T cells and immunity to the asexual blood stages of *Plasmodium chabaudi chabaudi* CB

by

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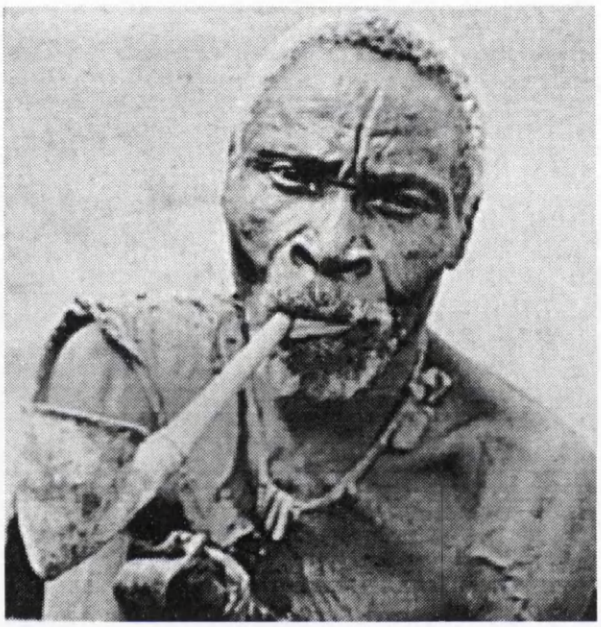
FOR MY PARENTS

TO WHOM I OWE EVERYTHING



SCIENCE MOVES BUT SLOWLY SLOWLY, CREEPING ON FROM POINT TO POINT

ALFRED, LORD TENNYSON

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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.

Kathleen E. Mathers
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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
ATS	Anti-thymocyte serum
B cell	B lymphocyte
BCG	Bacille Calmette Guèrin
BP	Blood passage
BSA	Bovine serum albumin
CD	Cluster designation
Ci	Curie(s)
CM	Cerebral Malaria
CMI	Cell-mediated immunity
Con A	Concanavilin A
cpm	Counts (of radioactivity) per minute
CSP	Circumsporozoite protein
d	day
DDT	Dichloro diphenyl trichloroethane
DNA	Deoxyribonucleic acid
DPI	Days post infection
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen-binding fragment of immunoglobulin
Fc	Crystalline fragment of immunoglobulin
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Acceleration in the earth's gravitational field
g	Gramme(s)
G	Gauge
Gy	Gray(s)
^{3}H-T	Tritiated thymidine
H-2	Mouse major histocompatibility complex
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
HLA	Human histocompatibility leucocyte antigens
hr(s)	Hour(s)
ICAM-1	Intercellular adhesion molecule-1

IFA	Indirect fluorescent antibody
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
Il	Interleukin
i.p.	Intraperitoneally
i.u.	International unit(s)
i.v.	Intravenously
kDa	Kilodalton(s)
l	Litre(s)
L-NMMA	L-N^G-monomethyl arginine
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mg	Milligramme(s)
µg	Microgramme(s)
min	Minute(s)
ml	Millilitre(s)
µl	Microlitre(s)
mm	Millimetre(s)
µm	Micrometre(s)
µM	Micromolar
MSP	Merozoite surface protein
MW	Molecular weight
ng	Nanogramme(s)
NK cell	Natural Killer cell
nm	Nanometre(s)
nmol	Nanomole
NMS	Normal mouse serum
NO	Nitric oxide
NO_2^-	Nitrite anion
NO_3^-	Nitrate anion
nRBC	Normal/uninfected red blood cell
NRS	Normal rat serum
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
PABA	Para-aminobenzoic acid

PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocytes
Pf EMP	*P. falciparum* erythrocyte membrane protein
PHA	Phytohaemagglutinin
PMN	Peripheral blood mononuclear cells
pRBC	Parasitised/infected red blood cell
RBC	Red blood cell
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RT	Room temperature
s	Second(s)
S.D.	Standard deviation
SICA	Schizont-infected cell agglutination test
S/N	Supernatant
T cell	T lymphocyte
T_C	T cytotoxic lymphocyte
T_H	T helper lymphocyte
TNF	Tumour necrosis factor
TNS	Tumour necrosis serum
T_S	T suppressor lymphocyte
u.v.	Ultraviolet
v/v	Volume per volume
WBC	White blood cell
WEP	Wellcome Experimental Parasitology (prefix used to describe numbered batches of stabilate)
WHO	World Health Organisation
w/v	Weight per volume
>	Greater than
<	Less than
%	Percentage point(s)

SUMMARY

It is now clear that the major protective immune mechanisms to the erythrocytic stages of *Plasmodium chabaudi chabaudi* AS require the presence of $CD4^+$ T lymphocytes. Both T_H1 and T_H2 malaria-specific, cloned T cell lines can protect immunocompromised mice upon adoptive transfer. Infection with *P. c. adami*, however, another subspecies of *P. chabaudi*, is thought to be resolved solely by antibody-independent mechanisms. In different host/parasite combinations, therefore, CD4-bearing lymphocytes mediate cellular protective functions and/or act as helper cells in protective antibody production. In addition, one report suggests that infection with *P. c. chabaudi* CB is controlled in an antibody-independent manner. Experiments were performed, therefore, to define further the role of T cells in immunity to rodent malaria using the CB strain of *P. c. chabaudi* in NIH mice. Although this strain is a virulent but usually resolving mouse malaria, it is often necessary to treat mice at the peak parasitaemia with subcurative doses of chloroquine. Such treatment, therefore, also allowed the effect of chemotherapy on the development of an immune response to be investigated.

Using the *P. c. chabaudi* CB/NIH mouse system, selective depletion of T cell subsets by treatment with rat monoclonal antibodies to murine CD4 and CD8 determinants revealed that the CD4-bearing population of lymphocytes is critically required for protection against erythrocytic infection. Mice lacking a $CD4^+$ cell compartment suffered very high primary parasitaemias, that recurred despite treatment of the mice with chloroquine on days 8 and 9 post infection. For 30 days post infection (DPI) parasitaemias were never reduced below 3% and mice developed complications of infection such as ascites and subcutaneous oedema. In contrast, the parasitaemias of mice after removal of CD8-bearing lymphocytes did not deviate from the parasitaemias of immunocompetent control animals, although in mice lacking both $CD4^+$ and $CD8^+$ T cells the parasitaemias were higher than in mice depleted of $CD4^+$ cells alone. This experiment demonstrated the crucial role that is played by *P. c. chabaudi* CB-specific $CD4^+$ lymphocytes in the protective immune response to the asexual erythrocytic stages of this malaria parasite.

Adoptive transfer experiments *in vivo* were carried out to determine if protection could be transferred with specific populations of enriched splenic lymphocytes, alone or in combination. Sublethally irradiated mice that received splenic cells taken from donor mice after clearance of a malarial infection (75 DPI) demonstrated a reduced level and quickened remission of primary parasitaemia and more rapid clearance of pRBCs from the bloodstream without recrudescence of infection, compared to control mice receiving unprimed splenic lymphocytes.

