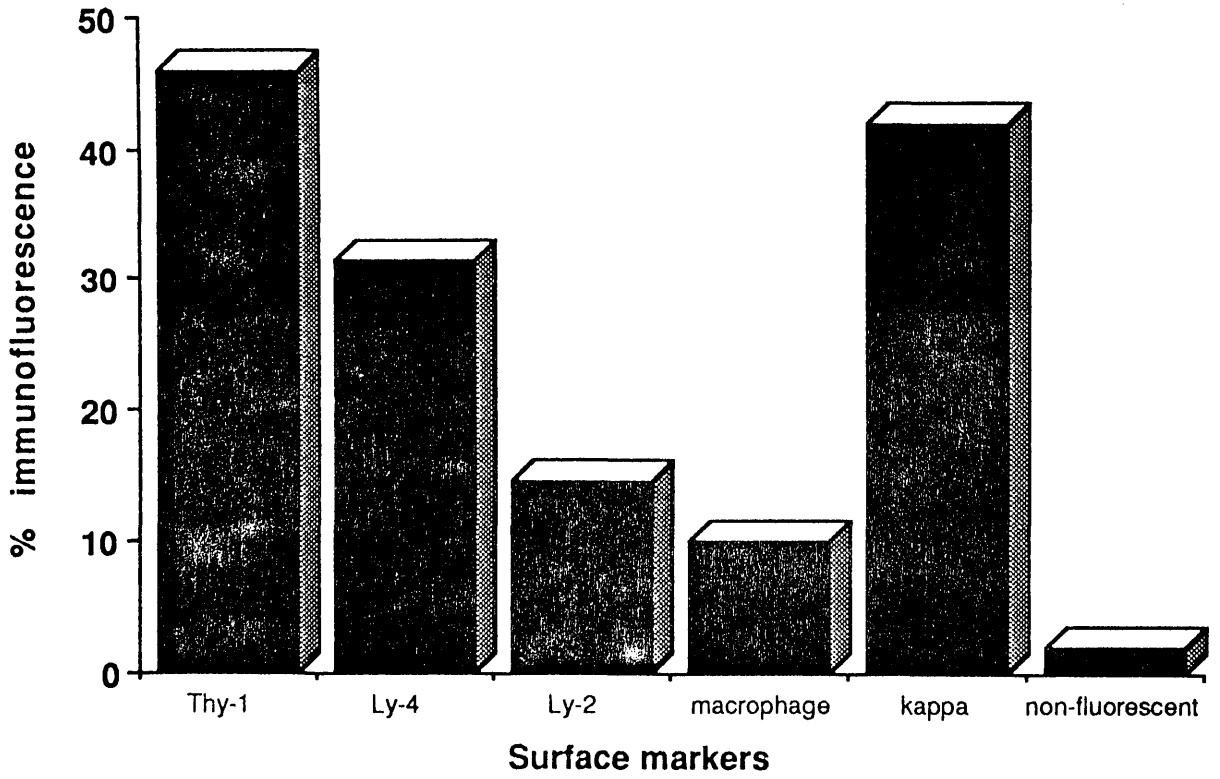

Fig. 9.2.2 Surface phenotypic analysis of a naive spleen cell population as a control for indirect immunofluorescence

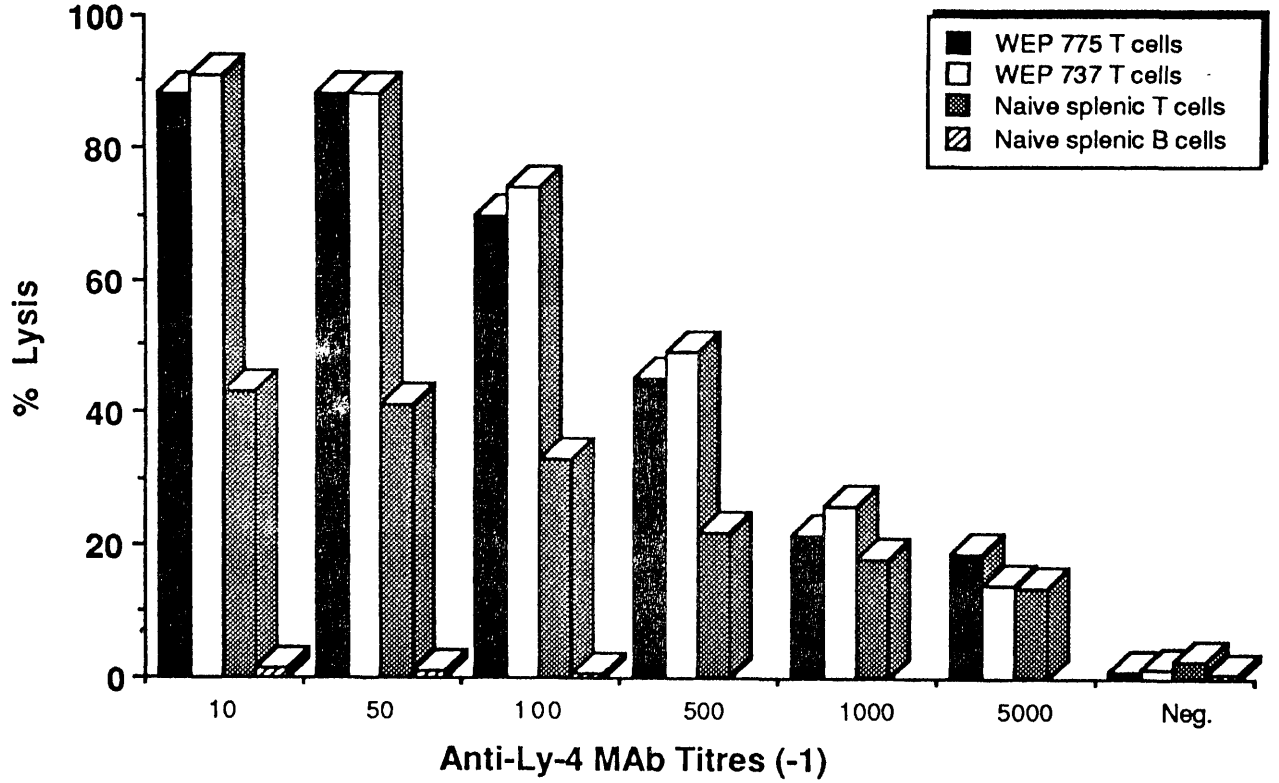

Fig. 9.2.3 Surface phenotyping of P. c. chabaudi AS-specific T lymphocyte lines by complement-mediated anti-Ly-4 cytotoxicity

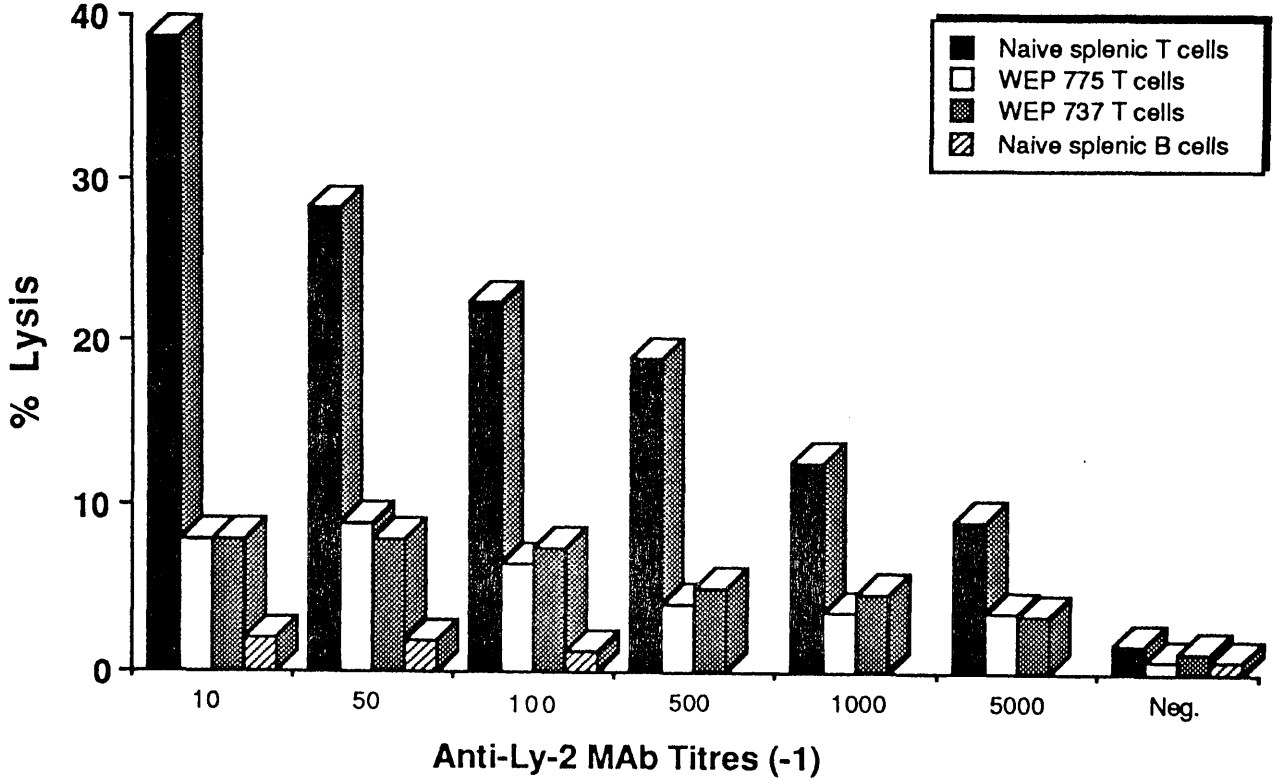

Fig. 9.2.4 Surface phenotyping of P. c. chabaudi AS-specific T lymphocyte lines by complement-mediated anti-Ly-2 cytotoxicity

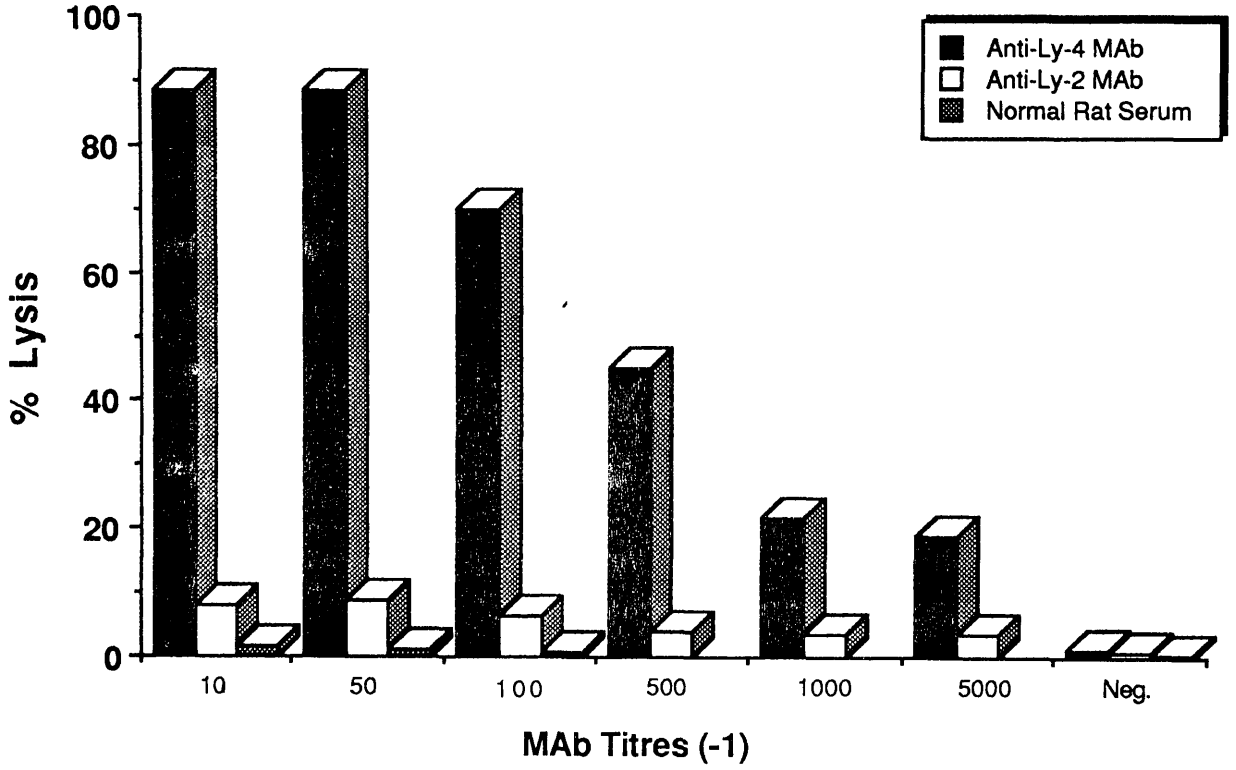

Fig. 9.2.5 Surface phenotyping of the WEP 775 P. c. chabaudi AS-specific T lymphocyte line by complement-mediated cytotoxicity

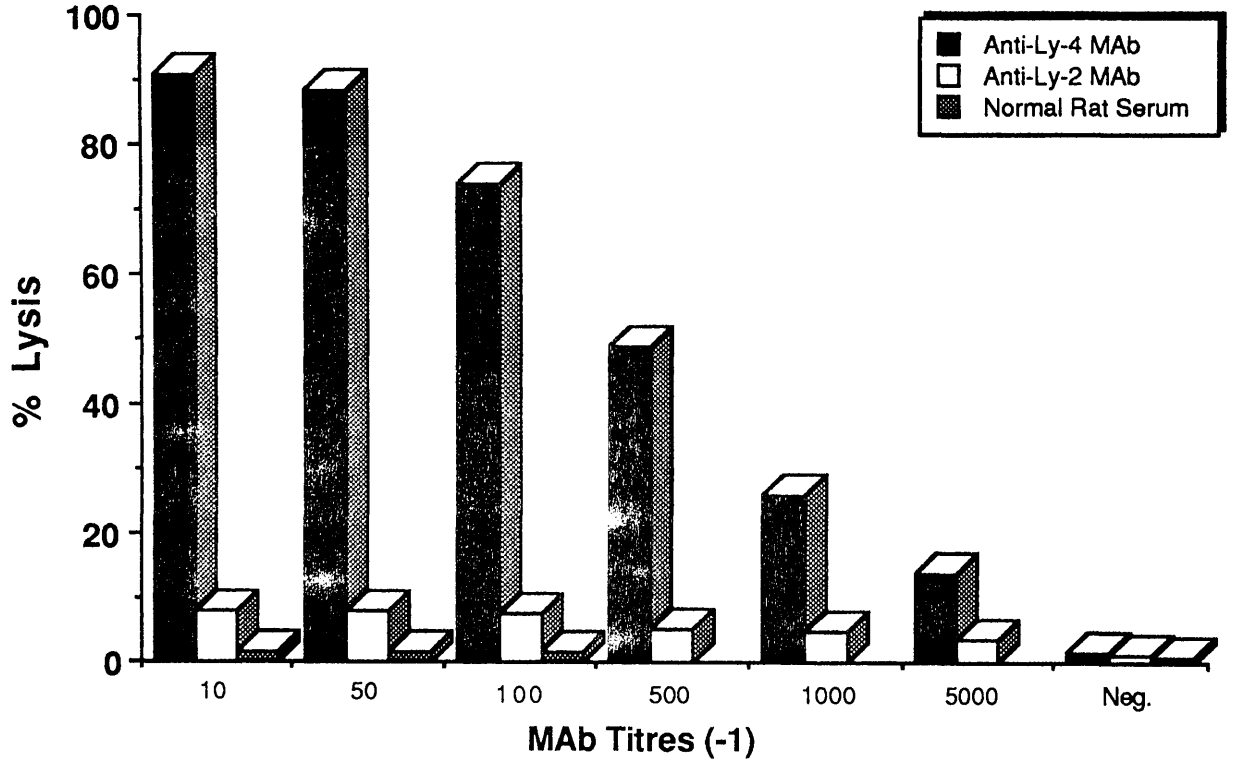

Fig. 9.2.6 Surface phenotyping of the WEP 737 P. c. chabaudi AS-specific T lymphocyte line by complement-mediated cytotoxicity

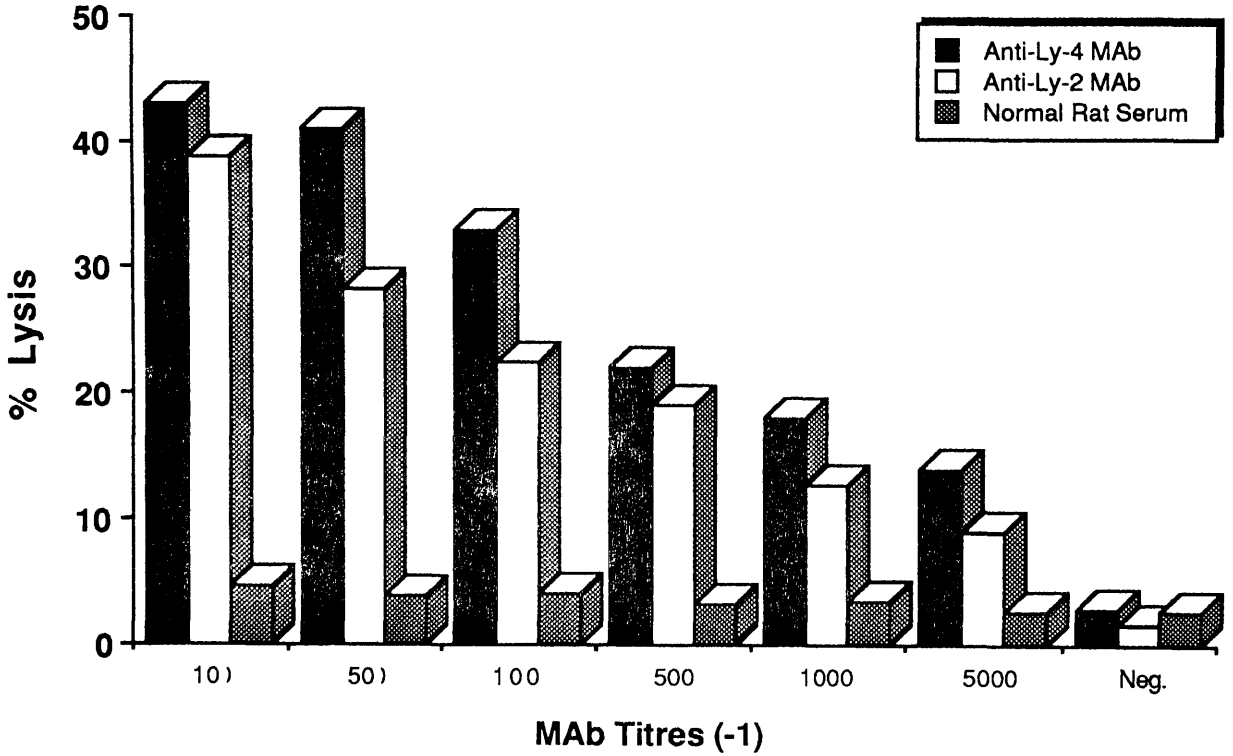

Fig. 9.2.7 Surface phenotyping of a naive splenic T lymphocyte population by complement-mediated cytotoxicity

Table 9.2.1 Phenotyping of T lymphocyte clones by surface immunofluorescence.