Transfers carried out 21 DPI when the primary patent parasitaemia was subpatent, resulted in the same pattern of protection by T cells and a population of unfractionated cells, although the transfer of B cells did not prevent a recrudescence from occurring. Splenic T cells, therefore, from donor mice 21 DPI produced a sterilising immunity which appeared to transcend antigenic variation. When donor mice were treated with a subcurative dose of chloroquine 10 DPI the ability of 75 DPI spleen cells to confer a similar level of protection was not affected. When T, B and unfractionated cells were taken from chloroquine treated donor mice at the time of subpatency (14 DPI) and adoptively transferred into irradiated recipients, they were able to confer a level of protection which was demonstrated by a reduced peak parasitaemia, quickened pRBC remission and a prolonged subpatent period compared to the transfer of naive spleen cells, but in each case a recrudescence did occur.

In all the transfers carried out, 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. Examination of serum antibody titres for recipients of T, B and unfractionated (T + B) spleen cells from donor mice 75 DPI, however, did not support this view, as titres did not differ significantly between recipients of enriched cells or a mixed splenic population.

Titration of the number of 21 DPI T lymphocytes necessary to produce a sterilising immunity showed that decreasing the number of cells below 2×10^7 cells/ml resulted in effective transfer of immunity upon adoptive transfer but a recrudescence occurred, the height and timing of which changed as the number of T cells decreased. Mice receiving 2×10^7 21 DPI T cells produced IFN-γ and NO, but not at levels significantly different from recipients of a smaller number of T cells or naive T cells, although an earlier and prolonged secretion of IFN-γ was detected. T cell recipients produced higher levels of malaria-specific antibody compared to naive T cell recipients but as the number of transferred T cells was decreased the level and pattern of antibody production was not affected.

The passive transfer of serum collected during subpatency in naturally recovering or chloroquine treated animals resulted in a delayed onset in patency in recipient mice. Recrudescent parasites collected from the former were equally susceptible to the antiparasite effects of subpatent serum as the infecting population, but recrudescent parasites collected from chloroquine treated mice were insensitive to 14 DPI subpatent serum. Whereas recrudescent parasites from naturally recovering mice did not differ with respect to the course of infection in naive mice compared to the infecting parasite population, recrudescences from chloroquine treated mice

showed a course of infection with a delayed onset and lower recrudescence. This indicated that following subcurative chloroquine treatment antigenic variation was demonstrated by serum sensitivity but this could not be demonstrated in untreated animals. Analysis of the recrudescent populations by live IFAT showed that all recrudescent populations from chloroquine treated mice were antigenic variants of the infecting parasite population, while only two out of four of the recrudescences from naturally recovering mice were shown to be antigenic variants.

The production of nitric oxide (NO) was examined during a *P. c. chabaudi* CB infection by measurement of the levels of nitrate in the serum of infected mice. A large peak of NO production coincident with the peak parasitaemia was produced in infected mice with background levels remaining throughout the rest of infection. In both chloroquine treated and naturally recovering mice there was no subsequent elevation of NO associated with the recrudescences. These results suggest that at the peak of infection with *P. c. chabaudi* CB non-specific immune mechanisms are operative to control a rapidly escalating acute parasitaemia. Chloroquine treatment during the ascending primary parasitaemia, one or two days prior to the peak parasitaemia, prevented any elevation in NO levels. Chloroquine treatment, *in vivo* at these times, did not appear to inhibit the pattern of IFN-γ production. *In vitro*, chloroquine inhibited the production of NO from J774 macrophage-like cells in a dose-dependent manner.

To dissect further the protective immune response in both naturally recovering and chloroquine treated mice, splenic T lymphocytes were taken from *P. chabaudi* CB strain-infected NIH mice at different time points during an infection. Each of these preparations was established as a cell line *in vitro* using pRBCs as the source of antigenic stimulation and irradiated spleen cells as antigen presenting cells (APCs). This was somewhat problematic and only one cell line was successfully raised. This line, prepared from mice at 60 DPI, was phenotypically characterised by surface immunofluorescence as $CD4^+$ i.e. belonging to the helper/inducer T cell subset. This line proliferated specifically *in vitro* in response to *P. c. chabaudi* CB antigen processed and presented by syngeneic APCs, although it also proliferated in response to nRBCs. *In vivo*, adoptive transfer of this $CD4^+$ line was effective in conferring protective immunity to immunocompromised mice. This was demonstrable, compared to control mice that were given naive splenic T cells, as both a reduced level and shortened duration of primary parasitaemia, and as a quicker parasite remission.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Historical Outline

The history of malaria is rooted in antiquity. The writings of the Sumerians and ancient Chinese, Homer, Aristotle, Plato and Shakespeare all described the intermittent fevers characteristic of malaria. In the 5th century B.C., Hippocrates, the great Greek physician, recognised the distinct features of the disease and linked it to the proximity of stagnant waters. Rome, ancient and modern, was one of the most malarious places in the world until the Pontine marshes were drained in the 1930's. St. Augustine, Dante, the Holy Roman Emperor Charles V, as well as Pope Sixtus V and his successor Urban VII, are all believed to have died from malaria. The Italians called the disease "Roman fever" and *mal'aria* - literally meaning bad air - on the theory that its cause was related to the foul air common near marshy areas.

No progress was made in the aetiology of malaria until 1847, when Meckel observed black pigment in the blood and spleen of a patient who died of the disease (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 Major Louis Alphonse Laveran, a French army physician. 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. This finding was followed by the discovery in 1898 by Dr Ronald Ross that the parasite was transmitted through the bite of a mosquito. The same year the renowned Italian zoologist Giovanni Battista Grassi found that malaria was transmitted only by the "dapple-winged" female *Anopheles* mosquito (reviewed by Garnham, 1966; Harrison, 1978). These discoveries marked the beginning of one of the most prolonged research efforts in the history of modern science.

Significant control of the disease became possible in the 1930's and 1940's with the development of new synthetic antimalarial drugs and modern-day insecticides. Consequently, 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). However, in the last 20 years there has been an alarming resurgence of malaria as mosquito vectors became resistant to DDT and malaria parasites developed resistance to prophylactic and therapeutic drugs. There has been an estimated 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, almost half the world's population is at risk from malaria, the disease remaining endemic in 103 countries (WHO, 1993). Since the areas involved are so vast, accurate figures regarding the levels of morbidity and mortality are subject to considerable errors. However, estimates suggest that 270 million people are infected with malaria each year with over 2.5 million deaths,

mostly of children and infants (Stürchler, 1989).

1.2 Classification

Malaria parasites are protozoans and members of the family Plasmodiidae within the order Coccidia, sub-order Haemosporina, which comprises various parasites found in the blood of reptiles, birds and mammals. The zoological family of Plasmodiidae includes the parasites which undergo two types of multiplication by asexual division (schizogony) in the vertebrate host and a single sexual multiplication (sporogony) in the mosquito host. The genus *Plasmodium* has been defined on the basis of one type of the asexual multiplication by division occurring in the parenchymal cells of the liver of the vertebrate host (pre-erythrocytic schizogony); the other characteristic of this genus is that the mosquito hosts are various species of *Anopheles.*

There are nearly 120 species of plasmodia, including at least 22 species found in primate hosts and 19 in rodents, bats or other mammals. About 70 other plasmodial species have been described in birds and reptiles. 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.