FITC-MAb		α-Thy-1	α-Ly-4	α-Ly-2	α-Θ	α-κ	non-fluor.
WEP	999	99.70	99.70	0	0	0	0.30
	996	98.98	98.98	0	0	0	1.02
	997	100	100	0	0	0	0
	998	99.68	99.68	0	0	0	0.32
	988	100	100	0	0	0	0
	985	99.31	99.31	0	0	0	0.69
	986	100	100	0	0	0	0
	987	99.68	99.68	0	0	0	0.32

% values

Table 9.2.2 Phenotyping of T cell clones by complement-mediated cytotoxicity.

Clone		MAb	Titre (-1)						
			10	50	100	500	1000	5000	Neg.
WEP	999	α-Ly-4	100	100	92.93	88.34	69.0	25.43	2.58
		α-Ly-2	1.08	0.56	0	0	0.53	0	0.58
		Rat serum	1.44	1.01	0.46				0.52
	996	α-Ly-4	99.46	100	90.61	82.05	60.70	23.27	1.04
		α-Ly-2	1.33	0.48	0	0	0.52	0	0.56
		Rat serum	1.84	0.95	0				0.93
	997	α-Ly-4	100	100	94.79	86.83	71.79	30.94	1.56
		α-Ly-2	0.49	0.51	0	0	0.57	0	0
		Rat serum	0.94	0.91	0.51				0.50
	998	α-Ly-4	99.51	99.50	92.86	88.72	64.90	30.00	1.52
		α-Ly-2	0.93	0.54	0	0.57	0	0	0.51
		Rat serum	1.05	0.57	0.49				0.50
WEP	988	α-Ly-4	100	100	96.15	84.86	67.84	28.04	1.05
		α-Ly-2	1.03	0.49	0.52	0	0	0	0
		Rat serum	1.38	0.93	0				0.47
	985	α-Ly-4	99.07	99.52	89.78	82.81	59.09	21.95	0.93
		α-Ly-2	0.97	0.51	0	0	0	0.46	0.50
		Rat serum	0.99	0.92	0.52				0.49
	986	α-Ly-4	100	99.07	95.90	86.19	74.09	34.63	1.05
		α-Ly-2	1.01	0.48	0.47	0	0.50	0	0.49
		Rat serum	0.97	0.87	0.56				0.48
	987	α-Ly-4	99.52	98.07	91.33	84.76	68.11	25.85	1.07
		α-Ly-2	1.04	0.48	0.50	0	0	0	0
		Rat serum	0.57	0.94	0				0.46

% lysis values

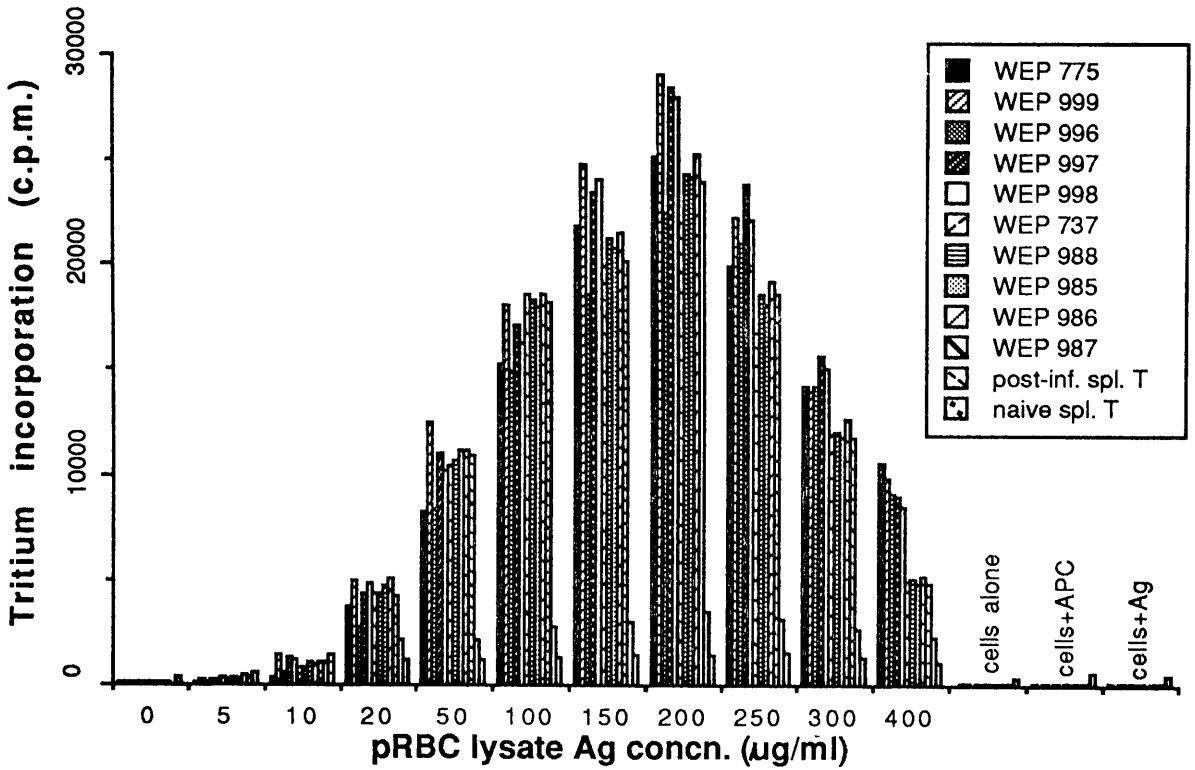

Fig. 9.3.1 Assay of proliferation of the P. c. chabaudi AS-specific T lymphocyte lines WEP 775 & WEP 737 and their respective daughter clones in response to stimulation with P. c. chabaudi AS pRBC lysate

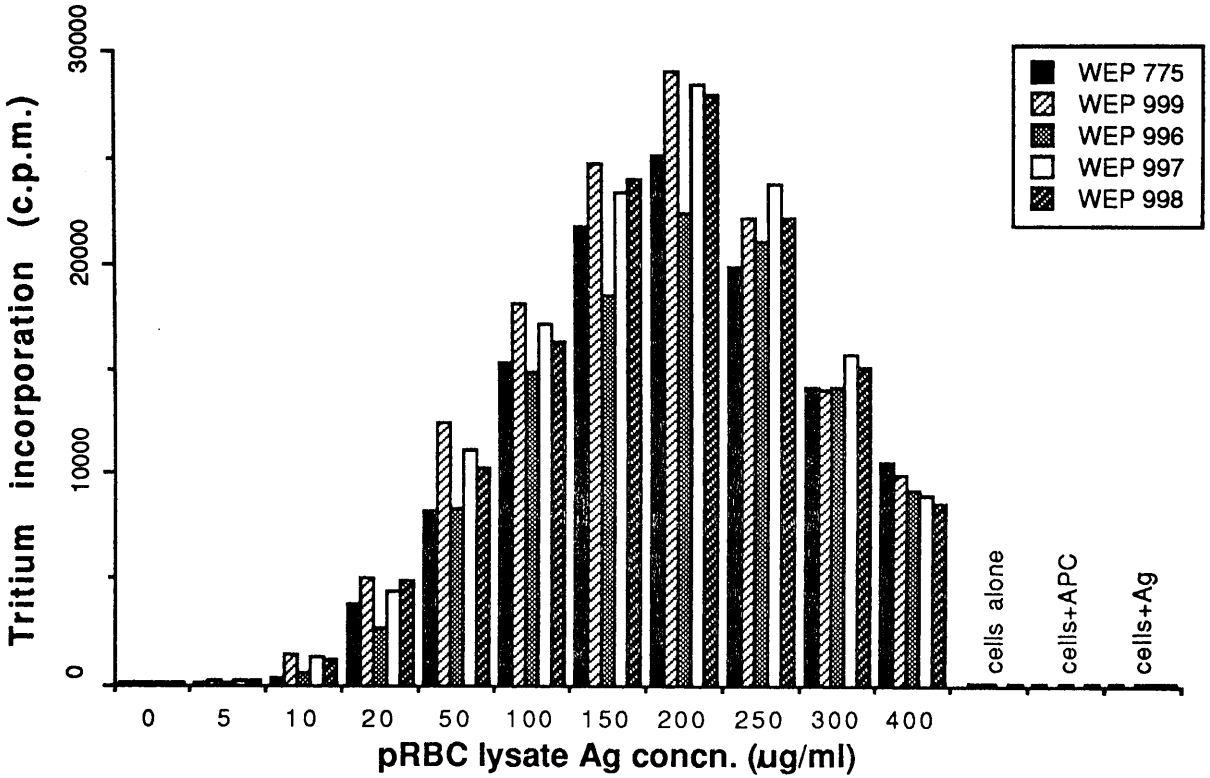

Fig. 9.3.2 Assay of proliferation of the P. c. chabaudi AS-specific T lymphocyte line WEP 775 and of its daughter clones WEP 996-999 in response to stimulation with P. c. chabaudi AS pRBC lysate

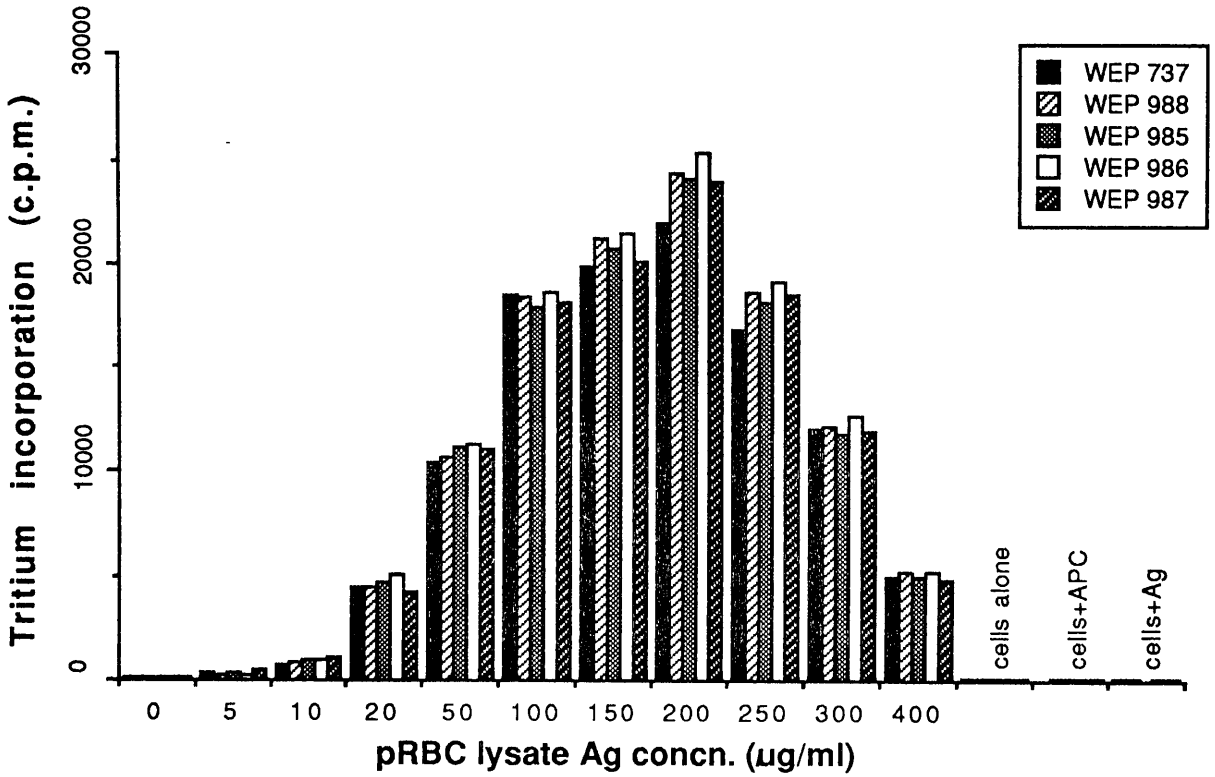

Fig. 9.3.3 Assay of proliferation of the P. c. chabaudi AS-specific T lymphocyte line WEP 737 and of its daughter clones WEP 985-988 in response to stimulation with P. c. chabaudi AS pRBC lysate

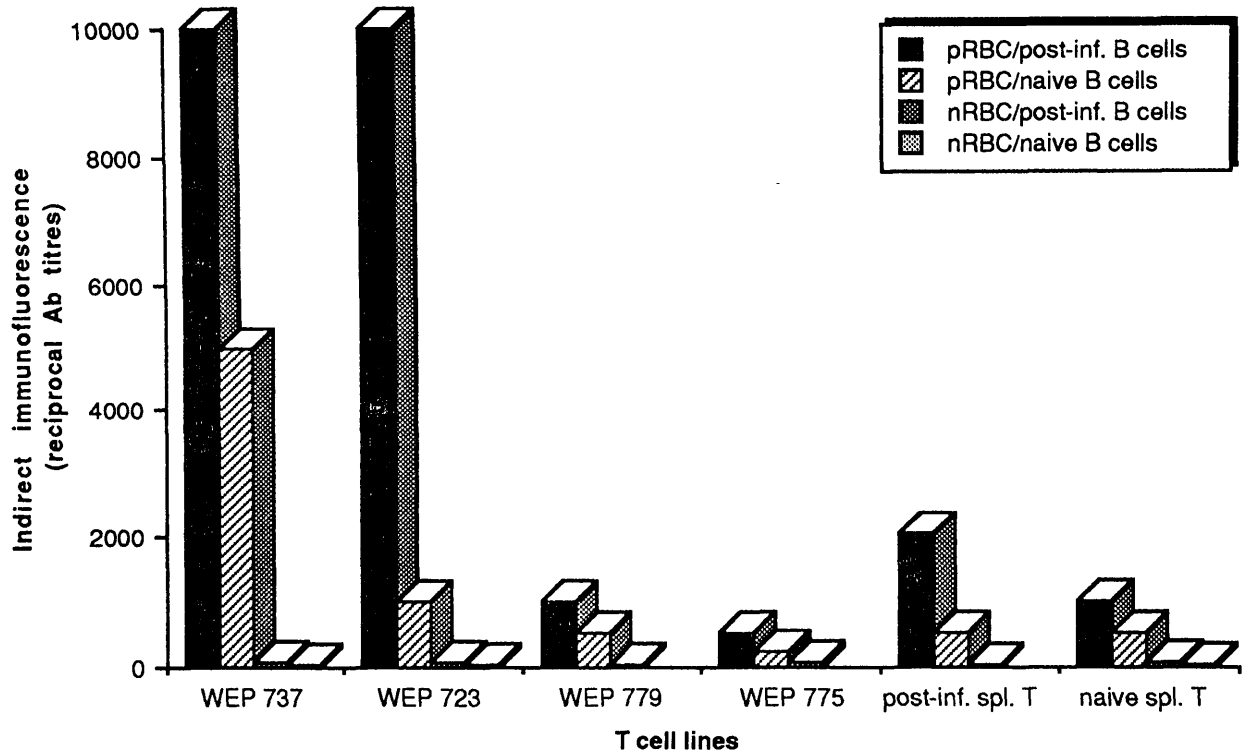

Fig. 9.4.1 Assay of the induction of antibody responses to P. c. chabaudi AS by T lymphocyte lines in vitro

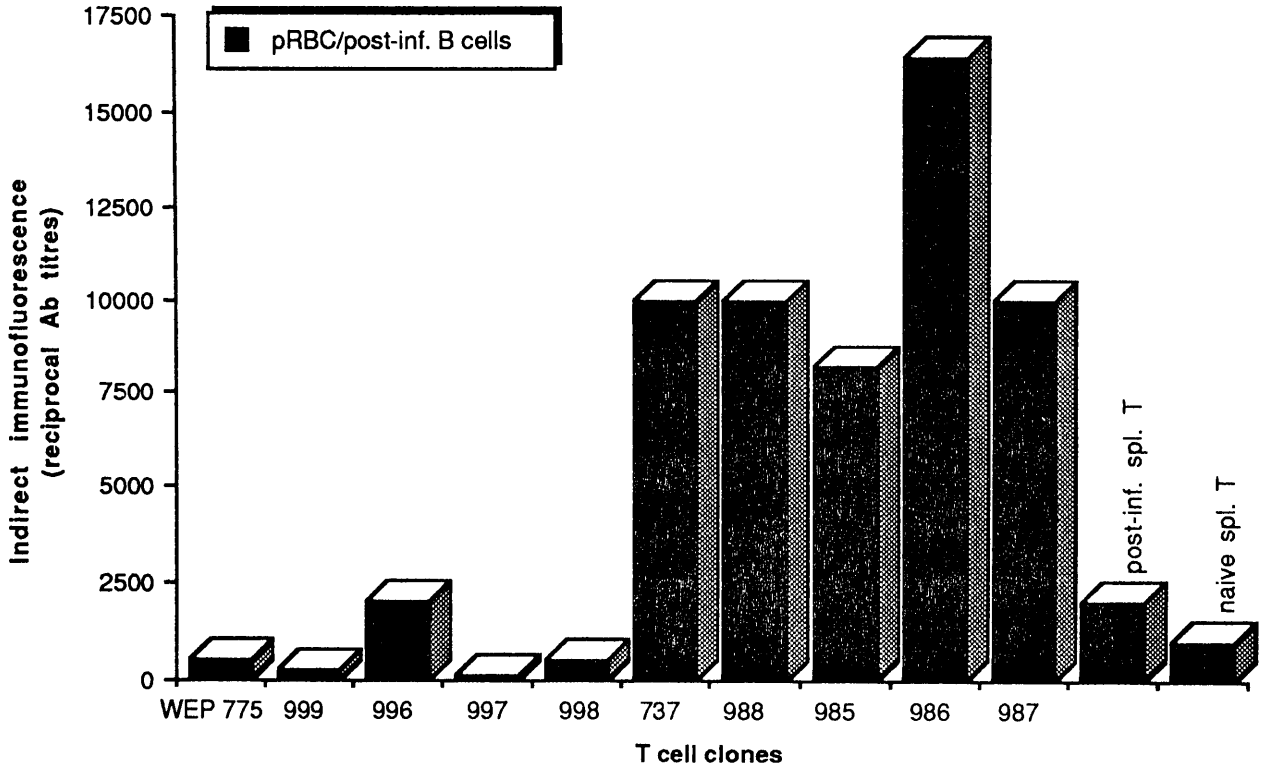

Fig. 9.4.2 Assay of the induction of antibody responses to P. c. chabaudi AS by T lymphocyte clones in vitro

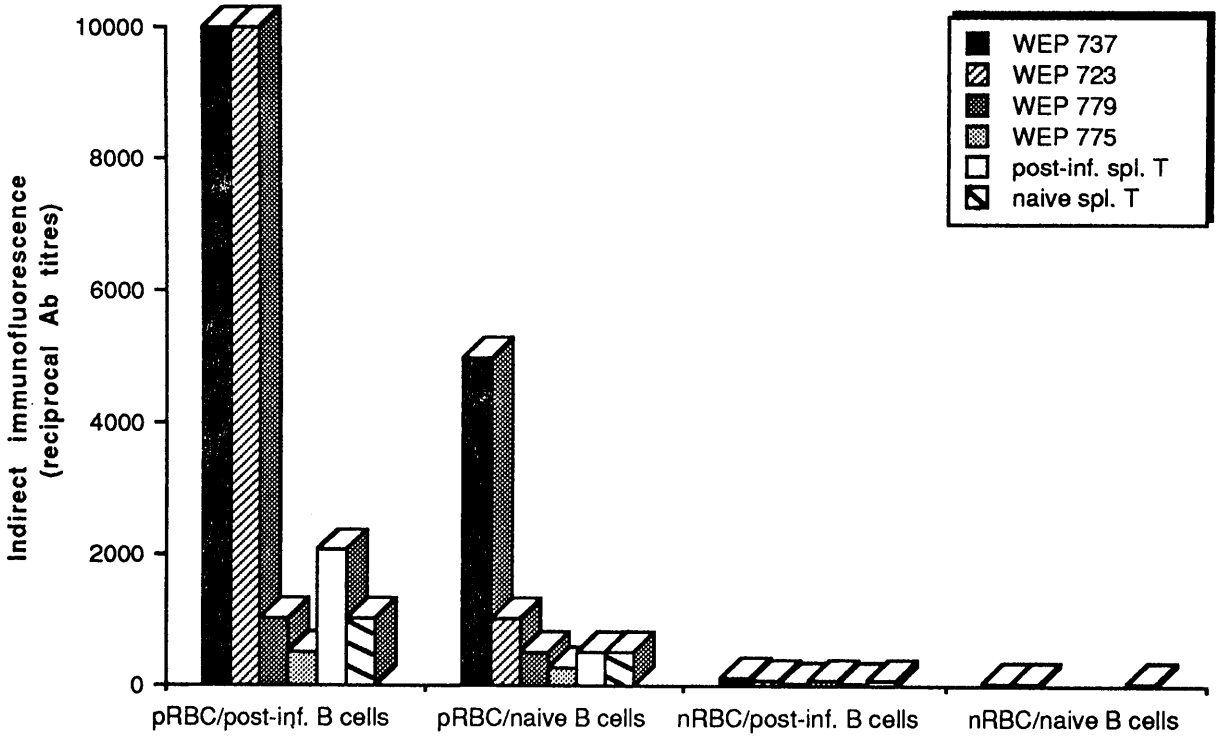

Fig. 9.4.3 Assay of the induction of antibody responses to P. c. chabaudi AS by T lymphocyte lines in vitro

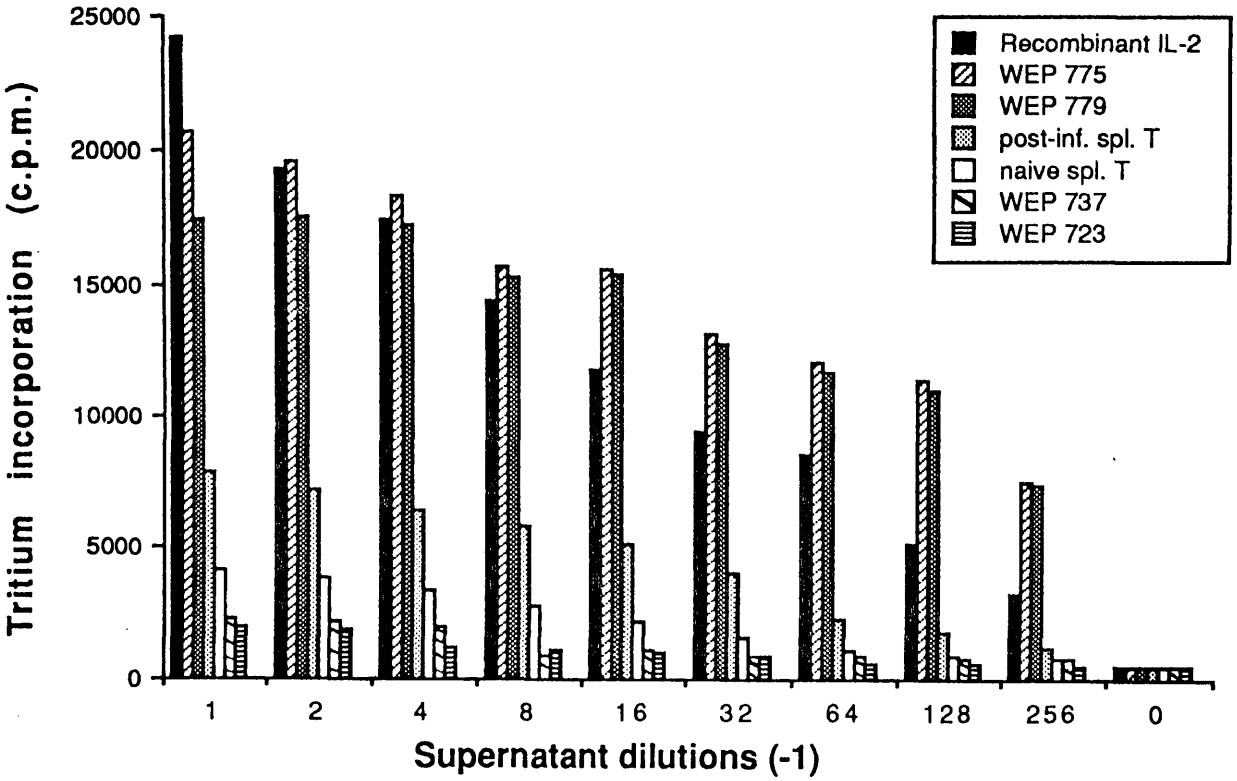

Fig. 9.5.1 Assay for IL-2 (IL-4) secretion by the P. c. chabaudi AS-specific T lymphocyte lines in vitro by proliferation of the CTLL-2 cell line

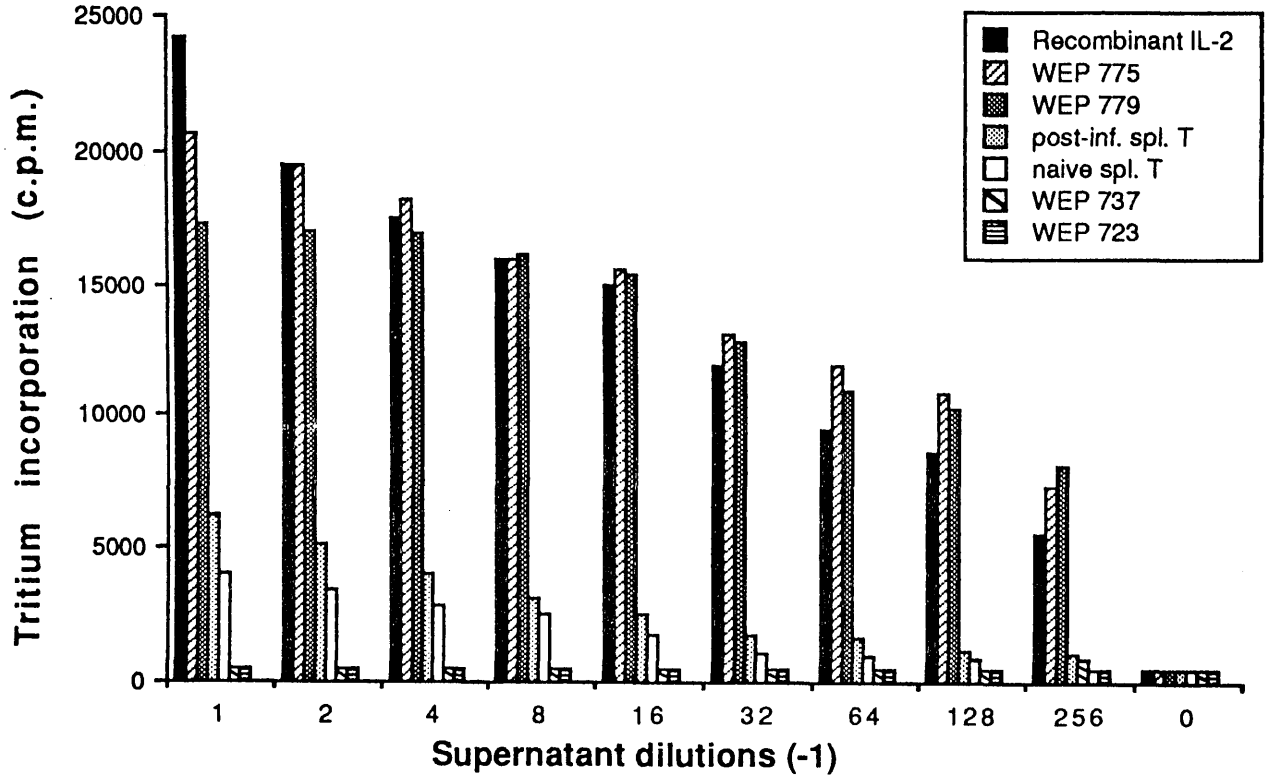

Fig. 9.5.2 Monospecific assay for IL-2 secretion by the P. c. chabaudi AS-specific T lymphocyte lines in vitro by proliferation of the CTLL-2 cell line in the presence of the anti-IL-4 MAb

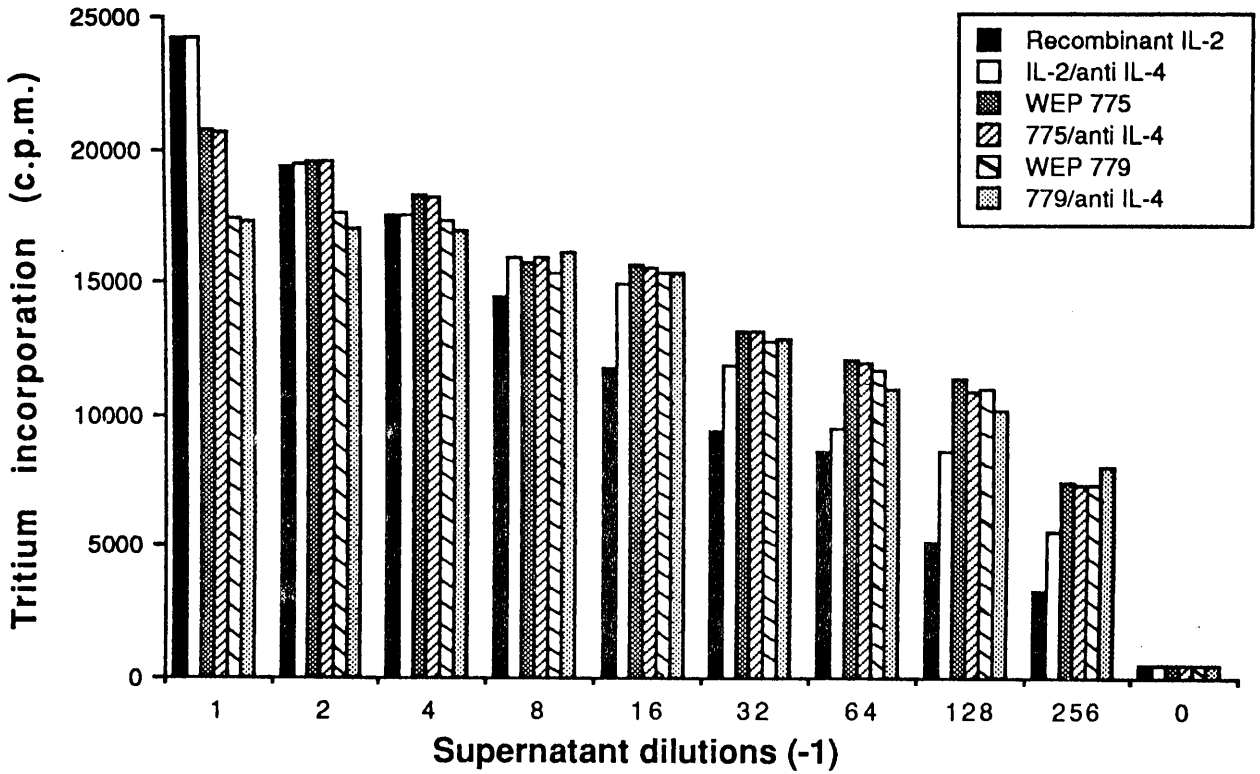

Fig. 9.5.3 Assay for IL-2 secretion by WEP 775 & WEP 779 <u>in vitro</u> by proliferation of the CTLL-2 cell line in the presence/absence of the anti-IL-4 MAb

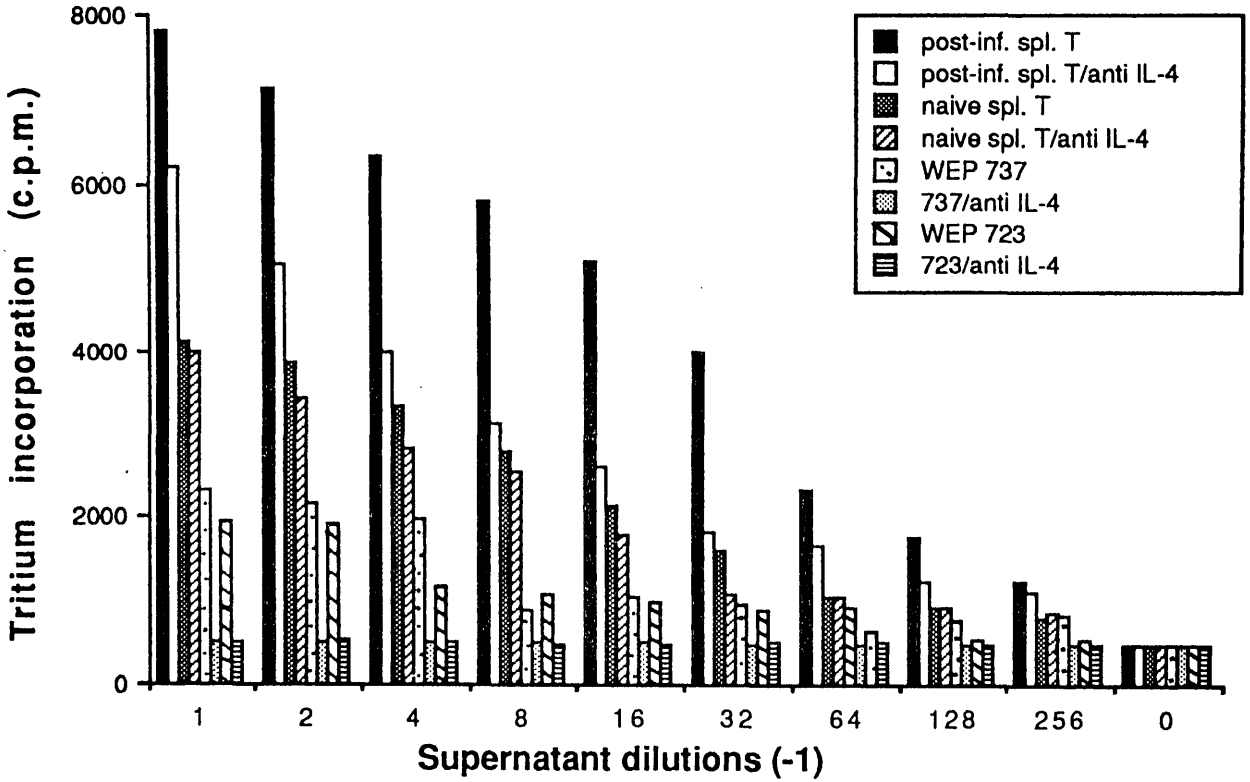

Fig. 9.5.4 Assay of IL-4 secretion by WEP 737 & WEP 723 in vitro by proliferation of the CTLL-2 cell line in the presence/absence of the anti-IL-4 MAb

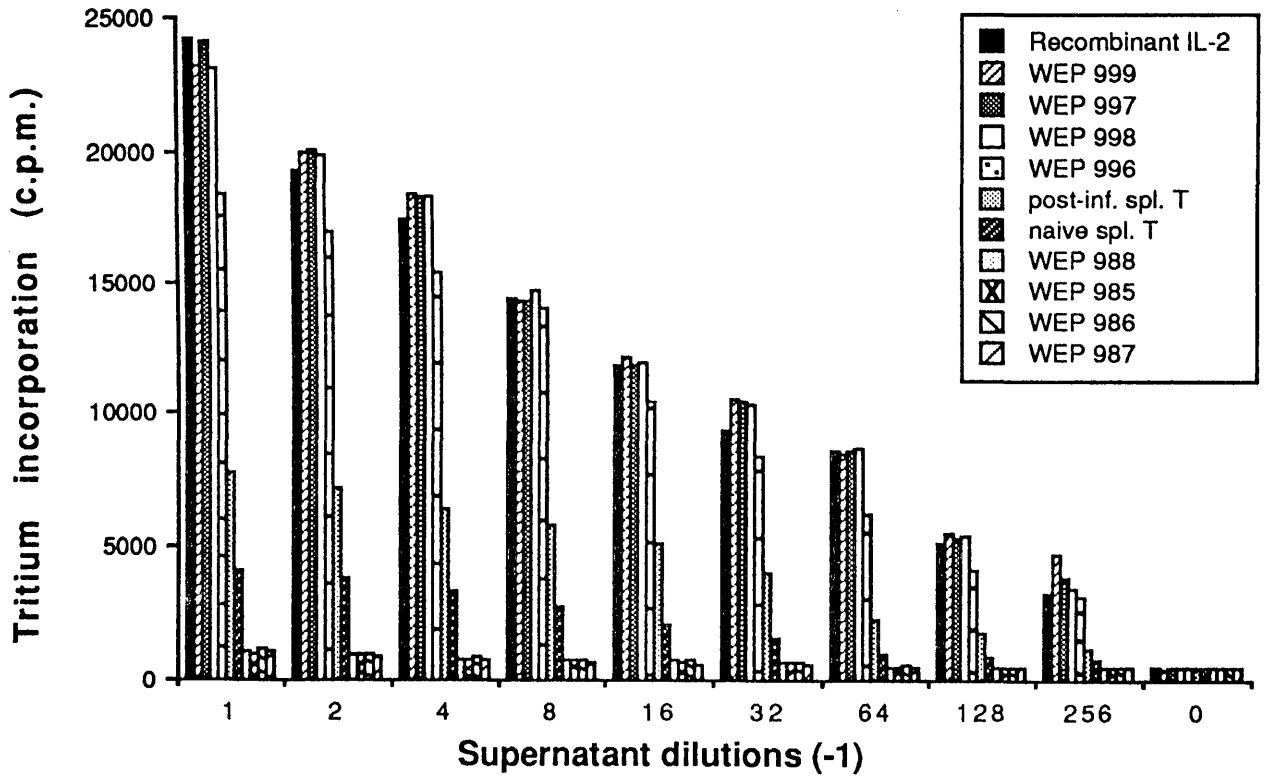

Fig. 9.5.5 Assay for IL-2 (IL-4) secretion by the P. c. chabaudi AS-specific T lymphocyte clones in vitro by proliferation of the CTLL-2 cell line