1.3 Life cycle

Infection with *Plasmodium* is initiated by the bite of an infected female anopheline mosquito. Motile sporozoites contained in the insect's saliva are inoculated into the bloodstream 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 mins (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). Within the hepatocyte, the parasites develop into pre-erythrocytic schizonts (Garnham *et al.*, 1966) by asexual multiplication. When mature, each schizont and its host cell rupture, releasing around 30,000 haploid merozoites in the case of *P. falciparum.* Mammalian malaria parasites are thought to undergo only one cycle of pre-erythrocytic multiplication, this taking between 5.5 and 15 days for human malarias, depending on the species. Tissue schizogony directly follows sporozoite invasion in *P. falciparum* and *P. malariae* infections, while 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 a cycle of asexual parasite multiplication. The merozoite attaches to a 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. falciparum* and *P. vivax*, these receptors are known to be associated with glycophorin (Miller *et al.*, 1977; Perkins, 1981) and the Duffy blood group Ags (Miller *et al.*, 1975b), 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.*, 1975a). 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 parasite species. In all cases, as many merozoites are formed as there were nuclei, each new merozoite regaining its surface coat (Bannister *et al.*, 1975; 1977). After vesiculation and swelling of the pRBC membrane, the erythrocytic schizont bursts, releasing the merozoites which invade further RBCs (Dvorak *et al.*, 1975). The length of the asexual erythrocytic cycle depends on the species of malaria parasite: one cycle takes 48 hrs in *P. falciparum*, *P. vivax* and *P. ovale*, and 72 hrs in *P. malariae*. In the rodent malaria, *P. chabaudi chabaudi*, one cycle takes 24 hrs. 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).

Rather than undergoing asexual replication, a proportion of merozoites, upon reinvasion, differentiate without cell division, into the sexual stages of 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 hrs 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 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 hrs, crosses the gut wall, usually passing intracellularly through the midgut 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, 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 it ruptures, with up to 10,000 sporozoites 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 doing so becoming infective to the vertebrate host (Vanderberg, 1975) and remaining viable indefinitely until discharge during one of frequent blood meals. Figure 1.3.1 illustrates schematically the life cycle of a mammalian malaria parasite.

P. falciparum infections tend to last no longer than 12 months in the human host, although infections of up to 4 years have been reported from Mauritius (Verdrager, 1964). Under natural conditions, the life span of *P. falciparum* must not be considered separately from immune factors, since observations of Bekessey *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. 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

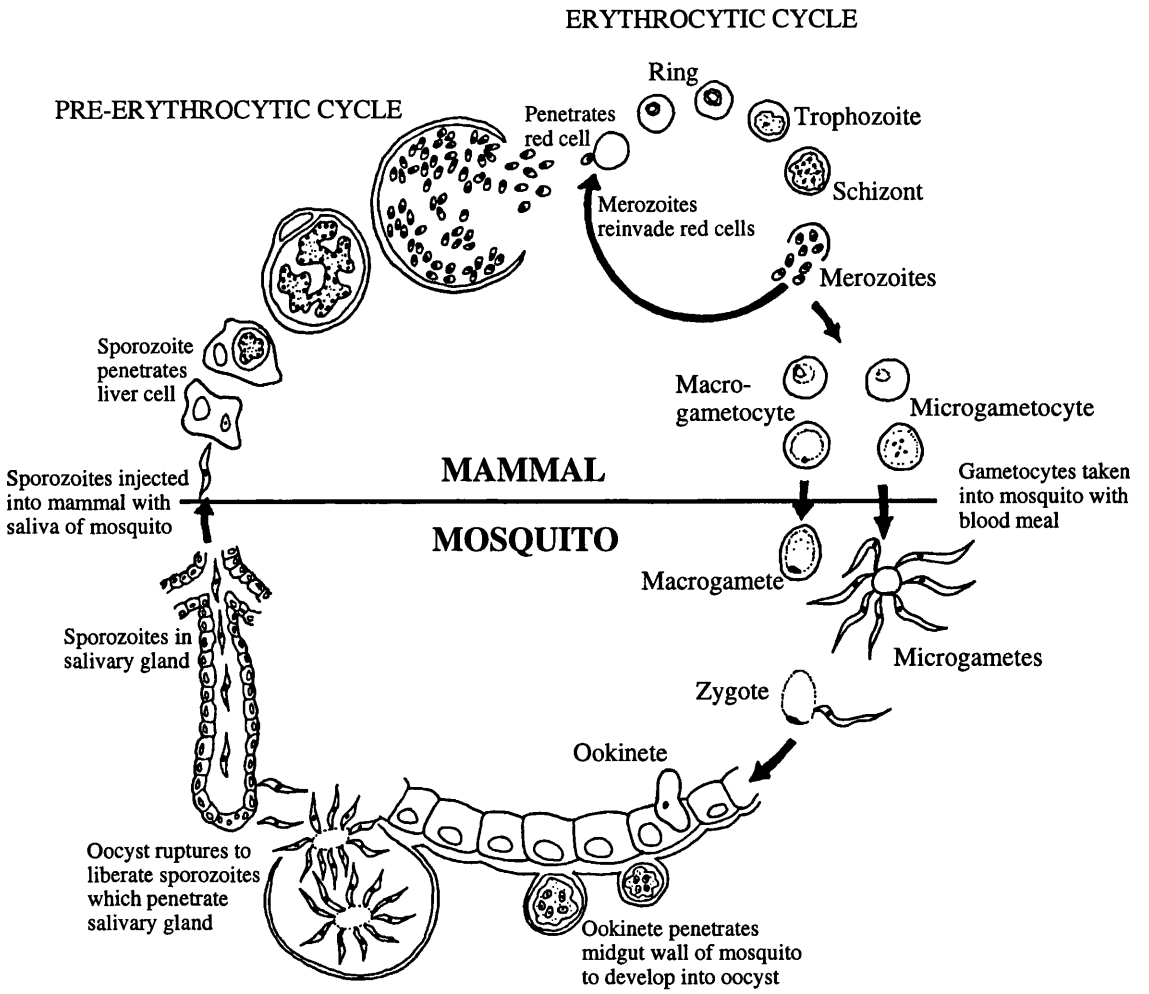


FIGURE 1.3.1 The life cycle of *Plasmodium* spp. in mammals (Adapted from Vickerman & Cox, 1967)

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. 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.

1.4 The clinical disease

The typical symptom of malaria is a violent fever lasting 6-8 hr, recurring every two or three days. The different species of *Plasmodium* cause two types of intermittent fever. A tertian fever (*P. falciparum, P. vivax* and *P. ovale*) has one day free of fever between paroxysms; a quartan fever (*P. malariae*) has two. Anaemia and enlargement of the spleen develop as the disease progresses.

These periodic paroxysms of prostrating fever distinguish malaria from other infections. Each attack is abrupt and often severe. After a variable incubation period, the patient feels very ill, with headache and backache. A feeling of unbearable cold comes on rapidly, and causes violent, uncontrollable shivering. Within an hour or so the body temperature rises to 40-41°C (105-106°F). This is followed by the hot stage, when the feeling of cold gives way to one of distressing heat, this lasting for another 1-2 hrs. Profuse sweating then ends the attack, restoring the body temperature some 5-8 hrs after it began to rise. Each attack exhausts the victim, but between paroxysms the patient may feel quite normal (Kitchen, 1949).

In all types of infection the periodic febrile response is related to the time of the rupture of a sufficient number of mature schizonts and consequent discharge of merozoites and much other antigenic and altered host material into the blood stream.

Most illness and death caused by malaria infection is associated with acute infection, although some fatalities result from unusual chronic immunologically based sequelae. *P. 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. Malaria infection has also been implicated in the incidence of tropical splenomegaly syndrome (reviewed by Greenwood, 1979), Burkitt's lymphoma (Dalldorf *et al.*, 1964; Burkitt, 1969), spontaneous abortion (Herd & Jordan, 1981) and the incomplete response to vaccination shown by malarious children (McGregor & Barr, 1962). However, there is as yet no evidence that immunosuppression by HIV leads to major complications of reactivations of malaria (Fleming, 1990; Lucas, 1990; Butcher, 1992). Unfortunately,

by being a potent cause of anaemia, malaria enhances transmission of HIV to children through blood transfusion (Greenburg *et al.*, 1988).

1.5 Chemotherapy

The choice of anti-malarial drug for use in man depends on whether it is for prophylactic or for curative purposes. In the latter case, the treatment priorities in severe and uncomplicated cases have to be distinguished. In life-threatening *P. falciparum* infections the objective of treatment is to save life. In uncomplicated malaria, prevention of late recrudescences or minor toxicity assumes the greatest importance, and may influence the choice of therapy. The type of infection also affects 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.

Drugs commonly used to treat malaria are summarised in Table 1.5.1.

GROUP	COMPOUND TYPES	STAGE OF LIFE CYCLE AFFECTED	ACTION ON PARASITES	ACTION ON MALARIAL PIGMENT
1	Chloroquine Mepacrine 4-Aminoquinolines	All asexual stages	Inhibition of haem polymerase (Slater & Cerami, 1992)	Rapid coarse clumping
2	Quinine Mefloquine Primaquine 8-Aminoquinolines	All stages except mature gametocytes of *P. falciparum* (but Primaquine active)	•Degeneration of nuclei •Vacuolation of cytoplasm	Slow, fine clumping
3	Antifolates: •Proguanil •Pyrimethamine •Sulphonamides	Schizogony	Maturation arrest producing large non viable parasites	-
4	Sesquiterpenes: •Artemisinine	Schizogony	-	-

TABLE 1.5.1 Classification of antimalarial drugs (adapted from Malaria (1991), Edited by A.J. Knell, Oxford University Press, Oxford).

Quinine in the form of *Cinchona* bark was introduced into Europe in the mid 1600's and remained the standard remedy for the disease till well into this century. It was largely replaced by chloroquine and other synthetic compounds, during the 1930's and 1940's, as these were more efficacious and less toxic (Geary & Jenson, 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.

Recently, observations by Picot *et al.* (1993) have suggested that chloroquine may help to prevent cerebral malaria, whatever the drug sensitivity of the parasite strain and regardless of the emergence of parasite multi-drug resistance, by inhibition of TNF (a cytokine implicated in having a major role in the pathology of cerebral malaria (Grau *et al.*, 1989b,c; Kwiatkowski *et al.*, 1990)). This provides an exciting prospect for new tools for anti-disease therapy.

1.6 Antimalarial Drug Resistance

Resistance to antimalarial drugs is spreading rapidly in most tropical areas. In South-East Asia, for example, strains of *P. falciparum* may be resistant to most or all conventional drugs, arising from the remarkable adaptability of this species, as well as the use of antimalarials for prophylaxis and for inadequate routine treatment of undiagnosed fevers in areas endemic for malaria.

1.7 Laboratory models for malaria research

Only in the final stages of vaccine or drug trials is research into human malarias involving the natural host sanctioned. 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 discovery of rodent malaria parasites in Katanga by Vincke and Lips was a major breakthrough in the study of malaria (Vincke & Lips, 1948). It was quickly followed by the isolation of other rodent malaria parasites from both native and rodent species as well as mosquitoes (reviewed by Balfort, 1971). These parasites have now been adapted to a range of laboratory rodents and guinea pigs which are convenient experimentally. In terms of their biological behaviour, the mouse malarias 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). These carefully defined rodent models allow experimental analysis of the mechanisms of immunity to malaria, in experiments that cannot be undertaken in humans, although much care must be taken when attempting to extrapolate results from these models to the human malarias.

An important technical step forward which has facilitated research in many areas of malaria including immunology and vaccine development, was the elucidation

of the conditions necessary for the continuous *in vitro* growth of the asexual erythrocytic stages of *P. falciparum* (Trager & Jenson, 1976; Haynes *et al.*, 1976). The candle jar method (Jenson & Trager, 1977) is now used routinely for *in vitro* cultivation of *P. falciparum*, although several modifications of this method are available.

1.8 Host Resistance

A prospective host's ability to control infection may take two forms - innate and acquired resistance. Innate resistance, which can be parasite specific, is expressed regardless of any previous contact, and has no immunological specificity. It may relate to a requirement of the parasite (for example, either nutritional or as a membrane receptor for invasion) or the presence of a deleterious substance within the host. Acquired resistance requires previous exposure to the parasite and is immunologic in nature. Between these two lies non-specific resistance which is immunologic in nature but requires exposure to a substance or organism (eg, concanavalin A or *Propionibacterium acnes*) which is unrelated to *Plasmodium* spp. but which nevertheless stimulates the host to kill parasites.

1.8.1 Innate Resistance

Increased resistance to malaria in individuals heterozygous for haemoglobinopathies such as thalassaemia, was first suggested by Haldane (1949). Subsequently, a number of conditions have been associated with protection from malaria. Substitution of valine for glutamic acid at position 6 of the β-globin chain produces the defective haemoglobin that is responsible for sickle-cell anaemia (reviewed by Weatherall, 1990). Association of sickle cell haemoglobin incidence and hyperendemic falciparum malaria has long been noted (Allison, 1954; Luzzatto, 1979). The mechanism of this innate resistance is related to the fact that ring-stage parasitized heterozygote cells have a far greater sickling rate than do non-parasitized cells (Luzzatto *et al.*, 1970; Roth *et al.*, 1978). Although no sickling occurs in cells with later-stage parasites abundant HbS polymer is formed and parasite development is retarded (Pasvol *et al.*, 1978). Two possible mechanisms have been proposed for parasite killing (Nagel, 1990): polymerised HbS may be a poor substrate for parasite proteases, or the polymer may physically damage and/or interfere with some critical function of the parasites. Polymerisation of HbS may be aided by the loss of water from the RBCs concomitant with potassium loss during sickling. However, potassium loss *per se* may not be the crucial cause of parasite death, since it has been shown that the parasite grows normally in ouabain-treated RBCs with high sodium and low potassium content (Ginsburg *et al.*, 1986; Tanabe *et al.*, 1986).

Other abnormal haemoglobin and erythrocyte phenotypes have also been associated with protection against malaria eg. imbalanced production of α- and β-chains (α- and β-thalassaemia), other changes in the β-globin chain structure (HbC and HbE), persistence of foetal haemoglobin, glucose-6-phosphate dehydrogenase deficiency, ovalocytosis and En(a^-) erythrocytes (glycophorin A negative individuals). Exactly how these phenotypes exert their protective effect remains unclear (Luzzatto, 1979) although Weatherall (1987) suggested that the flexibility of the erythrocyte membrane may be involved. It has been suggested that the protection provided by erythrocyte abnormalities may involve interactions with the host's immune system. Mediation of this effect could be by increased oxidative damage to intra-erythrocytic parasites in these cells (Clark & Hunt, 1983) or via exposure of parasite-dependent erythrocyte neoantigens (Marsh *et al.*, 1989).