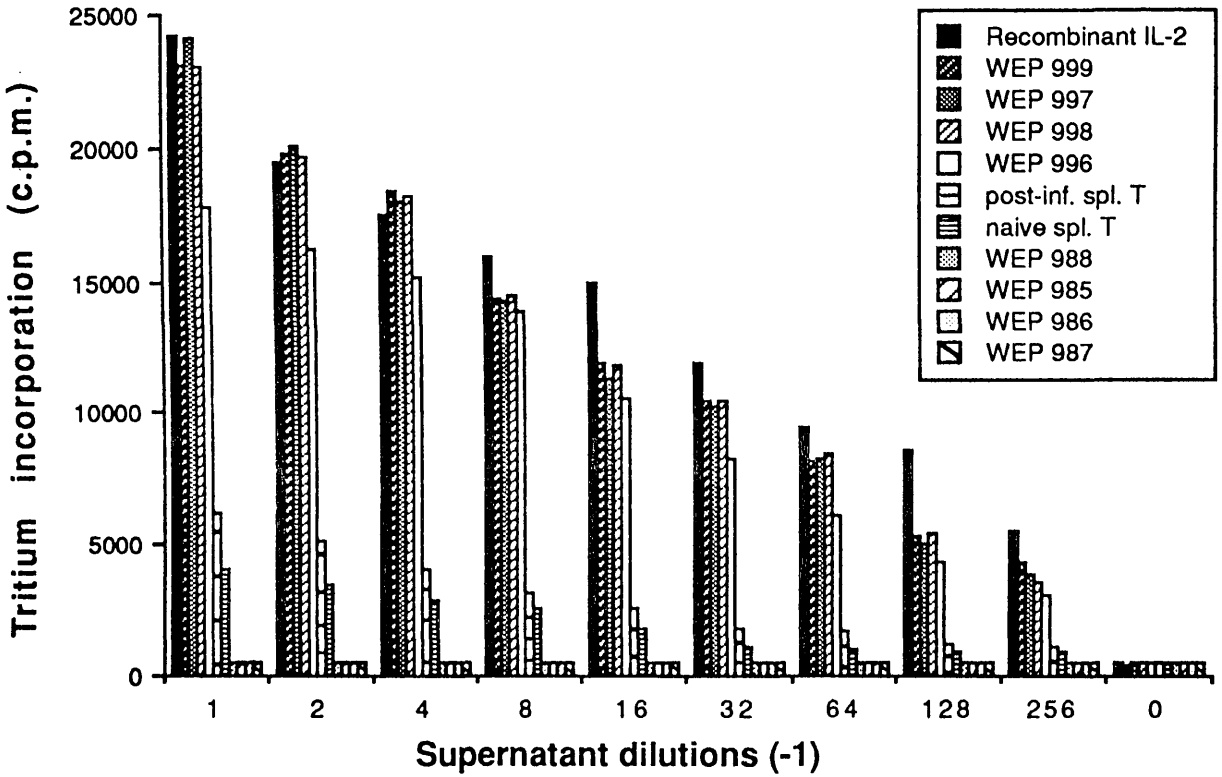

Fig. 9.5.6 Monospecific assay for IL-2 secretion by the P. c. chabaudi AS-specific T lymphocyte clones in vitro by proliferation of the CTLL-2 cell line in the presence of the anti-IL-4 MAb

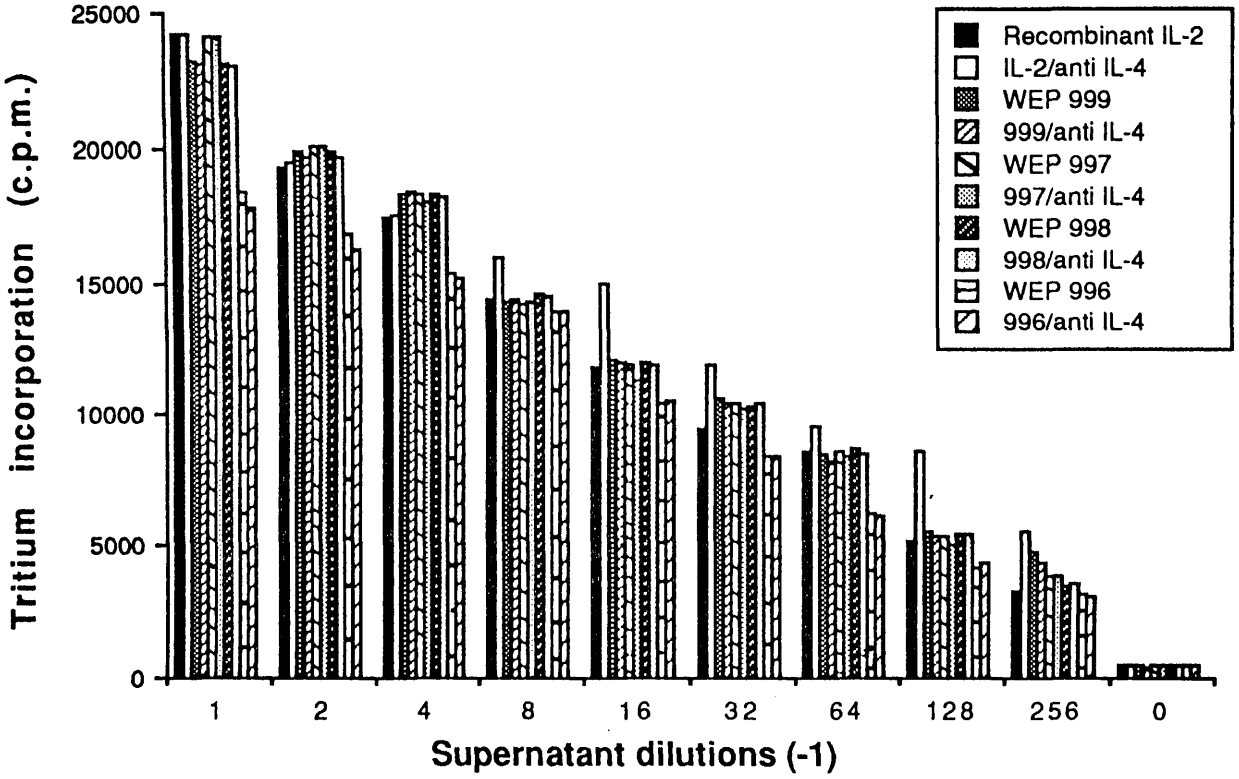

Fig. 9.5.7 Assay for IL-2 secretion by WEP 996-999 <u>in vitro</u> by proliferation of the CTLL-2 cell line in the presence/absence of the anti-IL-4 MAb

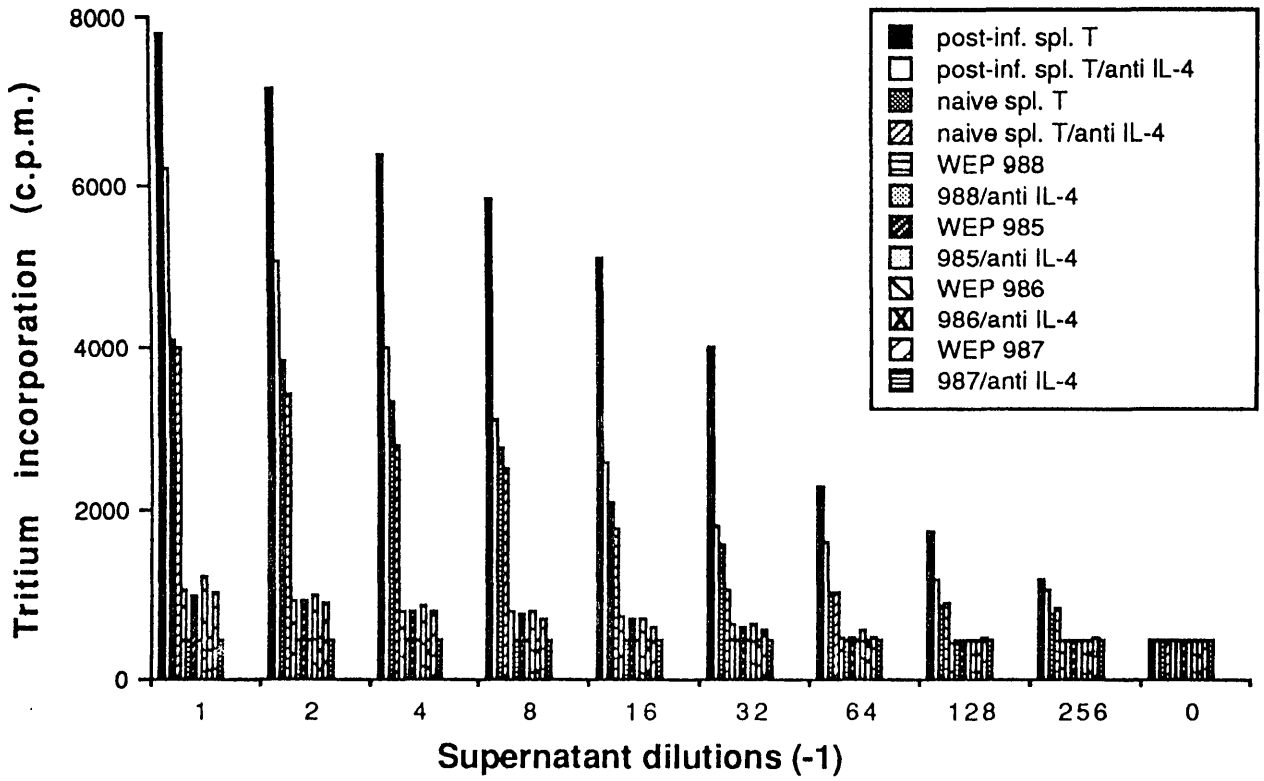

Fig. 9.5.8 Assay for IL-4 secretion by WEP 985-988 <u>in vitro</u> by proliferation of the CTLL-2 cell line in the presence/absence of the anti-IL-4 MAb

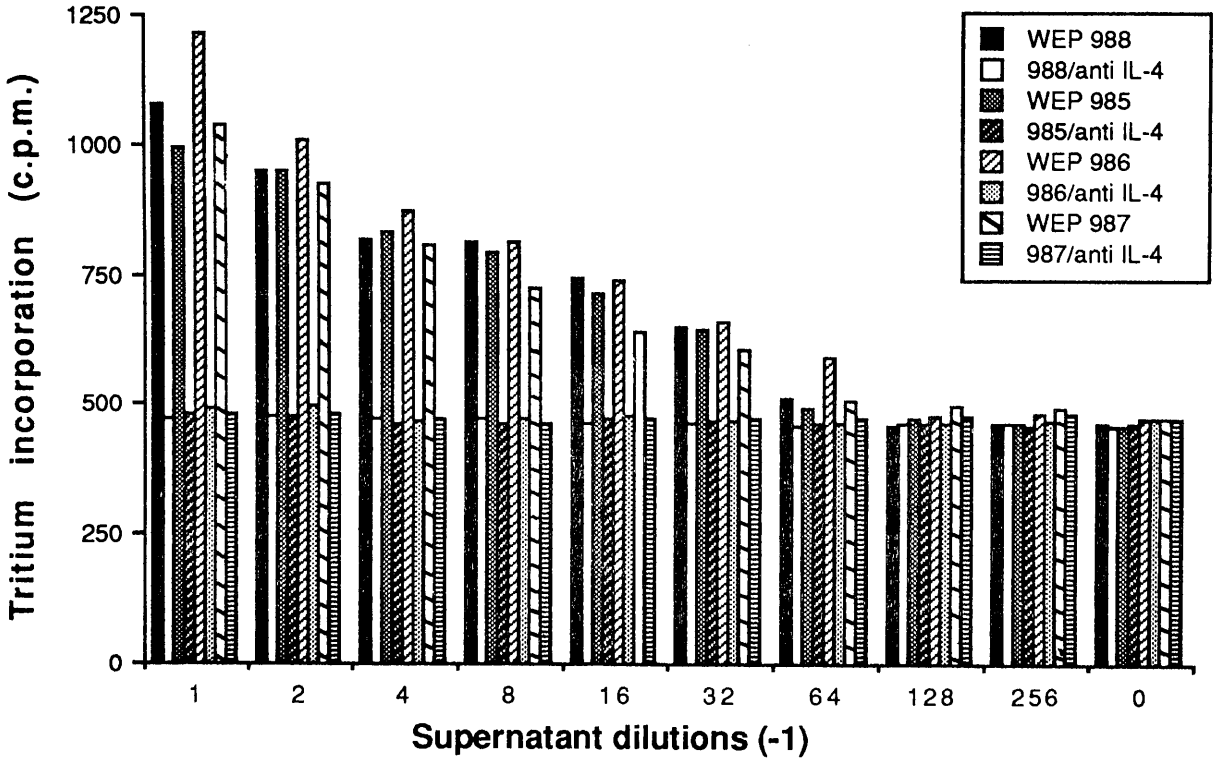

Fig. 9.5.9 Assay for IL-4 secretion by WEP 985-988 in vitro by proliferation of the CTLL-2 cell line in the presence/absence of the anti-IL-4 MAb

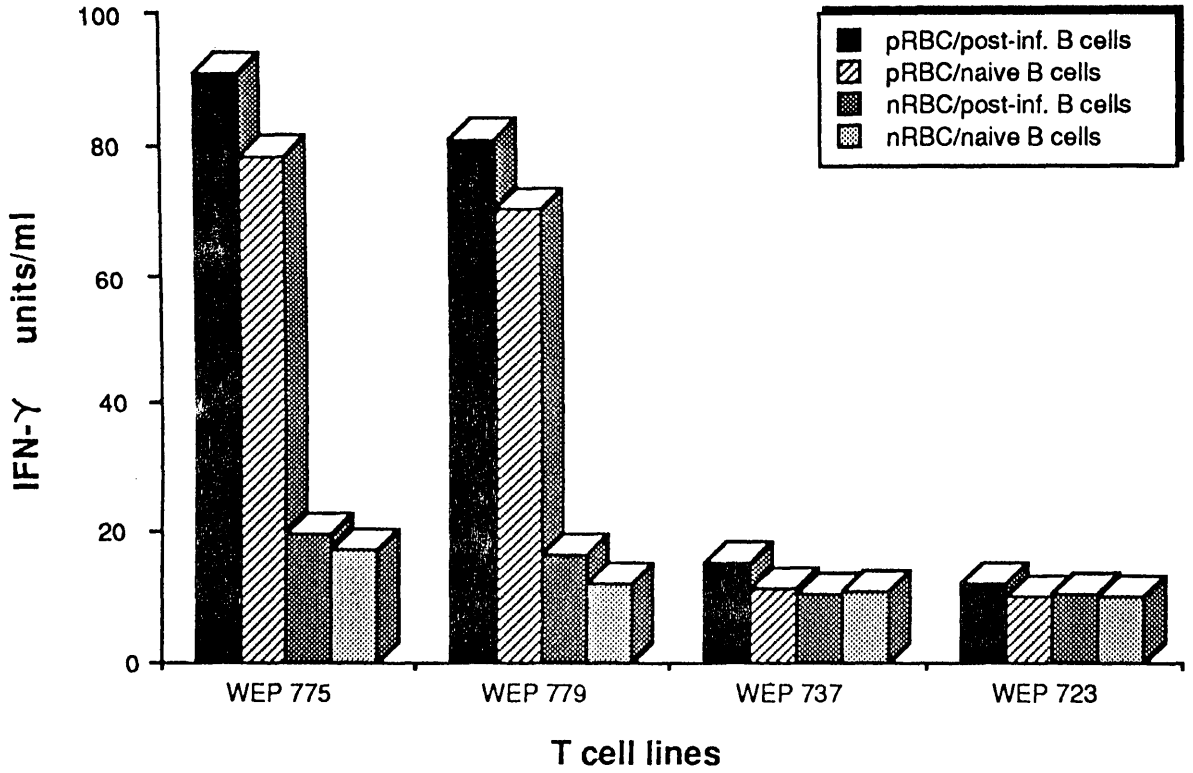

Fig. 9.6.1 Assay for IFN-γ secretion by the P. c. chabaudi AS-specific T lymphocyte lines in vitro by measurement of viral plaque inhibition

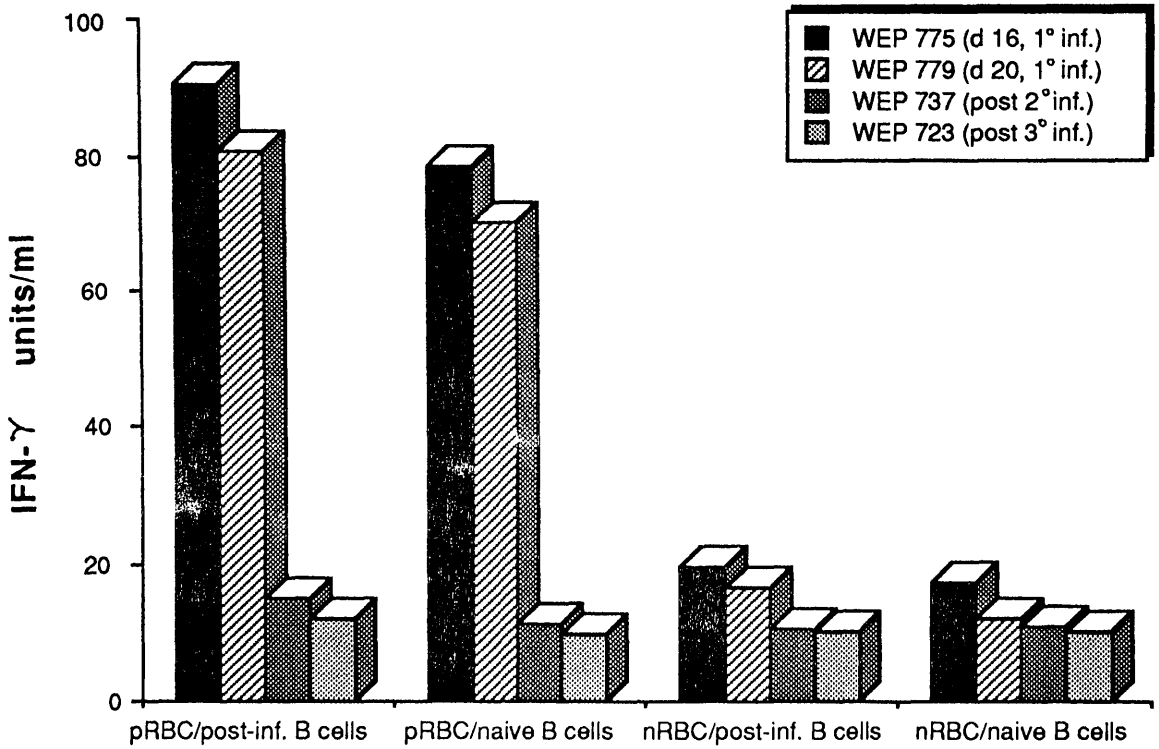

Fig. 9.6.2 Assay for IFN-γ secretion by the P. c. chabaudi AS-specific T lymphocyte lines in vitro by measurement of viral plaque inhibition

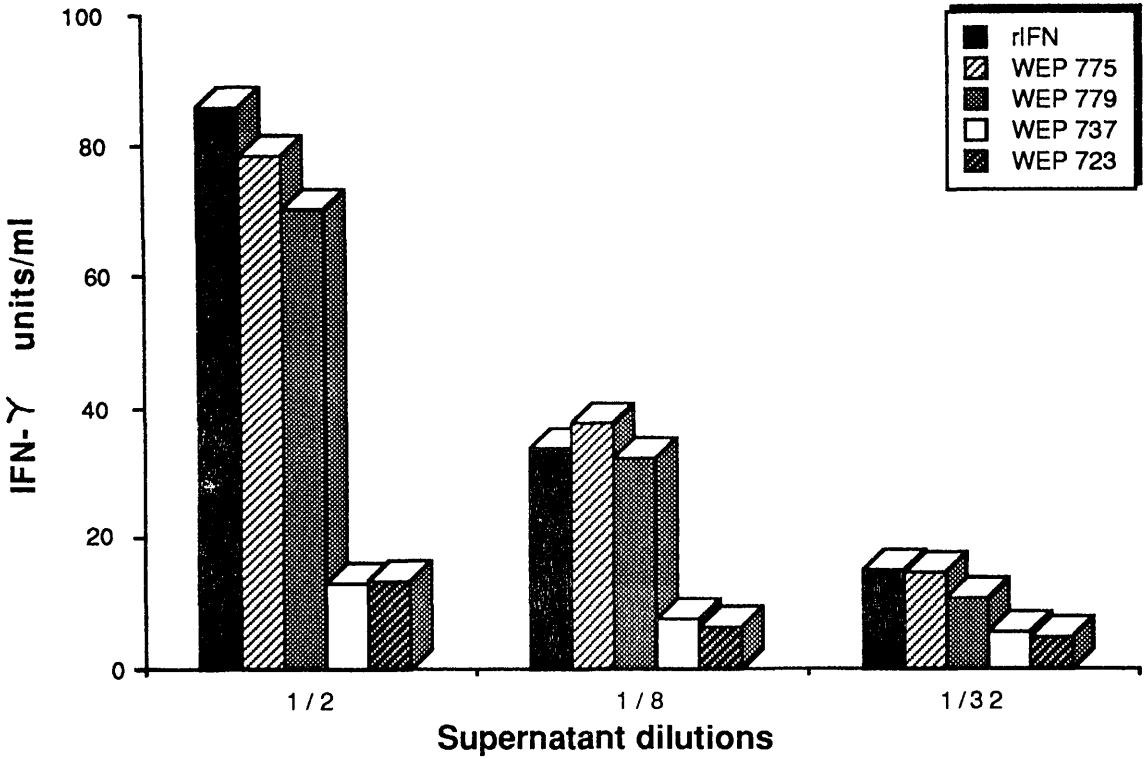

Fig. 9.6.3 Titration of IFN-γ concentrations in in vitro culture S/N of the P. c. chabaudi AS-specific T lymphocyte lines