At least one erythrocyte phenotype appears to be protective against malaria in the homozygous state. A significant proportion of African Americans are entirely resistant to *P. 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. 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, consequently, *P. vivax* is now absent. As *P. vivax* is not a lethal parasite, this fixation has probably arisen through spread of a fortuitous mutation rather than positive selection by the malaria parasites (Pasvol & Wilson, 1982).

Erythrocyte age also plays an important part in malaria infections with certain parasites showing profound preferences for young, or old cells. Of the human malarias, *P. vivax* preferentially invades reticulocytes (young RBCs), whilst *P. falciparum* invades both reticulocytes and normocytes (mature cells). Erythrocyte age preferences are also seen in the rodent parasites.

The genetic background of the host has also been shown to affect 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). 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). Recently, Hill *et al.* (1991) observed an association between certain HLA class I and II haplotypes and protection from severe malaria in the Gambia. Molecular analysis of the HLA B53 association has been studied by sequencing of peptides eluted from the B53 molecule followed by

screening of candidate epitopes from pre-erythrocytic stage antigens of *Plasmodium falciparum* (Hill *et al.*, 1992). Among malaria-immune Africans, HLA-B53-restricted cytotoxic T lymphocytes recognised a conserved nonamer peptide from liver-stage-specific antigen-1 (LSA-1). LSA-1, therefore, is an encouraging antigen for inclusion in a future subunit anti-malaria vaccine.

The effect of the host's diet, or nutritional state on malaria infections is not fully understood, and is little studied. A diet deficient in PABA causes a suppression of parasitaemia (Hawking, 1954; Gilks *et al.*, 1988). Indeed, 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. The overall nutritional status of an infected individual may affect the course and outcome of clinical disease. There may be a correlation between protein intake and parasitaemia since it is known that a protein-deficient diet can depress *P. berghei* infections in rats (Gilks *et al.*, 1989). This may in part explain why famine relief in humans is sometimes accompanied by outbreaks of malaria - so called 'feeding malaria' (Murray *et al.*, 1981).

1.8.2 Non-specific Immunity

Prior administration of some 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 manifest 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 known as *Corynebacterium parvum*) confers a degree of protection against subsequent challenge with *P. berghei* (Nussenzweig, 1967; Murphy, 1981), *P. vincke* (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, *Haemobartonella*, 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 agent 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 killing either through the release of superoxide ions (Allison & Eugui, 1982), or nitric oxide (Green *et al.*, 1990a). The mechanisms of this parasite destruction will be discussed in more detail later.

Some evidence that the growth of *P. falciparum in vitro* can be inhibited by a non-Ab, non-dialysible factor has been observed by Jenson *et al.* (1983). As pRBC death was caused during the resolution or crisis period of an acute, primary malarial infection in laboratory models, this factor was called 'crisis form factor'. Jenson *et al.* (1982; 1983) have since identified this factor in serum samples from immune Sudanese adults. They showed a stronger association between clinical immunity and *in vitro* serum inhibition with this crisis form factor than with IgG.

1.8.3 Acquired immunity

The immune response and acquisition of immunity to various species of *Plasmodium* has been extensively studied. 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 pre-erythrocytic stage, the asexual erythrocytic stage (against which immunity is mainly directed), and gametocytes (immunity to which would interrupt transmission).

A number of aspects of the host's immune response to malaria have been observed and their relative importance in parasite clearance and subsequent resistance to re-infection investigated. Methods 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 - some of which will be discussed in subsequent chapters in relation to *P. chabaudi* in NIH inbred mice.

Evidence of acquired immunity to malaria infection in humans starts to appear at the beginning of the second week of patent parasitaemia and is manifest as a reduction in the reproduction rate and the number of pRBCs in the blood. After a variable period, the immunity decreases the parasitaemia to undetectable levels (McGregor, 1956). However, there is not a direct correlation between parasitaemia and symptomatology as the immune response can occasionally diminish the clinical manifestations of infection even in the presence of considerable blood-borne parasite

burden. In malarious 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 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 (Cox, 1964; Playfair, 1977). The presence of sterile immunity in humans has not been investigated experimentally, but epidemiological studies do not indicate that it exists.

(a) Sporozoite Immunity

It is now known that viable sporozoites induce a detectable Ab response in both man and experimental animals. Nardin *et al.* (1979) were able to detect rising levels of anti-sporozoite Abs with increasing age in serum samples from Gambian children and adults. This infers that repeated exposure to sporozoites over many years is necessary to induce Ab formation in man. There is also indirect evidence that an acute blood stage infection suppresses the response to sporozoites in mice (Orjih & Nussenzweig, 1979).

Irradiated sporozoites have been shown to be strongly immunogenic. After three or more immunizations with irradiated sporozoites, more than 90% of mice challenged with *P. berghei* were immune to unattenuated sporozoite challenge and the protection was maintained for almost two months and then declined progressively (Nussenzweig *et al.*, 1969). Protection by repeated injection of irradiated sporozoites has also 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* immunization. Mice immunized with repeated inoculations of irradiated sporozoites clear challenge sporozoites from their blood more quickly than non-immunised mice (Nussenzweig *et al.*, 1972). Recipients of serum collected from immunised mice showed a similarly increased rate of clearance, and the prepatent period of the challenge infection was considerably prolonged (Nussenzweig *et al.*, 1972), implying a role for Ab in sporozoite immunity.

Protective mAbs have been produced against the sporozoite surface (CSP - see later) of *P. berghei* (Yoshida *et al.*, 1980; Potocnjak *et al.*, 1980) and the antigens to which they react have been separated (Vermeulen *et al.*, 1983). The antibodies and their Fab fractions prevent parasite entry into cultured hepatoma cells (Hollingdale *et al.*, 1982) and the antigen which they recognise is probably involved in the recognition and penetration of the hepatocyte by the parasite. mAbs recognising

similar antigens in *P. knowlesi* have been isolated and are protective (Cochrane *et al.*, 1982), while others have been produced which reduce the infectivity of *P. falciparum* sporozoites to splenectomised chimpanzees (Nardin *et al.*, 1982).

Spitalny *et al.* (1976) and Spitalny & Nussenzweig (1973) suggested a role for cell-mediated immunity in acquired resistance to sporozoite infection. A level of protective immunity in the absence of detectable antibody was observed during the early stages of immunisation in mice, or following vaccination with irradiated sporozoites in splenectomised chimpanzees. 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 (Spitalny *et al.*, 1977). Adoptive transfer of thymus cells restored the capacity of these animals to synthesise Ab and become immunised, concluding 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 immune after sporozoite vaccination (Chen *et al.*, 1977) and that adoptive transfer of T cells could transfer immunity (Verhave *et al.*, 1978). However, Tsuji *et al.* (1990) have reported the identification of a $CD4^+$ cytolytic T cell clone effective against sporozoites and liver stage parasites, that secretes IFN-γ and Il-2. *In vitro* studies performed with sporozoites of *P. yoelii* and murine hepatocytes have also demonstrated that Il-6 inhibits sporozoite penetration and development (Pied *et al.*, 1991; 1992)

(b) Immunity to pre-erythrocytic stages

It has long been held that it is only after pre-erythrocytic schizont rupture and merozoite release that this stage of the parasite provokes a cellular response. Phagocytic infiltration of infected livers and engulfment of large numbers of merozoites supported this view (Shortt & Garnham, 1948; Garnham & Bray, 1956; Lupascu *et al.*, 1967). However, evidence of some clinical immunity was reported by Beaudoin *et al.* (1975) who demonstrated that chloroquine treated rats, with suppressed blood stage malaria, showed fewer pre-erythrocytic stages of *P. berghei* when viable sporozoites were administered simultaneously.