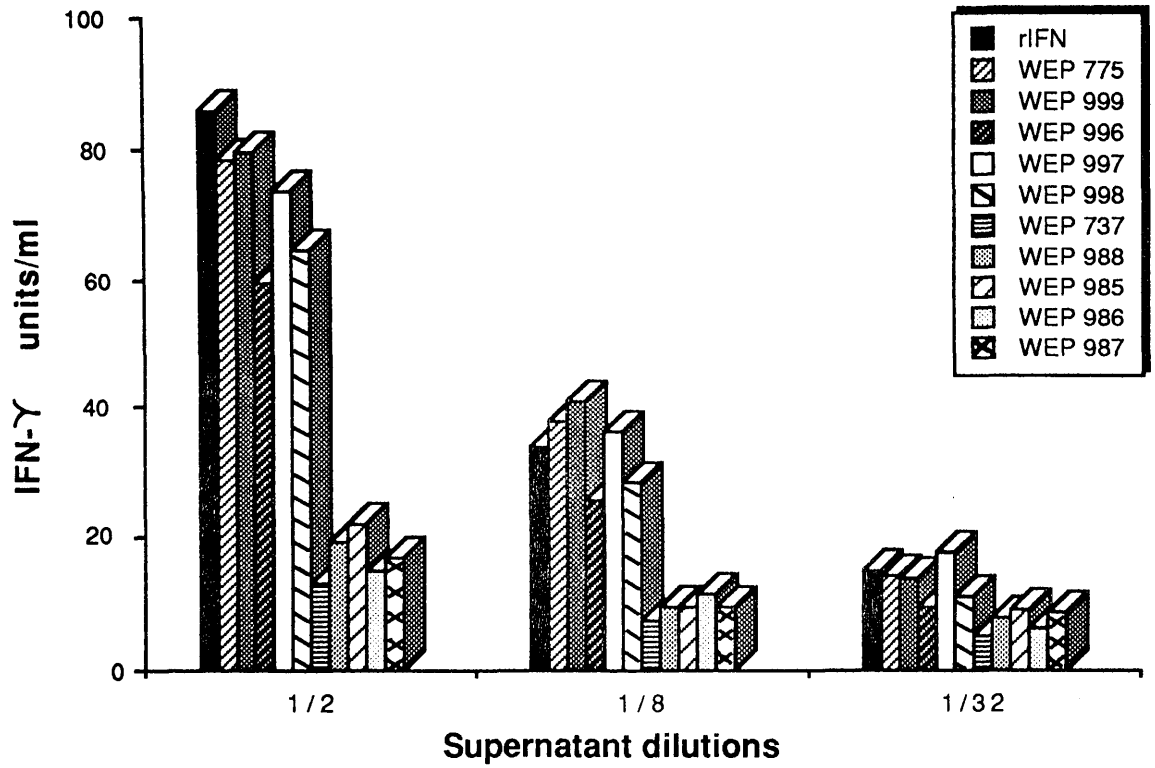

Fig. 9.6.4 Titration of IFN-γ concentrations in in vitro culture S/N of the P. c. chabaudi AS-specific T lymphocyte clones

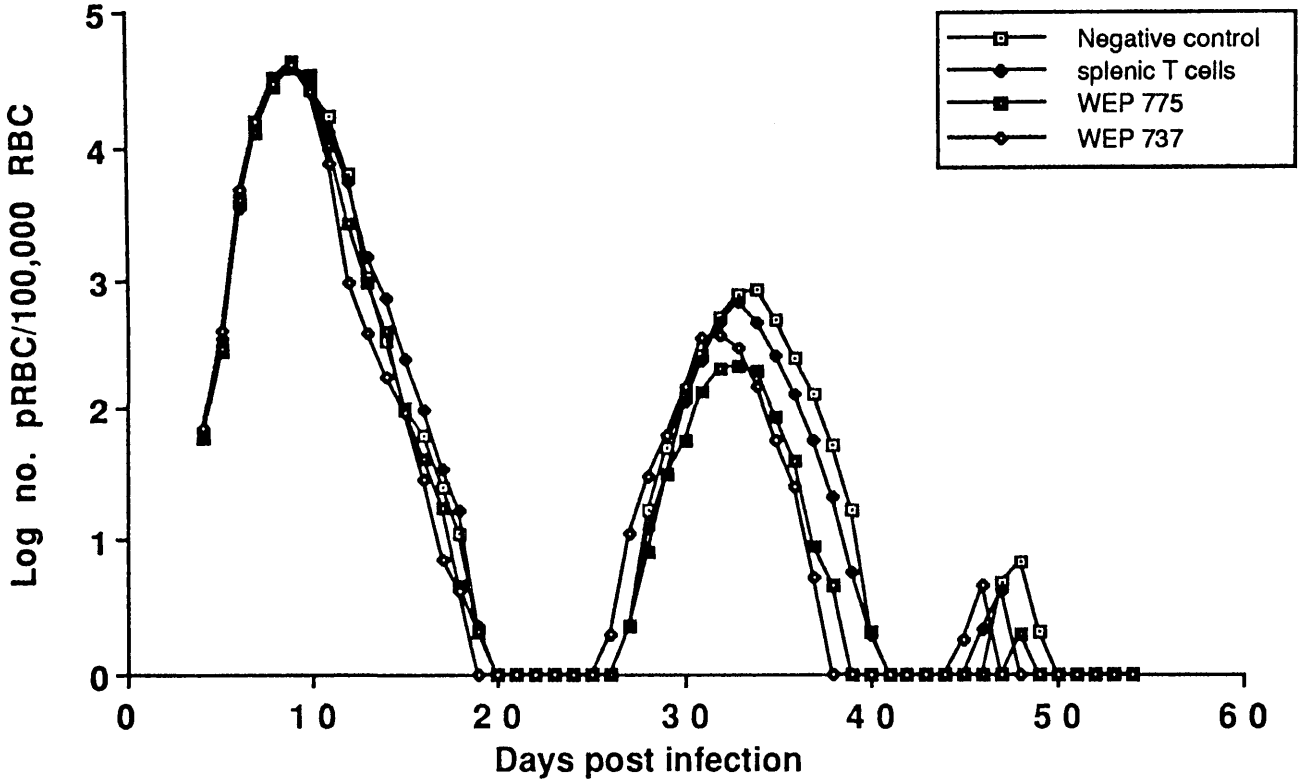

Fig. 9.7.1 Course of infection in NIH naive recipients of WEP 775 & WEP 737 T lymphocyte lines upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v..

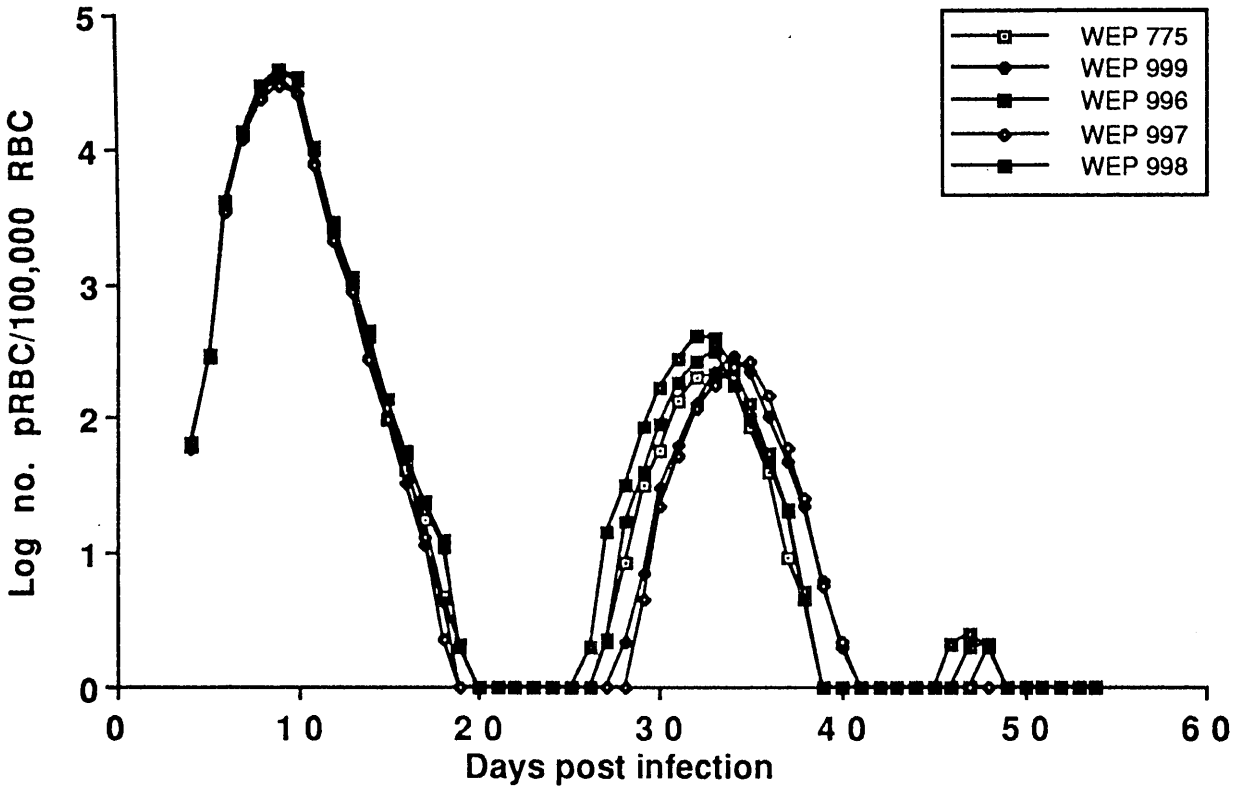

Fig. 9.7.2 Course of infection in NIH naive recipients of WEP 775 and of its daughter clones WEP 996-999 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v..

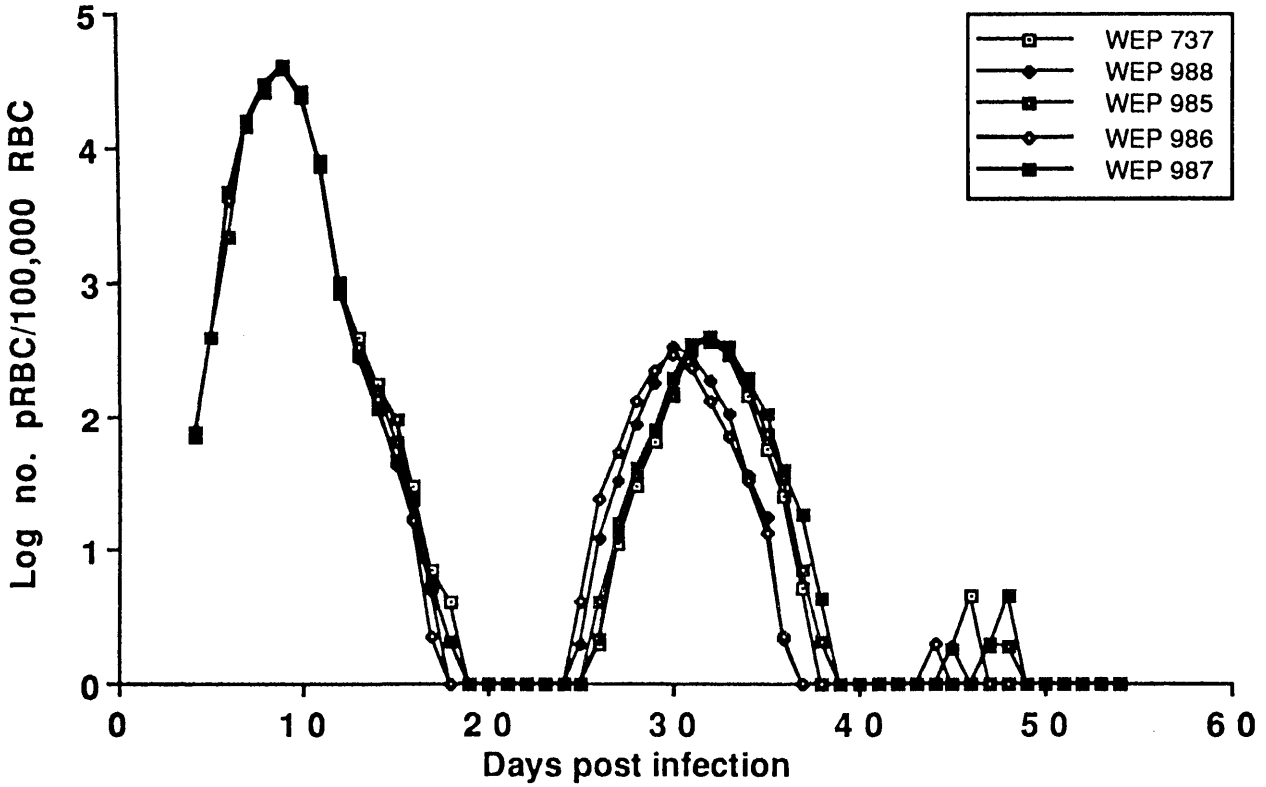

Fig. 9.7.3 Course of infection in NIH naive recipients of WEP 737 and of its daughter clones WEP 985-988 upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v..

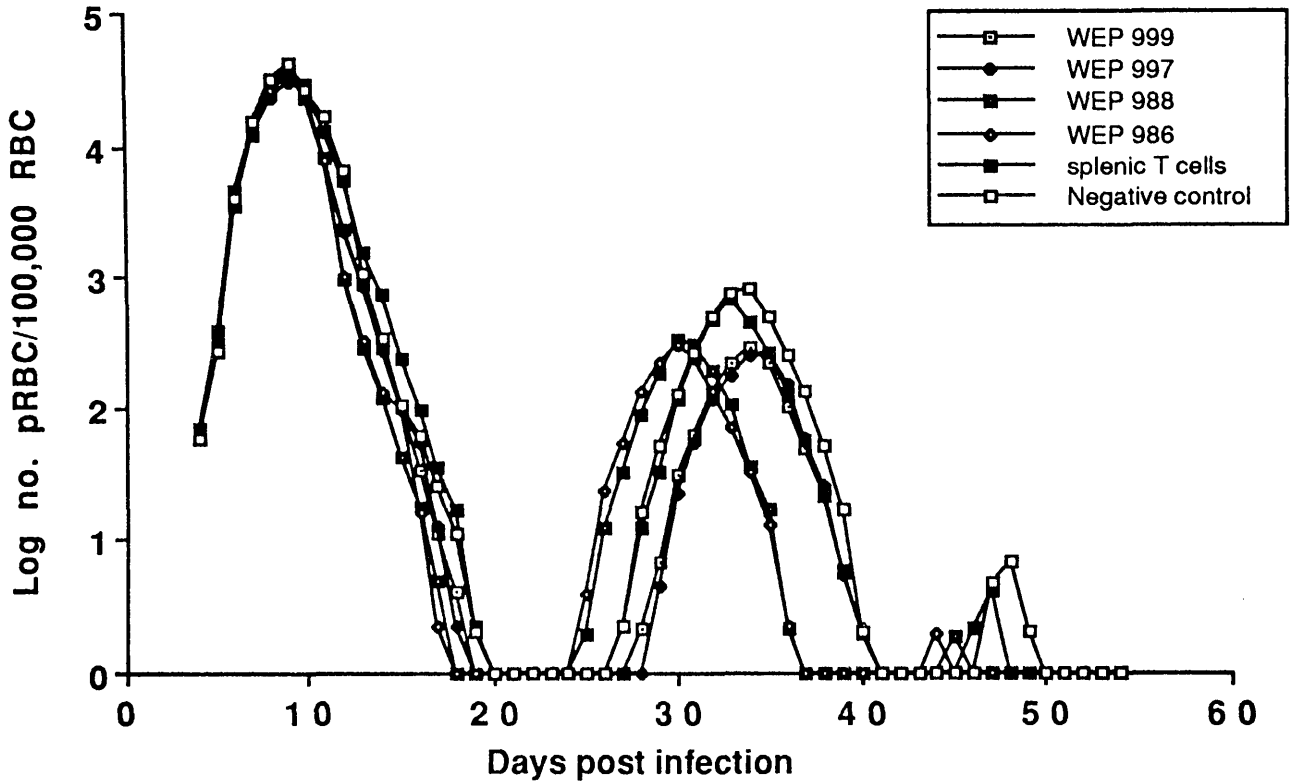

Fig. 9.7.4 Divergent courses of infection in NIH naive recipients of P. c. chabaudi AS-specific T lymphocyte clones of varying origin upon challenge with 1 x10^5 homologous pRBC i.v..

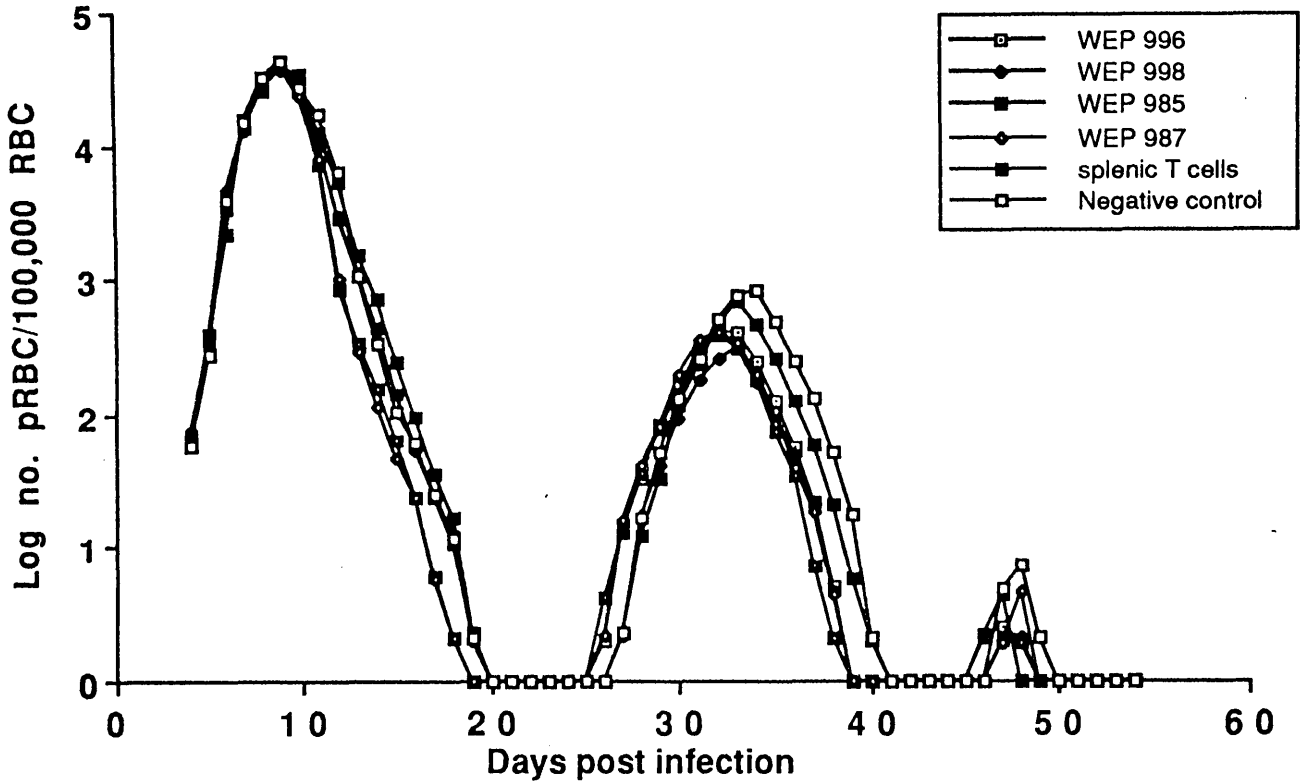

Fig. 9.7.5 Similar courses of infection in NIH naive recipients of <u>P. c. chabaudi</u> AS-specific T lymphocyte clones of varying origin upon challenge with 1 x10^5 homologous pRBC i.v..

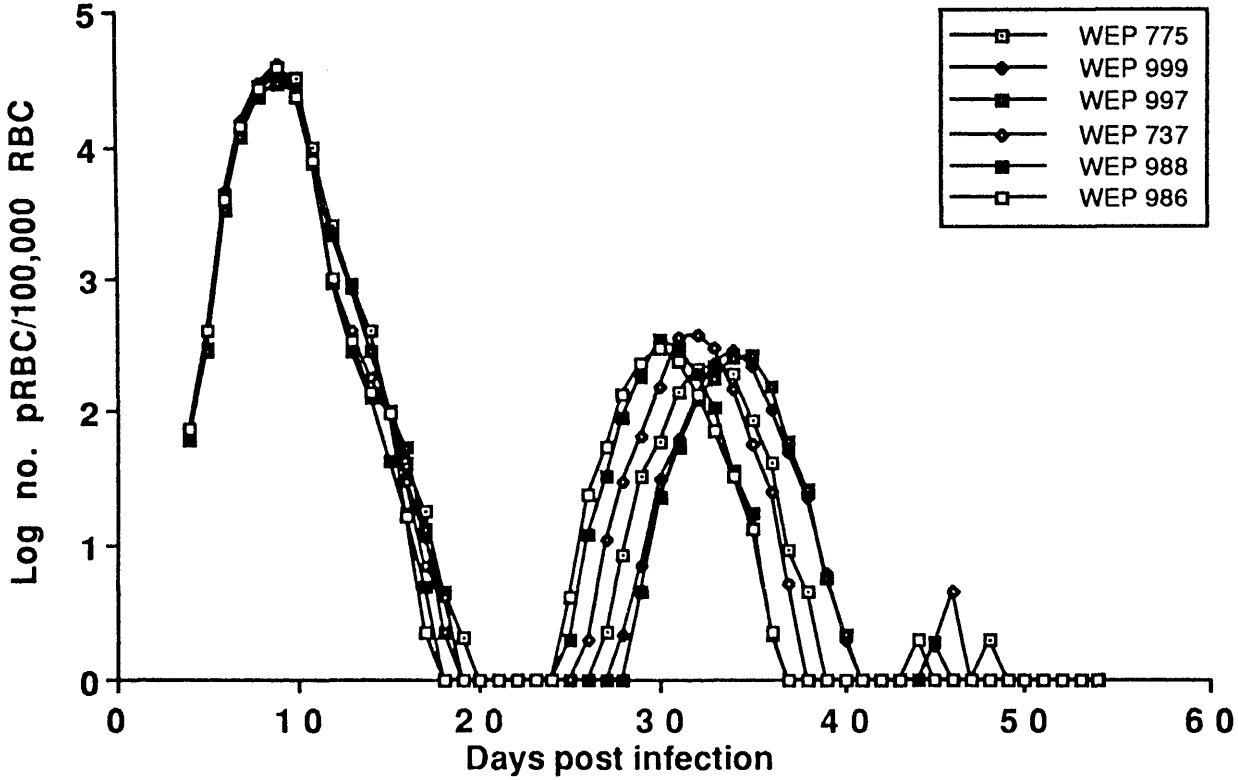

Fig. 9.7.6 Divergent courses of infection in NIH naive recipients of WEP 775- or WEP 737-derived T lymphocyte clones upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v..

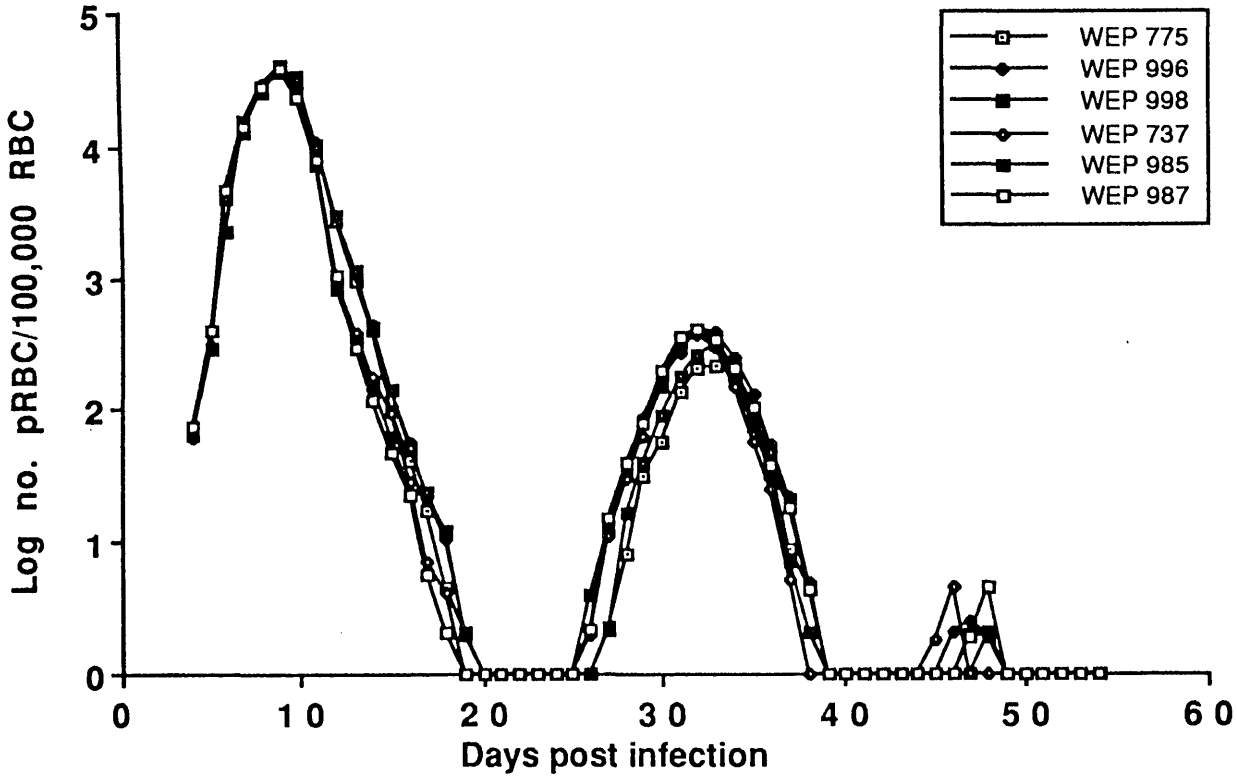

Fig. 9.7.7 Similar courses of infection in NIH naive recipients of WEP 775- or WEP 737-derived T lymphocyte clones upon challenge with 1 x10^5 P. c. chabaudi AS pRBC i.v..

CHAPTER 10

GENERAL DISCUSSION

THE PATHWAYS OF CELL-MEDIATED IMMUNE REACTIVITY LEADING TO PROTECTION AGAINST THE ASEXUAL ERYTHROCYTIC STAGES OF MALARIA

The experiments described in this thesis have demonstrated the importance of T lymphocytes carrying the Ly-4 cell surface marker in the development of protective immunity to the blood stages of Plasmodium chabaudi chabaudi AS. Rather than reiterating points made previously, this discussion is intended to speculate on the ways in which different Ly-4$^+$ T cell-dependent effector functions may be involved in the control or elimination of blood-borne malaria parasites. The Ly-4-bearing T lymphocyte can be characterised functionally as a cell which can mediate delayed-type hypersensitivity and which can also act as a helper cell for Ab production. Recent experiments (Mosmann et al 1986, Cherwinski et al 1987) have indicated that these functions may be performed by distinct subsets of Ly-4$^+$ cells, T_H1 and T_H2, respectively. Both types of lymphocyte recognise plasmodial peptides which have been processed and presented on H-2 class II molecules of APC (Chapter 4). When they are thus activated, T_H1 cells secrete exclusively IL-2 and IFN-γ, amongst other lymphokines (Mosmann & Coffman 1987, Chapter 9). IFN-γ is a potent activator of macrophages, which leads to their expression of raised levels of MHC class II determinants (King & Jones 1983, Fernandez-Botran et al 1988), and thereby their becoming more efficient APC (Zlotnik et al 1983). Macrophage activation results also in enhanced phagocytosis (Zlotnik et al 1983) and in the release of a variety of mediators, such as TNF (Dockrell & Playfair 1983), reactive oxygen radicals (Clark et al 1987) and toxic nitrogen oxides (Green et al 1990 b). There is mounting evidence for the effectiveness of such a pathway contributing towards clearance of malaria pRBC. As has been shown here (Chapter 9) and elsewhere (Langhorne et al 1989 a & b), the rapid host response to P. c. chabaudi AS is dominated by those Ly-4$^+$ cells that produce IFN-γ and IL-2, i.e., T_H1 cells. Activated macrophages phagocytose effectively Plasmodium-infected RBC (Ockenhouse & Shear 1983, Shear 1984) and inflammatory mediators, such as the products of activated macrophages, are known to be toxic for murine malaria pRBC in vitro (Ockenhouse et al 1984, Rockett et al 1988) and in vivo (Clark & Hunt 1983, Clark et al 1984). The detection of TNF in the serum of patients with acute malaria (Scuderi et al 1986) implies that these processes could also explain the toxicity of such serum to P. falciparum in vitro (Tharavanij et al 1984), Butcher et al 1985). The importance of these effector mechanisms in protective anti-malarial immunity has been supported by the reports that P. c. adami infection can be controlled by Ab-independent means (Grun & Weidanz 1981, 1983), and that administration of inducers of inflammatory responses, BCG, TNS and TNF, causes parasiticidal effects in

vivo (Clark et al 1977, Taverne et al 1987).

T_H1 Ly-4^+ lymphocytes, through their synthesis and secretion of IFN-γ (Chapter 9), are thus effective initiators of the activation of the mononuclear phagocytic system, and hence, indirectly, of Ab-independent pathways of cell-mediated immunity involving non-specific toxic effector molecules. The levels of IFN-γ detectable in the circulation of P. c. chabaudi AS-infected mice are maximal just before peak parasitaemia (Slade & Langhorne 1989, Stevenson et al 1990), i.e., at a time soon after challenge when Ly-4-bearing cells of the T_H1 subset are thought to predominate (Langhorne 1989). Thus, there is a direct correlation between the kinetics of production of IFN-γ in vitro (Slade & Langhorne 1989, Stevenson et al 1990) and in vivo (Meding et al in press) by spleen cells from infected animals and the requirement in vivo for the endogenous molecule during the acute phase of infection. This concurs with the claim that resolution of the primary parasitaemia of P. c. adami is independent of specific Ab production (Grun & Weidanz 1981). Moreover, T_H1 lymphocytes are thought to be unable to stimulate resting B cells (Boom et al 1988). As the frequency of T_H2 cells, which can induce the activation of resting B cells, is very low in the first two weeks following P. c. chabaudi AS challenge (Langhorne et al 1989 a), this may explain the slow appearance of significant levels of malaria-specific Ab (Langhorne et al 1984). What little Ab is detectable during this time is of the IgG_{2a} isotype, the synthesis of which is positively regulated by IFN-γ (Snapper & Paul 1987). In contrast, the suppressed levels of IgG_1 Abs elaborated during the primary patency (Langhorne et al 1984, Langhorne et al 1985, Langhorne & Asofsky 1986, Falanga et al 1987) may be due to the T_H1-produced IFN-γ; this is a potent inhibitor of IgG_1 synthesis, both in vitro and in vivo, probably by its antagonistic effects on IL-4-dependent responses (Rabin et al 1986, Snapper & Paul 1987, Snapper et al 1988). In this regard, it is relevant to consider that the Ly-4^+ lines and clones derived from P. c. chabaudi AS-challenged mice during the early stages of infection produce both IFN-γ and IL-2 in vitro (Chapter 9) and give protection in vivo (Chapters 5-9). Likewise, Brake et al (1988) found that the only one of 10 Ly-4-bearing lymphocyte clones raised in vitro to P. c. adami to confer protection upon adoptive transfer had a lymphokine secretion pattern of the T_H1 subset.

The Ab-independent responses described do not explain the protective immune response to an erythrocytic infection with all species of malaria parasite. Some plasmodia, such as P. yoelii 17X cannot be controlled in mice lacking B cells and specific Ab (Weinbaum et al 1976 b), and others, e.g., P. yoelii 17XL and P. berghei, are fully virulent in

animals pretreated with non-specific inducers of inflammatory responses (Weidanz & Long 1988, Long 1988). Even for host immune responses shown to comprise a cell-mediated element to control pRBC multiplication, such as that to P. c. chabaudi AS involving IFN-γ, data show that parasite elimination and resolution of infection require the activation of alternative effector functions (Slade & Langhorne 1989, Stevenson et al 1990, Meding et al in press). Moreover, there is a pronounced species-specific element of protective immunity in rodent models of malaria. This is manifested as the attainment of effective protection normally only against challenge with homologous species, strains or variants (McLean et al 1982 a, Jarra & Brown 1985, Jarra et al 1986, Chapter 6). These findings are not reconciled readily with the Ly-4$^+$ cell-dependent pathway of protection characterised by specifically-induced but non-specific toxic effector molecules.

The other major pathway of Ly-4$^+$ cells in acquired resistance to malaria is the induction of the T_H2-driven Ab helper function. Activation of cells of the T_H1 Ly-4$^+$ subset triggers the release of IL-2 and the subsequent activation of macrophages to produce IL-1 (Mosmann & Coffman 1989 a & b). Both these lymphokines act as paracrine growth factors for T_H2-type Ly-4-bearing lymphocytes (Powers et al 1988), which then secrete their own growth factor, IL-4. After encounter with the same or different malaria Ags as those that stimulated a T_H1 response, T_H2 cells provide help in the form of IL-4 and IL-5 for B lymphocytes to produce specific Ab (Mosmann & Coffman 1987). Ig specific for blood stage malaria parasites has been shown to serve several functions. Circulating Abs against pRBC membrane-bound determinants may cause elimination of infected RBC through agglutination (Brown et al 1970 b) or complement-mediated lysis, or alternatively, function in a neutralising capacity by blocking reinvasion of merozoites into RBC (Butcher et al 1978). In addition, circulating Ig acts as an opsonin for the Fc receptor-mediated phagocytosis of pRBC (Ockenhouse & Shear 1984). In turn, this signals further mononuclear phagocytic cell activation and the subsequent release of toxic mediators into the surrounding localities of liver and spleen. Alternatively, B lymphocyte membrane-bound Ig specific for surface Ags of free merozoites or pRBC may function as a reservoir for parasite material, thereby facilitating the uptake, processing and presentation of plasmodial Ags by available APC to Ly-4$^+$ cells (Langhorne 1989). This would serve to recruit further effector T cells to the parasite-directed immune response.

The present study showed that Ly-4$^+$ populations specific for P. c. chabaudi AS obtained

from immune mice which had no detectable pRBC belonged to the T_H2 subset and provided considerable help for specific Ab synthesis (Chapter 9). This agreed with the findings of Langhorne et al (1989 a) that it was only after three weeks of primary infection with P. c. chabaudi AS, or during further challenges, that the predominant Ly-4^+ response was that of T_H2 cells. Furthermore, only with this increased frequency of T_H2 lymphocytes could Abs of the IgG isotype be detected (Langhorne et al 1984, Langhorne & Asofsky 1986). For host immunity to P. c. chabaudi AS, which appears to combine Ab-dependent and Ab-independent mechanisms, the sequential appearance of the two subsets of Ly-4-bearing cells may reflect the requirement for an Ab-independent effector function to control parasitaemia in the period prior to the relatively slow induction of malaria-specific IgG Abs. Although this is no doubt true, the temporally-regulated activation of different Ly-4^+ subsets may, in part, be a result of their varying requirements for developing into lymphokine-secreting cells. Powers et al (1988) and Swain et al (1988 b) reported independently that , in contrast to IL-2 secretion, readily detectable levels of IL-4 and IL-5 production are found only after T_H2 lymphocyte populations are primed and restimulated. Furthermore, both the expansion of T_H2 cells and their secretion of IL-4 in vitro upon repeated exposure to Ag is dependent on the presence of IL-2 (Powers et al 1988). From these findings it can be inferred that the delayed development of IL-4-producing helper T cells is by no means unique to the acquired immune response to malarial infection, and suggests that T_H2 Ly-4-bearing lymphocytes may play a particularly important role in immunity to Ags that persist or that are encountered repeatedly. Another implication of these data is that the activation of T_H1 cells, and the resultant elaboration of IL-2, is a necessary prerequisite for stimulation of cells of the T_H2 subset. It would thus appear that the protective immune response to P. c. chabaudi AS in vivo provides an ideal example of the way in which the diverse activities of T_H1 and T_H2 Ly-4^+ cells are coordinated to achieve the appropriate regulation of the immune system.

Several studies have indicated the important role played by Ab in the elimination of plasmodia in vivo. Successful passive transfer experiments have been undertaken using rodent models and immune serum or MAbs specific for merozoite or pRBC surface determinants (Freeman et al 1980, Yoshida et al 1980, reviewed by Deans & Cohen 1983). For a P. c. chabaudi AS infection, the ability to control parasitaemia coincides with the production of specific IgG but not IgM Abs, and the predominance of helper T lymphocytes of the T_H2 Ly-4^+ subset (Langhorne et al 1989 b). This is consistent with

the hypothesis of Grun & Weidanz (1981, 1983) that although acute parasitaemia can be cleared by Ag-independent mechanisms, B cells are necessary to eliminate peripheral blood infection. Taken together with other experiments illustrating the need for an intact and activated spleen for successful pRBC clearance (Grun et al 1985, Kumar et al 1989), Langhorne (1989) proposed that the available data would suggest that a major role for specific anti-malaria Ab is in the promotion of phagocytosis, and the focussing of asexual stage plasmodia to those areas of the spleen where they would be brought into close proximity to activated macrophages or other mediators of effector function. Certainly, this view explains the apparent anomaly between the exquisite specificity of the acquired immune response elicited by a given species or subspecies of Plasmodium (Jarra & Brown 1985) and the efficacy of a protective effector mechanism characterised only by non-specific inflammatory mediators released by IFN-γ-stimulated macrophages.