Jahiel *et al.* (1968a,b) were the first to demonstrate that pre-erythrocytic malaria parasites could be killed by non-specific immune mediators. Later recombinant IFN-γ was shown to inhibit the *in vitro* growth of liver stage parasites of *P. berghei, P. chabaudi* and *P. vivax* (Ferreira *et al.*, 1986; Maheshwari *et al.*, 1986), relatively small quantities of IFN-γ being required. Schofield *et al.* (1987a) showed that IFN-γ was acting hormonally, by binding to specific hepatocyte receptors and inducing intracellular death. Treatment of sporozoite-immunised mice and rats with a

neutralising mAb to rodent IFN-γ abrogated pre-erythrocytic immunity, thereby showing that IFN-γ secretion is required for this immunity (Schofield *et al.*, 1987b). Moreover, immunity was abolished by depleting immunised mice of $CD8^+$ cells (Schofield *et al.*, 1987b; Weiss *et al.*, 1988) but not CD4 cells. It is considered that upon parasite challenge, $CD8^+$ T cells release IFN-γ which then inhibits pre-erythrocytic development, although $CD8^+$ T cells may also be directly cytotoxic for liver stage parasites (reviewed by Schofield, 1989).

Weiss (1990) has suggested the presence of a CD8-independent mechanism in cellular immunity to liver stage parasites, since host control of protective immunity to pre-erythrocytic stages is different in *P. yoelii* and *P. berghei*. Therefore, depending on the mouse strain, $CD8^+$ cells may or may not be critical effectors, and a second, undefined, immune effector arm may protect infected animals (Weiss *et al.*, 1989). *In vivo* induction of Il-6 dramatically inhibits the development of *P. berghei* pre-erythrocytic forms (Vreden *et al.*, 1992) and *in vitro* studies have shown that in addition to Il-6, both Il-1 and TNF-α (Pied *et al.*, 1991; Nussler *et al.*, 1991a) inhibit pre-erythrocytic development. Blocking studies with mAbs showed that both TNF-α and Il-1 exert their effect through Il-6 (Pied *et al.*, 1991; Nussler *et al.*, 1991b). Acute phase reactants, reactive oxygen metabolites like H_2O_2, superoxide anions (Pied *et al.*, 1991), and nitrogen oxides (Nussler *et al.*, 1991b) have been shown to act as effectors in reducing the number of pre-erythrocytic forms *in vitro* after incubation with Il-6 or TNF-α. Interestingly, blocking of NO synthesis in naive hepatocytes enhances the number of pre-erythrocytic stages, suggesting that nitrogen oxides are part of the physiological defence mechanism of the hepatocyte (Vreden *et al.*, 1992).

In αβ T cell deficient mice, an immune response to sporozoite challenge is induced that is inhibited by an *in vivo* depletion of γδ T cells. Moreover, γδ-T cell clones upon adoptive transfer can protect normal mice against the development of liver stage *P. yoelii* parasites (Tsuji *et al.*, 1994).

(c) Immunity to asexual erythrocytic stages

Much information is available on various aspects of the host's immune response to erythrocytic stages of the parasite. However, the roles that each plays in the resolution of, and subsequent protection from, reinfection are still open to debate. 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 immune response, the relative importance of various immune effector mechanisms very probably varies widely between different host-parasite models, and at different times during infection.

(i) The role of the humoral response

The blood stages of *Plasmodium* 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 from their hosts (eg. 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 RBCs, complement, rheumatoid factor, and nuclear components (Deans & Cohen, 1983). The frequent presence of autoantibodies is related to the immunopathology of malaria infection (see later). In general, the correlation between total anti-malarial Abs and protective immunity and/or clinical status is poor, indicating that many of the Abs do not 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 to correlate Ab levels with protection comes from passive transfer experiments. The passive transfer of protection with immune sera has been demonstrated. In 1937, Coggleshall & Kumm transferred protection with sera from rhesus monkeys with chronic *P. knowlesi* malaria to naive monkeys. Cohen *et al.* (1961) and Cohen & McGregor (1963) demonstrated protective activity in serum of humans infected with *P. falciparum*, the effective component consisting of the IgG fraction. More recently, others have shown that this protective effect may be mediated by cytophilic antibodies in co-operation with monocytes (Druilhe & Khusmith, 1987; Bouharoun-Tayoun & Druilhe, 1992). A variety of passive Ab studies using rodents has also demonstrated immune IgG mediated protection (Diggs & Osler, 1969; Stechschulte *et al.*, 1969; Diggs *et al.*, 1972; Phillips & Jones, 1972; Green & Kreier, 1978; Reese & Motyl, 1979). Treatment of serum with anti-IgG removes the protective activity of rat hyperimmune serum in the *P. berghei* system (Diggs & Osler, 1969). The passive transfer of serum collected from hosts recovering from 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 cells has been shown in some host-parasite relationships. Mice congenitally deficient in B cells have higher and more prolonged infections than normal controls (Jayawardena *et al.*, 1979) and in some cases infections became lethal (Hunter *et al.*, 1979a). Mice made B cell deficient by treatment with anti-μ-chain Ig,

died from a normally self-limiting infection with *P. yoelii* (Weinbaum *et al.*, 1976; Roberts *et al.*, 1977). After drug cure and rechallenge, B cell deficient mice suffered a long lasting low grade parasitaemia (Roberts & Weidanz, 1979). B-cell enriched populations of immune spleen cells are effective in adoptively transferring protection to malaria. This has been observed in mouse-*P. chabaudi* (McDonald & Phillips, 1978), mouse-*P. berghei* (Ferranoni & Speer, 1982) and rat-*P. berghei* (Gravely & Kreier, 1976) systems. Recent reconstitution experiments using SCID and nude mice have shown that both B and T cells are necessary for the transfer of protective immunity to *P. c. chabaudi* (Meding & Langhorne, 1991). However, there is little or no apparent B-cell involvement in the resolution of *P. c. adami* infection (data of Van der Hyde, cited in Greiner *et al.*, 1992).

Antibodies could be effective against the parasite in a number of ways. Prevention of merozoite invasion or retardation of intraerythrocytic development are possibilities (Phillips *et al.*, 1972; Miller *et al.*, 1975a; Mitchell *et al.*, 1976; Cohen *et al.*, 1977; Epstein *et al.*, 1981; Revin *et al.*, 1981; Deans *et al.*, 1982; Miller *et al.*, 1984; Saul *et al.*, 1984; 1985; Banjal & Inselburg, 1985; Schmidt-Ulrich *et al.*, 1986; Udomsangpetch *et al.*, 1986). *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). Apart from the agglutinating activity of inhibitory Ab, other serum Abs offer protection against asexual parasites. Opsonising Abs, which promote phagocytosis of pRBCs, have also been detected *in vitro* (Zuckerman, 1945; Khusmith *et al.*, 1982). Opsonins against *P. knowlesi* have been shown to be variant specific (Brown *et al.*, 1970a). While opsonisation may be important *in vivo* in the early stages of infection, phagocytosis may be inhibited later in infection by serum factors (Shear *et al.*, 1979; Brown & Kreier, 1982).

(ii) The role of complement

In both experimental and naturally occurring human malarias depressed complement levels are observed (Dulaney *et al.*, 1948; Neva *et al.*, 1974; Greenwood & Brueton, 1974; Srichaikul *et al.*, 1975). This decrease was correlated directly with the degree of parasitaemia and the presence of complement fixing Ab, with the lowest detectable complement titres observed within a few hours of schizont rupture and during peak fever (Neva *et al.*, 1974). Infection of mice congenitally deficient in complement factors appears to have no effect on the course of *P. berghei* infection. It appears that complement has a negligible role in the immune response to malaria

infection and may not contribute to any extent to parasite clearance. The depletion of complement components after schizont rupture is thought to be due to complement fixation by Ab-Ag 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

The presence of a functional thymus plays a major part in the development of immunity to malaria. Congenitally athymic nude (nu/nu) mice were unable to clear an infection with a strain of *P. yoelii* from which intact normal mice recover (Clark & Allison, 1974; Weinbaum *et al.*, 1976b; Roberts *et al.*, 1977). 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. This has been observed in *P. berghei* infections in thymectomised rats (Brown *et al.*, 1968; Stechschulte, 1969), 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. c. chabaudi* and *P. c. adami* infections in 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). A conflicting finding was reported by Wright (1968) who reported that thymectomy of golden hamsters increased their survival time on infection with *P. berghei.* This model system is unusual, however, in that normal golden hamsters die from *P. berghei* infection when the parasitaemia is very low. Post mortem reveals cerebral haemorrhages. Thymectomised animals die when the parasitaemia is much higher and show no cerebral complications at post mortem. Thus, this early observation implicated T cells in the occurrence of cerebral malaria, which has since been attributed to the toxicity of a key mediator of inflammation, TNF (Clark, 1987).

Potential animal hosts have also been made T-cell deficient by treatment with anti-thymocyte serum (ATS). ATS treatment of rats prior to infection suppressed both natural and age resistance to *P. berghei*, while ATS treatment after the infection had cleared failed to enhance rat susceptibility to reinfection (Spira *et al.*, 1970). In contrast, Brown & Phillips (1971) found that rats with chronic *P. berghei* had a marked recrudescence following ATS treatment. Eling (1979) showed that ATS

treatment had maximal effect when administered 6 hours after challenge of *P. berghei* infected, drug cured mice, implicating a role for T cells early in challenge infections.

The role of T cells in the immune response to malaria infections has been examined by looking at differences in the T-cell response to non lethal and lethal malaria infections. Massive T cell mitosis has been observed in the spleens of mice infected with a non lethal strain of *P. yoelii*, but not in lethal *P. berghei* infections (Jayawardena *et al.*, 1975). Early in lethal *P. yoelii* strain infections there was a reduced splenic T cell response to malaria antigen *in vitro*.

Adoptive transfer experiments using rodent models of infection have indicated that animals receiving T cell enriched spleen cell preparations are protected against subsequent challenge (eg. McDonald & Phillips, 1978). Although some immunity to the blood stages of malaria can be transferred to irradiated recipient mice by immune T cells alone, better protection has been obtained when both T and B cells are transferred (Brown *et al.*, 1976a; 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.*, 1976a,b). This was confirmed by Jayawardena *et al.* (1977), who found that intact mice produce high levels of specific anti-malarial Abs while T cell deficient mice have considerably reduced levels of IgG_1, IgG_2 and IgM. The normal pattern of functional immunity was restored by reconstitution with syngeneic thymus cells. Adoptive transfer experiments will be discussed further in Chapter 4.

Additional support for the role of T cell mediated immune mechanisms in malaria has come from adoptive transfer studies in athymic nude mice. Immune T cell recipients resolve their infections more rapidly and demonstrate lower peak parasitaemias than recipients of non-immune T cells. Protection was best achieved with the T cell subsets expressing the $CD4^+$ phenotype (McDonald & Phillips, 1980; Cavacini *et al.*, 1986; Vinetz *et al.*, 1990) or with *in vitro*-propagated Il-2 dependent and *P. c. adami*-specific T cell lines or clones of the $CD4^+$ phenotypes (Brake *et al.*, 1986; 1988). Taylor-Robinson & Phillips (1992; 1993; 1994a) have also demonstrated the protective activity of $CD4^+$ T cell lines and clones of both T_H1 and T_H2 phenotypes, reactive to *P. c. chabaudi*. Moreover, studies have also shown that the depletion of the $CD4^+$ T cell subset *in vivo* by treatment with mAbs renders mice incapable of clearing an infection (Süss *et al.*, 1988; Kumar *et al.*, 1989; Langhorne *et al.*, 1990; Podoba & Stevenson, 1991 - see also Chapter 3).

Chickens rendered agammaglobulinaemic by combined immunological and chemical bursectomy (Rank & Weidanz, 1976; Roberts & 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). Cavacini *et al.* (1990) furthered these studies by demonstrating that acute infections of *P. c. chabaudi*, *P. vinckei petteri* and *Babesia microti* are controlled by Ab-independent, T cell-mediated immune mechanisms.

It is therefore evident that T cells are important in the development of immunity against blood stage parasites (Jayawardena, 1981) but it must also be noted that the mechanisms by which T cells mediate clearance and provide protection are not identical in different rodent models. In general, however, $CD4^+$ cells appear to be the more dominant T cell subset in conferring protection against asexual parasites (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; Jayawardena *et al.*, 1977; Roberts *et al.*, 1977; McDonald & Phillips, 1978; Playfair, 1982). $CD4^+$ cells that act as helper cells for Ab production belong exclusively to the T_H2 subset (Langhorne *et al.*, 1989b). However, it has recently been shown that T_H1 as well as T_H2 cells are important during infection (Langhorne, 1989; Langhorne *et al.*, 1989a,b; Langhorne *et al.*, 1990; Taylor-Robinson & Phillips, 1992; 1993; 1994a). It appears that soon after challenge with *P. c. chabaudi*, T_H1 cells predominate, producing an inflammatory type response with the major mechanism for parasite destruction being via toxic mediators secreted by activated macrophages. Later during infection, the proportion of T_H2 cells increases and Ab-mediated immunity begins to operate, so facilitating resolution of patent infection (Langhorne, 1989). In this model of infection, therefore, first T_H1 cells activate various non-specific immune mechanisms such as nitric oxide production (Taylor-Robinson *et al.*, 1993) or may in fact produce nitric oxide themselves (Taylor-Robinson *et al.*, 1994), to control a rapidly escalating acute parasitaemia. This paves the way for the T_H2-promoted specific immunoglobulin response to effectively clear the blood infection.

An increase in the number and proportion of peripheral blood $\gamma\delta$ T cells has been observed during acute *P. falciparum* infections of human patients (Ho *et al.*, 1990). It has been suggested that these $\gamma\delta$ cells might exhibit anti-parasitic effects via secretion of cytokines or as non-MHC-restricted cytotoxic cells (Ho *et al.*, 1990). In non-immune individuals, a vast majority of T cells vigorously proliferating in response to freeze-thawed extracts of *P. falciparum* blood stage parasites *in vitro* have been shown to be $\gamma\delta$ T cells (Goerlich *et al.*, 1991; Behr & Dubois, 1992; Goodier *et al.*, 1992), and are the source of a significant fraction of TNF-α and IFN-γ that were produced in these cultures (Langhorne *et al.*, 1992). Perera *et al.* (1994) have shown

that in *P. vivax* infections, the proportion and absolute numbers of γδ cells increase, and that this increase is associated with the time of schizont rupture and the event of paroxysm. These findings support the view that γδ T cells may also be involved in the pathogenesis of malaria (Langhorne *et al.*, 1992) via the secretion of TNF. γδ T cell deficient mice, however, clear the blood stages of non-lethal *P. yoelii* infection, while αβ T-cell-deficient mice failed to control the parasitaemia (Tsuji *et al.*, 1994). Similar results have been observed for *P. c. chabaudi* AS (A.W. Taylor-Robinson, personal communication).