The relative contributions of the Ab-dependent and Ab-independent pathways operating during an in vivo immune response to P. c. chabaudi AS will be a reflection of the balance between the two subsets of Ly-4-bearing lymphocytes at any given time. It has been argued (Langhorne 1989) that soon after challenge when T_H1 cells predominate, a major mechanism for parasite destruction will be via oxidative and non-oxidative toxic mediators secreted by stimulated macrophages. As Ly-4^+ cells of the T_H2-type increase both in numbers and proportion in the later stages of infection, Ab-mediated mechanisms of protective immunity will begin to operate. As peripheral blood parasitaemia falls, Ab will become necessary not just to neutralise circulating pRBC, but also to trap residual parasite populations in the spleen and liver by means of opsonising or surface Ig, so enabling the clearance, to subpatent levels at least, of the challenge infection. The divergent reactivities of the different Ly-4^+ lines and clones raised to P. c. chabaudi AS (summarised in Table 10.1), both at a cellular level (Chapter 9) and in vivo (Chapter 7), is compatible with a change in the mechanism of protection with time following infection, and in showing that this switch is dependent upon the type of Ly-4^+ subset present at a particular point in time. This view of the roles of T_H1 and T_H2 cells in host immunity to malaria infection concurs with the current level of understanding of the contribution of these two subsets of the Ly-4-bearing lymphocyte to immune responses to a range of antigenic stimuli (Mosmann & Coffman 1989 b). It is postulated that during an immune response that involves T_H1 but not T_H2 cells, as specifically exemplified by the early stages of P. c. chabaudi AS blood-

borne infection, delayed-type hypersensitivity (DTH) should be induced strongly, but B cell activation would probably not occur due to the inhibitory effects of an excess of IFN-γ produced in an overwhelming T_H1 response without T_H2 involvement.

Furthermore, a strong T_H2 response accompanied by a moderate T_H1 response, as typified by the later phases of infection with P. c. chabaudi AS pRBC, would be expected to lead to considerable Ab production. This is because of a predicted secretion of IL-4, IL-5 and IL-6 production, with a possible bias away from IgG_{2a} towards IgG_1, induced by IL-4. DTH should be detectable but weakly so, since T_H2 lymphocytes appear to inhibit this T_H1 function. This classical model of T cell interaction would therefore appear to predict most of the immunological features of an ongoing infection of P. c. chabaudi AS, as described by Langhorne et al (1989 a) and herein (Chapters 7 & 9). Manipulation of this model in favour of a strong T_H2 response without T_H1 activation, or vice-versa, would provide a plausible explanation of the host immune responses to infections by two other rodent plasmodial species, P. yoelii 17X and P. c. adami, respectively. These appear to represent extremes in the repertoire of protective immunity to malaria, one predominantly humoral and the other predominantly cell-mediated.

Although this hypothetical model explains satisfactorily the behaviour of different Ly-4^+ cell types once they have become established, it does not explain the signal for the switch from a T_H1- to a T_H2-dominated response or the activation requirements of either cell type. Many data are compatible with the concept that T_H1 and T_H2 lymphocytes represent the mature stages of different developmental pathways of Ly-4-bearing cells (reviewed by Janeway et al 1988, Mosmann & Coffman 1989 a & b). It is also possible, however, that the sequential appearance of T_H1 and T_H2 cells specific for P. c. chabaudi AS pRBC represents the different developmental stages in the course of differentiation taken by every activated Ly-4^+ lymphocyte (Janeway et al 1988).

In either case, despite the fact that T_H 1 and T_H2 subsets may respond to the same antigenic stimulus (Mosmann & Coffman 1987, Liew, F.Y., personal communication), it is also possible that particular Ags trigger one or other type of response. Observations by Scott et al (1988, 1990) on the nature of the Ly-4^+ T cell response to leishmanial Ags suggest this may occur. It was shown that T_H1 cells responded to a particular cell fraction and could protect against infection, whereas cells of the T_H2-type responded to a different fraction and exacerbated the disease. Infection of mice with L. major provides the clearest example of the divergent consequences of a T_H1 or a T_H2 response to a

pathogen, these having profoundly different effects on the outcome of disease. Although the effect of stimulation is not as marked as that observed in leishmaniasis, it is important to determine the activation requirements in response to plasmodial Ags of different malaria-specific Ly-4^+ populations. This is not only to elucidate the mechanisms of protective immunity of anti-plasmodial T cell clones more fully, but to evaluate their worth with a view to subunit vaccine development. At present, the determining factor in the differential induction or selective activation of one or other of the two Ly-4-bearing subsets has not been established. The ratio of T_H1 to T_H2 cells produced in various immune responses appears to be controlled tightly, as assessed both by the types of clones generated in tissue culture, and by the characteristic responses elicited by particular Ags or modes of immunisation. This control is considered by some to be very probably genetically-restricted, though evidence for this is not forthcoming (Liew, F.Y., personal communication). Whether or not the responding Ly-4^+ lymphocytes are already precommitted to the T_H1 or T_H2 profiles, mechanisms must presumably exist for selectively activating, expanding or differentiating precursor T cells into one of either T_H1 or T_H2 subset. The APC is a probable candidate for the cell influencing the T_H1: T_H2 ratio, although the precise nature of its involvement is not clear. It has been proposed that T_H1 lymphocytes may be activated and expanded selectively by B cells, whereas the secretion of IL-1 by macrophages causes clonal expansion of T_H2 cells (Janeway et al 1988). This possibility notwithstanding, T_H1 cells are probably more effective at activating macrophages, whereas cells of the T_H2-type are undoubtedly the major B cell helper population (Alexander, J.H., personal communication), leading to the proposal that the most important interaction of T_H1 and T_H2 cells during an immune response are with macrophages and B cells, respectively (Boom et al 1988).

These two divergent views could represent a real dichotomy between the most advantageous T cell-APC interaction for T cell proliferation, as distinct from the optimal interaction for activation of the non-T cell partner. It is known for the response of Ly-4^+ clones to malarial Ags that proliferation and lymphokine gene expression are not necessarily interlinked (Troye-Blomberg & Perlmann 1988). Alternatively, each proposal may be partially correct, since B cells can enhance Ag presentation to proliferating T cells (probably T_H1) by direct presentation as well a by producing Ab that enhances the ability of non-B cells, notably macrophages, to also present Ag (Kurt-Jones et al 1988). Conversely, T_H2 activation can be mediated by macrophages, and by

B cells if IL-1 is added exogenously or supplied by bystander macrophages (Rock et al 1986). Thus, the distinction between IL-1-producing and non-producing APC may be important in vivo only when systemic or local IL-1 concentrations are limiting. A variety of quite different cell types can act as APC, each of which may also influence the T_H1: T_H2 ratio by providing different accessory signals to Ly-4-bearing lymphocytes, depending on the physical nature of the Ag encountered. For example, Janeway et al (1988) have suggested that T_H1 cells can be activated only by a high density of Ag on the surface of the corresponding APC. This model is consistent with the observation that many intracellular pathogens, the Ags of which should be presented at high density by infected cells, elicit strong T_H1 responses, including DTH, IgG_{2a} synthesis and IFN-γ production (e.g. Finkelman et al 1988 a, reviewed by Janeway et al 1988). With regard to the protocol followed here to raise Ly-4^+ populations specific for P. c. chabaudi AS pRBC, total spleen cell populations were used as a source of APC throughout the study. Such a heterogeneous pool of cells would contain B cells, macrophages, endothelial cells and dendritic cells, all of which are capable of performing an Ag-presenting function. It is unlikely, therefore, that the mixed APC population present in in vitro culture would bias the selection of T_H1 or T_H2 types towards one or other subset. It is more probable that under the immunological pressure represented by a malarial challenge, a biased response is induced in favour of an initially strong T_H1 cell reactivity. Induction of T_H1 function causes specific secretion of the lymphokines IL-2 and IFN-γ, the former acting as an autocrine growth factor and the latter promoting the activation of non-specific anti-malarial effector mechanisms. The release of toxic mediators into the localised environments of the lymphoid organs into which pRBC pass kills blood stage parasites so enabling adequate control of parasitaemia prior to the mounting of a humoral response. In a previously uninfected animal, the production of specific anti-P. c. chabaudi Ab, in particular IgG, would be characterised by the relatively slow kinetics of a primary Ab response. The reason for this is that the clonal expansion and differentiation necessary to supply a population of Ab-secreting plasma cells takes a finite time (up to two weeks). During this intervening period, there would be little point in T_H2 cells contributing to the Ly-4^+ response, since there would be negligible Ab function in which to assist.

Thus, the proportion of the Ly-4-bearing population comprising T_H2-type lymphocytes during the acute phase of a P. c. chabaudi AS infection is low (Langhorne et al 1989 a), and rises only later at a rate seemingly commensurate with its requirement to provide

help for specific Ig production. Although the mechanism by which the frequencies of T_H1 and T_H2 cells change has not been demonstrated unequivocally, in light of the lack of requirement of T_H2 cells till after resolution of primary parasitaemia, it may be that the delayed development of Ly-4^+ cells of the T_H2-type observed in other systems (Powers et al 1988, Swain et al 1988 a) may be significant. Not only do T_H2 lymphocytes require activation by IL-2 prior to secretion of their specific autocrine growth factor IL-4, but naive cells can expand only once stimulated with Ag. For malaria, one could envisage a situation in which T_H2 cells are not produced immediately upon challenge, not only because they are dependent upon a T_H1 cell product, IL-2, for initial activation, but also because, like the B cells with which they interact to produce an effective immune response, they require prior priming to plasmodial Ags. Speculation this may be, but if such a scenario were to occur in the host immune response to P. c. chabaudi AS, it would not only explain the observed frequencies of Ly-4^+ cells during a primary infection, but provide a possible mechanism by which the levels of T_H1 and T_H2 cells are regulated during infection with this particular malaria parasite. This temporal regulation of activation of the two subsets of Ly-4-bearing lymphocytes is not the only example of a biased immune response induced by infectious agents. A notable example of this phenomenon is the resistance acquired by rodent hosts to infestation with the intestinal helminth Nippostrongylus brasiliensis. Infestation induces substantially raised levels of IgE, usually accompanied by eosinophilia and mucosal tissue mast cell hyperplasia (Ogilvie & Jones 1969, Kelly & Ogilvie 1972). All three effects are T cell-dependent (Jarrett & Ferguson 1974) and can be explained by the preferential activation or selective amplification of T_H2 cells. It has recently been found that for spleen and lymph node cells from N. brasiliensis-infected mice, the levels of IL-2 and IFN-γ are suppressed below normal and that IL-4 and IL-5 levels are greatly elevated (Street & Mosmann in press), giving support to this interpretation of the regulation of the immune response to this metazoan parasite.

Although the T_H1 and T_H2 patterns of lymphokine expression are distinct when long term murine T cell clones are examined, as exemplified by the P. c. chabaudi AS-specific populations described herein (Chapter 9), it is not yet known if normal resting mouse lymphocytes are already committed to these patterns. Whilst the T_H1 and T_H2 profiles of lymphokine synthesis are found commonly among murine T cell clones of the Ly-4^+ phenotype, several lines of evidence suggest that precursor stages may exist. Examination of the lymphokine secretion profiles of Ly-4^+ clones 2-10 weeks after

establishment in culture revealed that early patterns were not recognisably T_H1 or T_H2 but sometimes changed to unambiguous T_H1 or T_H2 expression upon continued growth in vitro (Mosmann & Coffman 1989 a). Monoclonal populations from immunised mice gave rise to clear-cut T_H1- and T_H2-type cultures notably more rapidly than did those from naive mice. Further evidence for precursors of T_H1 and T_H2 cells comes from experiments in which mixed Ly-4^+ splenic cell populations, after polyclonal or Ag-specific stimulation, produce large amounts of IL-2 but little IFN-γ, IL-4 or IL-5 (Swain et al 1988 a & b, Street et al 1990). Furthermore, after prolonged incubation in vitro of Ly-4^+ clones stimulated with Con A, it was found that a high proportion of cultures secreted a broad spectrum of lymphokines, including IL-2, IL-3, IL-4, IFN-γ and TNF (Firestein et al 1989). These patterns of secretion cannot be explained simply by some mixture of T_H1 and T_H2 lymphocytes, suggesting that there are at least two precursor stages in the development of the T_H1 and T_H2 differentiation states, termed T_HP and T_H0, respectively. If this is so, T_H1 and T_H2 cells may be thought of as the mature stages of development of the Ly-4-bearing lymphocyte, and, as such, analogous to the plasma cells of the B cell lineage which produce different isotypes of Ig. It remains to be determined whether the precursors are committed to a particular development before expressing the mature lymphokine pattern, i.e., before exposure to Ag, or whether a common precursor (T_HO?) can be induced to differentiate into either T_H1 or T_H2 cells. Current models of Ly-4^+ lymphocyte differentiation (Mosmann & Coffman 1989 b, Street et al 1990) envisage the IL-2-secreting T_HP cell giving rise to a cell, T_HO, exhibiting an unrestricted pattern of lymphokine secretion. Additional unidentified differentiation states may exist, but it is agreed that T_H1 and T_H2 cells represent the final activated state of the Ly-4^+ lymphocyte lineage. It is thought that the T_HP and T_HO precursors may be equivalent to the non-recirculating and short-lived T1 cells that are rapidly lost in vivo after adult thymectomy (Araneo et al 1975), whereas the long-lived T2 population includes both mature T_H1 and T_H2 cell types (Mosmann & Coffman 1989 a & b). This matching of two different divisions of Ly-4^+ T lymphocytes shows that it is the stimulated, fully differentiated effector T cells, T_H1 and T_H2, which are relatively resistant to T cell depletion by surgical procedures. This highlights the importance of the additional irradiation or MAb treatments used to produce mice of immunologically compromised status prior to the adoptive transfer of various P. c. chabaudi AS-primed Ly-4^+ populations at homologous parasite challenge (Chapters 7 & 8). The mice used were naive and therefore not previously exposed to

malarial Ags, yet even in unprimed animals, the balance of T_H1 to T_H2 cells upon subsequent infection may be biased in either direction, depending on the background immunity that would be attained by animals exposed to a large number of infectious agents, as is the case in any mouse colony (Mosmann & Coffman 1989 a). Depletion of circulating Ly-4-bearing lymphocytes removes any possibility of an unbalanced residual immune potential affecting the course of infection of P. c. chabaudi AS in these animals first immunologically crippled by thymectomy, then repopulated with pRBC-specific preparations of either T_H1 or T_H2 Ly-4^+ cells.

In spite of the clear dichotomy of murine T_H1 and T_H2 clones, as exemplified by the monoclonal populations raised against P. c. chabaudi AS described herein, and the accumulating evidence for their involvement in a range of immune responses, including to malaria (Chapter 9), the segregation of human $CD4^+$ cells into two comparable subsets has not been established. Although T_H1 and T_H2 clones have been reported (Maggi et al 1988, Umetsu et al 1988), the majority of CD4-bearing monoclonal populations secrete a mixture of the two lymphokine patterns, i.e., IL-2, IL-4 and IFN-γ (Paliard et al 1988, Maggi et al 1988). These results can be reconciled with data from murine studies if it is assumed that in vitro human lymphocytes tend to persist as the earlier, mixed lymphokine secretion cell type, T_HO, whereas clones from mice tend to differentiate more rapidly into either T_H1 or T_H2 populations. What would therefore appear to be contradictory findings between human and murine systems can thus be explained in terms of the varying stability of different developmental stages of the $CD4^+$ or Ly-4^+ lineage in vitro. Until recently, there was no evidence for the existence of T_H1 and T_H2 cells in malarious humans. Preliminary results from patients convalescing from a recent bout of falciparum malaria showed that all the pRBC-specific $CD4^+$ clones recovered produced IL-2, and most IFN-γ, upon stimulation with homologous Ag (Ho & Webster 1990 b). These findings suggest that these CD4-bearing clones are compatible with the T_H1 characterisation. Troye-Blomberg et al (1990) have analysed human $CD4^+$ responses to defined immunodominant T cell epitopes of the P. falciparum Ag Pf155/RESA. Available evidence indicates the activation of cells from individual donors producing either IFN-γ or IL-4. Interestingly, the induction of IL-4, but not that of proliferation or IFN-γ synthesis, correlated well with elevated levels of Abs to the activating peptide in the serum of lymphocyte donors. These results indicate that IL-4-secreting $CD4^+$ cells, similar to cells of the T_H2-type in mice, are involved in the induction of Pf155/RESA-specific Abs in people who have acquired functional immunity

to falciparum malaria after prolonged natural exposure to the parasite. Thus, it may transpire that in the host immune response to P. falciparum, there may be a regulatory compartment of CD4-bearing lymphocytes that at rest is composed of cells with diverse lymphokine secretion profiles, similar to the T_H0 cell type found in mice (Firestein et al 1989). Upon stimulation, these cells may be driven to adopt lymphokine expression patterns analogous to those of the murine T_H1 or T_H2 Ly-4^+ subsets observed for clones isolated from mice infected with P. c. chabaudi AS (Chapter 9).

While CD8- or Ly-2-bearing lymphocytes appear to play a significant role in immunity to sporozoites (Schofield et al 1987 b, Weiss et al 1988), evidence supporting the notion that Ly-2^+ cells also play an essential part in resolving blood stage malaria is contradictory. Studies of P. yoelii in the mouse have suggested that Ly-2^+ cells can protect against challenge infection (Mogil et al 1987). This ability was correlated with an increase of expression of class I MHC molecules on infected reticulocytes and led to the postulation that lymphocytes of the Ly-2^+ phenotype may be directly cytotoxic for pRBC (Jayawardena et al 1982, 1983). However, when Vinetz et al (1990) attempted to repeat this finding in the same system, they were unsuccessful. Similarly, depletion experiments using anti-Ly-2 and anti-Ly-4 MAbs have shown little indication that Ly-2^+ lymphocytes have any significant role in resisting challenge with either P. c. chabaudi AS (Süss et al 1988, Langhorne et al 1989 b, Chapter 8) or P. vinckei (Kumar et al 1989), since, in their absence, parasitaemias are only marginally higher and the infection is cleared.

However, it is possible that under the conditions of these experiments, Ly-2-bearing cells could not be activated appropriately. This may provide an explanation for the recent report that C57BL/6 mice depleted of Ly-2^+ cells cannot eliminate P. c. chabaudi AS infection as efficiently as intact animals (Stevenson, M.M., personal communication, Podoba & Stevenson in press). In this regard, it is relevant to note that similarly contradictory findings with a totally unrelated infectious agent, Listeria monocytogenes, were clarified only when Ly-2^+ lymphocytes were activated prior to adoptive transfer (Bishop & Hinrichs 1987). There is the suggestion, therefore, that suitably activated Ly-2^+ cells elicited during malaria infections could perform a parasiticidal function. However, these data and those from adoptive transfer experiments in P. c. chabaudi AS infection (McDonald & Phillips 1978, Chapters 5-9) indicate that it is the Ly-4-bearing T cell subset that performs the major protective role against this particular parasite. If Ly-2^+ lymphocytes do play an ancillary part in acquired resistance to the

asexual erythrocytic stages of P. c. chabaudi AS, it is unlikely that they are directly cytotoxic, since this species of malaria preferentially invades mature RBC which present little or no class I MHC molecules. It is known, however, that activated Ly-2^+ cells produce a spectrum of lymphokines similar to that for T_H1 Ly-4-bearing cells, i.e. IL-2 and IFN-γ (Fong & Mosmann 1990). From this, Ly-2^+ lymphocytes responding to a multiplicity of plasmodial Ags could serve to amplify markedly the activity of Ly-4^+ cells in the triggering of non-specific effector mechanisms responsible for the elimination of blood stage parasites. Although there is no direct evidence for this forthcoming, Weidanz et al (1990) pointed to the observations of a disproportionate increase in Ly-2^+ lymphocytes in the spleens of P. berghei- and P. c. adami-infected mice at the time of maximal pRBC destruction (Gross & Frankenburg 1989, Weidanz et al 1990). As the number of Ly-2-bearing cells decreases after the acute phase of infection, this transient flux in the Ly-4^+: Ly-2^+ ratio may indeed be of significance. There is a corollary here with respect to P. falciparum in humans, where the development of $CD8^+$ clones from donors with acute infection (Troye-Blomberg et al 1983 a, 1984, Sinigaglia et al 1987) may be a reflection of the increased number of such T cells found in the blood of acutely ill patients as contrasted to clinically immune donors (Troye-Blomberg et al 1984). If Ly-2-bearing cells do interact with T_H1 Ly-4^+ cells to control parasitaemia prior to the induction of Ab-mediated mechanisms of protective immunity, as is proposed, it could be envisaged that the rise in levels of Ly-2^+ lymphocytes at this time is due to the activation of these cells. As clonal expansion of Ly-2^+ cells is an IL-2-driven process (Janeway et al 1988), it is highly probable that growth of this T cell population in response to malarial Ag is dependent upon IL-2 secreted by T_H1-type Ly-4-bearing lymphocytes, in much the same way as would appear to be the case for the initial activation of T_H2 cells (Powers et al 1988).

For P. falciparum malaria in man, with which murine models must stand comparison, cloning technology has been applied recently to human peripheral blood T cells taken from donors of varying immunological status (Sinigaglia & Pink 1985, Chizzolini & Perrin 1986). While both $CD4^+$ and $CD8^+$ clones have been isolated, the latter have been recoverable only from patients with, or recovering from, clinical malaria (Sinigaglia et al 1987). Not only would this appear to agree with the finding that CD8-bearing cells are activated in vivo in some patients with acute falciparum malaria (Troye-Blomberg et al 1984), it would suggest that for studies of murine malarias which showed that Ly-2^+ lymphocytes do not contribute significantly to the protective

immune response to blood stage parasites (Süss et al 1988, Langhorne et al 1989 b, Kumar et al 1989, Vinetz et al 1990, Chapter 8), this may have been due to a failure to fulfill the activation requirements of this T cell phenotype. All $CD8^+$ clones analysed to date have been shown to produce IFN-γ (Sinigaglia & Pink 1985, Pink et al 1987), a finding which would support the notion from murine studies that Ly-2-bearing lymphocytes may act by boosting the IFN-γ-mediated activation of macrophages by $Ly\text{-}4^+$ cells (Weidanz et al 1990). It would be reasonable to assume, therefore, that the principal contribution of cells bearing either the Ly-2 or CD8 marker towards a protective immune response to malaria is through the promotion of Ab-independent effector functions. However, Sinigaglia et al (1987) have reported that the $CD8^+$ clones which they isolated successfully from acutely ill donors displayed MHC restriction when activated with P. falciparum Ag in vitro but showed an MHC-unrestricted cytotoxicity towards unrelated tumour cells. Thus, CD8-bearing monoclonal populations raised against P. falciparum pRBC do exhibit a cytotoxic potential, albeit not in an Ag-specific manner. This behaviour is different to that thought by some workers to be shown by $Ly\text{-}2^+$ cells in the immune response to P. c. chabaudi AS (Süss et al 1988, Langhorne, J., personal communication). This distinction can be explained by the fact that P. falciparum has a predeliction for invading metabolically young RBC (Phillips 1983), whereas P. c. chabaudi AS tends to prefer mature RBC. Reticulocytes (very young RBC) express MHC class I determinants at their cell surface, so enabling P. falciparum-infected RBC to perform an Ag-presenting role to $CD8^+$ cells for which they themselves are the target of cytotoxic activity. Since the MHC class I receptor is lost from the RBC membrane as the cell matures, P. c. chabaudi AS pRBC would not normally express this molecule, hence making them inaccessible to direct $Ly\text{-}2^+$ lymphocyte cytotoxicity. It would therefore appear that this possible mechanism of immunity to asexual stage plasmodia is related to MHC class I expression on reticulocytes, and is thus restricted to acquired resistance to those species of malaria, such as P. falciparum, P. vivax, P. ovale, P. berghei and P. vinckei, which commonly invade reticulocytes. It would be thought that a mechanism of protective immunity of such limited application would not constitute the major effector function of $Ly\text{-}2^+$ or $CD8^+$ lymphocytes towards clearance of blood stage malaria parasites. However, the extent to which CD8-bearing cells play a role in immunity to malaria remains to be determined.

The relatively recent development of methods enabling long term culture and cloning of T cell lines with preserved antigenic specificity has been important to the understanding

of the specificity and functioning of T cells in immunity to malaria. Several investigators have reported the generation of human or rodent continuous T lymphocyte lines (Gross et al 1984, Chemtai et al 1984 a & b, Brake et al 1986) and clones (Sinigaglia & Pink 1985, Chizzolini & Perrin 1986, Sinigaglia et al 1987, Pink et al 1987, Good et al 1987 a, Simitsek et al 1987, Brake et al 1988). In the present study, P. c. chabaudi AS pRBC-reactive Ly-4-bearing T cells were maintained in vitro as lines in the presence of plasmodial Ag and APC and periodic boosting with IL-2 (Chapter 4). The stable lines were also cloned by limiting dilution to generate malaria-specific Ly-4^+ clones that were then maintained in the same fashion (Chapter 4). All these populations have been characterised in vitro for their proliferative responsiveness, lymphokine secretion and helper activity (Chapters 4 & 9), studies which have provided ample indirect evidence for the participation of the Ly-4^+ subset of T lymphocytes in protective immunity to P. c. chabaudi AS. Similar analyses have been performed on human T cells responding specifically to asexual blood stages (Sinigaglia & Pink 1985, Chizzolini & Perrin 1986, Sinigaglia et al 1987, Pink et al 1987, Simitsek et al 1987) or gametocytes (Good et al 1987 a) of P. falciparum found to be present in the peripheral blood of individuals with or without previous exposure to malaria. An advantage of studying murine models of malaria rather than P. falciparum directly is that it allows adoptive transfer experiments to be performed, which, for ethical and practical considerations, may not be permitted in humans. The undertaking of the adoptive transfers described herein demonstrated unequivocally the protective activity in vivo of selected T_H1 and T_H2 populations of P. c. chabaudi AS-specific Ly-4^+ lines (Chapter 5-8) and clones (Chapter 9). Inoculation of uncloned preparations of Ly-4-bearing cells into challenged mice showed that such lines were able to confer enhanced immunity to naive mice (Chapters 5 & 6) and selectively lymphocyte-depleted recipients (Chapters 7 & 8) against P. c. chabaudi AS infection. Similarly, Brake et al (1986) were able to protect both nude and sublethally irradiated mice against homologous challenge by the adoptive transfer of P. c. adami-reactive Ly-4^+ lines. Upon cloning of these populations, it was found that only one of 10 clones could adoptively transfer protection (Brake et al 1988), this clone belonging to the T_H1 subset of Ly-4^+ cells. In contrast, the present study showed that each of the four monoclonal populations of either T_H1- or T_H2-type were capable of conferring protection upon challenge of recipient animals with the same P. c. chabaudi AS isolate to which they had been raised (Chapter 9). For both these studies, the plasmodial Ags responsible for stimulating

protective T cell immunity have yet to be identified. However, Good & Miller (1989) have argued that if non-specific effector mechanisms of acquired resistance can protect from blood stage malaria , one would think that most Ags capable of stimulating T cells would be suitable for vaccine design. Unfortunately, the finding of Brake et al (1988) using a P. c. adami model in which T_H1 Ly-4-bearing lymphocytes play a primary role in recovery from blood-borne infection independent of Ab production (Weidanz & Long 1988, Long 1988), suggests that this may not be true. The successful transfer of protection with all eight P. c. chabaudi AS-specific clones, however, would offer hope that it may not prove too difficult to identify and isolate malaria-reactive T cell epitopes. Analysis of these clones in vitro has revealed that whilst some are of the T_H1 subset and thus mediate protection by activating non-specific effector functions, others belong to the T_H2 subset noted for providing help for specific Ab production. This finding is of particular importance for it shows that Ly-4^+ lymphocytes of either type may confer protection against P. c. chabaudi AS infection, and thus indicates that T_H2 as well as T_H1 epitopes may be worth consideration for inclusion in a subunit vaccine candidate. As the immunogens included in such a construct should contain B cell epitopes as well as T cell-reactive antigenic sites, it would be hoped that either a single epitope would be recognised by both B and T_H2 cells, or that once stimulated by different antigenic fragments, T_H2 cells would amplify the induced Ab response. This possibility serves to illustrate the central role of the Ly-4^+ or $CD4^+$ T cell system for the induction and maintenance of protective immunity to malaria and should be taken into account in vaccine development.

With regard to the construction of subunit vaccines, the work presented here and by Brake et al (1988) has given credibility to this approach to vaccine design. In both studies, it was shown that a single Ly-4^+ lymphocyte clone could transfer protection adoptively against challenge with a murine malaria parasite, either P. c. adami (Brake et al 1988) or P. c. chabaudi AS (Chapter 9). The inference that can be made from this is that the recognition by a T cell clone of the Ly-4^+ phenotype of a single protective epitope expressed on the pRBC surface is sufficient to cause the induction of protective immunity against blood stage malaria infection. Due to the problem of MHC restriction of the immune response, for practical purposes, the identification of any one T cell epitope will be unlikely to be sufficient for vaccine development. Therefore, to be fully effective, an anti-malarial vaccine should contain several T cell antigenic determinants, at least one of which would be recognised by any given individual receiving the vaccine.

The reason for the varying success of generating clones engendering a protective activity in vivo is not clear, but may not necessarily be due to the recognition of non-protective pRBC determinants by most of the P. c. adami-specific Ly-4$^+$ populations (Brake et al 1988). Not only may differences in Ag processing between the situation in vitro and in vivo, or variation in lymphokine secretion profiles, provide plausible explanations of the differences in protection conferred by the various malaria-specific clones, so too may potentially critical differences in homing ability between the clones. Many in vivo studies have been hampered by the aberrant trafficking patterns of such cultured cells, a phenomenon which may be due, in part, to the loss of homing receptors from the lymphocyte surface membrane (Gallatin et al 1986). These receptors are thought to be needed by lymphocytes in order to recirculate through the lymphatic system, a capacity which may or may not be required for their protective function. It may be, however, that there are critical antigenic determinants, the recognition of which is required to induce a Ly-4$^+$ lymphocyte response. If this is the case, it would appear that all the P. c. chabaudi AS-specific clones recognised the same or different critical Ags (Chapter 9), whilst only a single clone raised against P. c. adami did likewise (Brake et al 1988). Although this may suggest that there is a limited pool of suitable epitopes, it is still likely that there will be many different Ags that could successfully stimulate protective Ly-4- or CD4-bearing T cells.

Further characterisation of the protective Ly-4$^+$ lymphocyte clones described herein and by Brake et al (1988) is required to attain a better understanding of the host immune responses involved in acquired resistance to malaria. In particular, as T cell clones such as these provide a unique tool for the identification and characterisation of protective T cell-reactive epitopes, identification of the Ags to which these clones react is a major priority. The method which is being employed either currently or in the future to identify the Ags reactive to P. c. chabaudi AS and P. c. adami Ly-4$^+$ clones is the screening of λ-gt 11 expression libraries with specific T cells (Phillips, R.S., personal communication; Long, C.A., personal communication)(Mustafa et al 1986). An alternative approach that could be used is that of the T cell Western blot, where a Ly-4-bearing clone is tested for reactivity to a panel of pRBC Ags separated on the basis of mass or charge (Lamb & Young 1987, Abou-Zeid et al 1987). After identifying the protein that stimulates a given clone, it can then be used to immunise mice and to screen an expression library with the immune sera collected. When such Ags are identified in murine malarias, it may be possible to identify the homologous antigenic determinants of

P. falciparum that could similarly induce a protective $CD4^+$ lymphocyte response in humans.

A recent advance in vaccine technology has been the use of a live vector as a carrier for an anti-pRBC vaccine (Kumar & Miller 1990). The carrier used was a strain of Salmonella typhimurium, SL3235, the virulence of which attenuates in vivo, but persists long enough in mice to induce immunity to a virulent strain of the same bacterium (Hoiseth & Stocker 1981, Killar & Eisenstein 1985). These properties lend this strain of S. typhimurium to being a vehicle to deliver recombinant Ags. In the experiments of Kumar & Miller (1990), despite the fact that immunity to the normally lethal P. vinckei could be achieved with live pRBC challenge and drug cure, immunisation of BALB/c mice with killed parasites with various adjuvants failed to protect recipients from live challenge. In contrast, immunisation with parasite Ags from killed P. vinckei pRBC in combination with attenuated S. typhimurium SL 3235 induced a high degree of protective immunity. These results, and previous ones by this group (Kumar et al 1989), suggest that induction of immunity against P. vinckei and other virulent malarias using this immunisation procedure requires both the induction of $Ly\text{-}4^+$ T cells and certain splenic modifications of either parasite or carrier origin that occur during infection. Kumar & Miller (1990) proposed that successful vaccination against asexual stage malaria parasites may require a combination of plasmodial Ags chosen to stimulate a protective $Ly\text{-}4^+$ response, and an agent which could have a similar modifying effect on the host spleen as does malarial infection. Although studies to date have used P. vinckei, the ultimate aim of such research is to engineer a vaccine comprising an attenuated strain of Salmonella expressing P. falciparum pRBC genes that may induce the protective immunity and splenic changes necessary to provide protection for vaccinated humans.

Table 10.1 Functional heterogeneity of the *Plasmodium chabaudi chabaudi* AS-specific Ly-4$^+$ T cell response

Cell line	*in vivo* priming	*in vivo* protection	Lymphokine secretion	B cell help
775	d.16, 1° inf.	B cell independent	IL-2 IFN-γ	-
779	d.20, 1° inf.	B cell independent	IL-2 IFN-γ	-
737	post 2° inf.	B cell dependent	IL-4	+
723	post 3° inf.	B cell dependent	IL-4	+

APPENDICES

APPENDIX A

Isotonic Buffers

PBS

Stock solution

60.0 g $Na_2HPO_4 . 12H_2O$

13.6 g $NaH_2PO_4 . 2H_2O$

8.5 g NaCl

Made up to 1 l with distilled water.

0.9% saline

9.0 g NaCl

Made up to 1 l with distilled water

Buffer

40 ml stock

Made up to 1 l with 0.9% saline and adjusted to pH 7.2

Giemsa's stain

3.0 g Na_2HPO_4

0.6 g KH_2PO_4

Made up to 1 l with distilled water and adjusted to pH 6.8

APPENDIX B

Media

RPMI

Stock

10.39 g RPMI 1640 powdered medium (with L-glutamine) (Gibco)

5.94 g N_2-hydroxyethylpiperazine-N'-2 ethane sulphonic acid (Hepes) (Sigma)

Made up to 960 ml with distilled water, filter-sterilised (Millipore filter size 0.22 μm) and aliquoted into 100 ml bottles.

Incomplete RPMI

100 ml RPMI

4.2 ml 5% w/v $NaHCO_3$ (filter-sterilised)

2.5 mg gentamycin sulphate (Sigma)

Complete RPMI

90 ml Incomplete RPMI

10 ml FCS (Gibco), unless otherwise stated

Sterile FCS was heat-inactivated at 56°C for 30 min and stored at -70 °C until use.

All supplements to RPMI stock solution were added immediately before use.

Minimal Essential Medium (MEM)

Stock

9.70 g MEM powdered medium (with Earle's salts, non-essential amino acids & L-glutamine) (Gibco)

5.94 g Hepes (Sigma)

2.20 g $NaHCO_3$

Made up to 1 l with distilled water (pH 7.2), filter-sterilised (Millipore filter size 0.22 μm) and aliquoted into 100 ml bottles.

Incomplete MEM

100 ml MEM

1.0 mg (100,000 i.u.) penicillin-G (Sigma)

2.0 mg (200,000 i.u.) streptomycin sulphate (Sigma)

Complete MEM

90 ml Incomplete MEM

10 ml FCS (Gibco)

Iscove's Modified Dulbecco's Medium (IMDM)

Stock

9.60 g IMDM powdered medium (with L-glutamine) (Gibco)

5.94 g Hepes (Sigma)

Made up to 1 l with distilled water (pH 7.2), filter-sterilised (Millipore filter size 0.22 μm) and aliquoted into 90 ml bottles.

Supplement of 1% FCS (0.9 ml) (Gibco) before use.

APPENDIX C

Miscellaneous Reagents

Sorbitol-glycerol (for cryopreservation of parasites)

380 g glycerol (Sigma)

39 g sorbitol (BDH)

6.3 g NaCl

White cell diluting fluid

490 ml distilled water

10 ml glacial acetic acid (BDH)

Few drops crystal violet stain (Gurr, BDH)

Tris-ammonium chloride (for RBC lysis)

0.17 M Tris(hydroxymethyl)aminomethane (20.60 g/l)

0.16 M ammonium chloride (8.30 g/l) (BDH)

10 ml Tris added to 90 ml ammonium chloride and the 0.83% stock solution adjusted to pH 7.4.

APPENDIX D

Density Gradient Centrifugation Media

Ficoll Hypaque

Stock

33.9% w/v Isopaque (Triosil 75)

20.0 ml Isopaque (Nycomed UK Ltd.)

23.9 ml sterile distilled water

9.0% w/v Ficoll

9.0 g Ficoll 400 (Pharmacia)

Made up to 100 ml with distilled water and autoclaved

Gradient

10 ml 33.9% Isopaque mixed with 21 ml 9% Ficoll, filter-sterilised and stored at 4 °C.

Percoll

10 x PBS

40 ml PBS stock (see Appendix A)

48 ml 18% saline (18.0 g NaCl made up to 100 ml with distilled water)

12 ml distilled water

90% stock

90 ml stock isotonic Percoll (Pharmacia)

10 ml 10 x PBS

Sterilised by autoclaving and stored at 4 °C.

Gradients

Dilutions	Volumes (ml)	
% Percoll	90% stock	Incomplete RPMI 1640 (see Appendix B)
30	3	6
40	4	5
50	5	4

APPENDIX E

Recombinant Mouse Interleukin-2

Quantity

10,000 BRMP units/vial

One unit of IL-2 bioactivity is that amount of IL-2 which is required to support half-maximal [^{3}H] incorporation by CTLL-2 cells (the BRMP unit system is an interim standard for IL-2 bioactivity established by the NIH).

Formulation

IL-2 was lyophilised from solution in sterile PBS containing 0.1% highly purified BSA

Reconstitution

Each vial was reconstituted in 1 ml incomplete RPMI 1640 medium. After reconstitution, the product was stable for 30 d at -70 °C.

APPENDIX F

Pierce BCA Standard Protein Assay

Stock

2 mg/ml BSA standard

Dilutions

Protein (µg/ml)	Volume (ml)	
	BSA standard	PBS
1500	0.375	0.125
1000	0.25	0.25
500	0.125	0.375
200	0.05	0.45

APPENDIX G

Anaesthetic Agents

Hypnorm (Janssen Animal Health)

10.0 mg/ml fluanisone (a neuroleptic of the butyrophenone group)

0.315 mg/ml fentanyl citrate (an analgesic of the morphine type)

Diluted 1: 10 with sterile distilled water for use, and 0.1 ml/30 g mouse bodyweight administered i.p.

Valium (Roche)

5.0 mg/ml diazepam

Diluted 1: 5 with sterile distilled water for use, and 0.2 ml/30 g mouse bodyweight administered i.p.

The combined use of these drugs gave satisfactory pre-operative sedation and muscle relaxation for surgical anaesthetic purposes.

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