(iv) The role of the reticulo-endothelial system

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 principle mechanism by which immunity was effected (Taliaferro, 1929). Increased phagocytic activity has been observed during malaria infection. An increased carbon clearance rate has been observed during *P. vinckei* and *P. chabaudi* infections of 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 added particles has been observed during malaria infections; these include ^{51}Cr-labelled sheep RBCs in mouse-*P. berghei* infections (Loose & DiLuzio, 1976) and ^{125}I-labelled microaggregated human serum albumin in human malaria infections (Sheagren *et al.*, 1970).

Peritoneal macrophages of mice with *P. berghei* infections showed enhanced phagocytic activity towards pRBCs and nRBCs 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 pRBCs 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 pRBCs (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 will be discussed elsewhere. The recruitment of macrophages and monocytes and their activation are mediated by cytokines such as IFN-γ, macrophage chemotactic factor, IL-2 and Il-6 (F.E.G. Cox, personal communication) secreted by T cells, which are themselves activated by plasmodial mitogens as well as specific malarial antigens (Wyler & Gallin, 1977; Allison & Eugui, 1983; Ockenhouse & Shear, 1983).

Both pRBCs and nRBCs have been observed within splenic macrophages *in vivo* in human malaria infections (Pongponratn *et al.*, 1987). Splenic filtration is increased by splenomegaly. Both enhanced phagocytosis and splenomegaly in malaria infections have been found to be thymus-dependent responses (Roberts & Weidanz, 1978). This is unsurprising 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 animals, however, increased clearance of pRBCs cannot be demonstrated in normal animals given hyperimmune serum (Quinn & Wyler, 1979a,b) although *in vitro* immune serum has been shown to facilitate the phagocytosis of merozoites (Khusmith & Druilhe, 1983). In Thai patients with falciparum malaria the activity of monocytes from cases of uncomplicated malaria was significantly increased compared to healthy controls (Ward *et al.*, 1984). In contrast, the activity of monocytes from cerebral malaria sufferers was within normal limits. In another study, the *in vivo* clearance of IgG-coated RBCs was accelerated in some but not all patients (Ho & Webster, 1990) with a significant positive correlation between the half-time for clearance of sensitised RBCs from the circulation and the level of parasitaemia. 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

The importance of $CD4^+$ T lymphocytes in the resolution of a primary infection with blood stage malaria parasites has been described above. It is thought that soluble macromolecules (cytokines) such as interleukins derived from lymphocytes and other cells are instrumental both in the T cell-dependent regulation of the immune reponse and its effector phase. Most of these processes probably reflect the function of cytokines secreted by activated T cells responding to malarial Ags.

The first direct support for cytokine production in response to malarial Ags was provided by Wyler & Gallin (1977) who identified a mononuclear cell chemotactic factor in spleen cell extracts from malarious mice and monkeys. Infected nude mice lacked significant activity, therefore, it was concluded that the chemotactic activity was secreted by, or was dependent upon, T cells and their precursors. Lelchuk *et al.* (1984) showed that the ability of spleen cells from *P. yoelii* or *P. berghei* infected mice to produce Il-2 upon Con A stimulation varied according to the time following infection. An increase in the capacity to release Il-2 was seen early in both infections,

a finding also shown for *P. c. chabaudi* infection (Langhorne *et al.*, 1989a,b).

Interferons are increasingly being considered important in acquired immunity to malaria blood stage parasites. Administration of exogenous IFN-γ inducers or IFN-γ containing serum was found to delay the progress of *P. berghei* infection in mice (Jahiel *et al.*, 1968b, 1970). Treatment with anti-mouse IFN globulin accelerated *P. berghei* infections in mice (Sauvager & Fauconnier, 1978). Eugui & Allison (1982) and Rhodes-Feuillette *et al.* (1985) have reported the presence of IFN-γ in the sera of infected humans as well as mice. T cells from malarious patients and immune individuals from endemic areas can secrete IFN-γ and Il-2 upon stimulation with homologous Ag (Troye-Blomberg *et al.*, 1985; 1987). This has been confirmed by other workers (Sinaglia & Pink, 1985; Riley *et al.*, 1988a). In contrast to its toxicity to the pre-erythrocytic stages, IFN-γ by itself has no effect on the erythrocytic stages of plasmodia (Ferreiera *et al.*, 1986). However, IFN-γ is capable of activating macrophages. IFN-γ (and Il-2) secretion is a unique property amongst T cells of the $CD4^+$ T_H1 subset (Mosmann & Coffman, 1987). This has been confirmed for malaria by Langhorne *et al.* (1989b) and Taylor-Robinson & Phillips (1992) using *P. c. chabaudi* AS. Intraerythrocytic *P. yoelii* parasites can be killed by normal macrophages after incubation with IFN-γ containing S/N (Ockenhouse & Shear, 1984). This killing is blocked with the addition of anti-IFN-γ Ab (Ockenhouse *et al.*, 1984). The same investigators showed that rIFN-γ activated macrophages to induce the appearance of crisis forms of *P. falciparum* in cultures of human pRBCs.

Inflammatory mediators such as TNF can be induced in macrophages activated by IFN-γ (Mosmann & Coffman, 1987) in response to malaria parasite stimulation (Bate *et al.*, 1988). TNF may contribute to protective immune mechanisms but it is also linked to the pathology of cerebral malaria (Grau *et al.*, 1987). *In vivo*, treatment of mice with exogenous IFN-γ has been reported to have a protective effect during the blood stages of various rodent malarias (Clark *et al.*, 1987a; Bienzle, 1988; Shear *et al.*, 1989) and also enhanced antimalarial chemotherapy of *P. vinckei* malaria when given prophylactically or in the early phase of blood-stage malaria (Kremsner *et al.*, 1991). In *P. c. chabaudi* AS-infected mice, endogenous IFN-γ has been detected in serum 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.*, 1990a). *In vivo* depletion of IFN-γ by mAb treatment exacerbates infection (Slade & Langhorne, 1989; Stevenson *et al.*, 1990c). However, administration of IFN-γ in combination with chloroquine in the late phase of *P. vinckei* malaria induced lethality despite effective parasite clearance by chloroquine (Kremsner *et al.*, 1992).

Stevenson & Tam (1993) have shown that both susceptible and resistant strains

of mice are capable of producing IFN-γ in response to both Con A and malarial antigen. However, the response is greater in resistant mice. Moreover, susceptible mice produce higher levels of Il-5, a marker of T_H2 cells, earlier in infection, indicating that early activation of T_H2 cells may be associated with severe and lethal malaria, as occurs in A/J mice infected with *P. c. chabaudi* AS. In addition, it seems that cerebral malaria in certain host-parasite combinations 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 can vary from one mouse strain to another, may determine susceptibility or resistance to infection.

Based on serum cytokine profiles in patients with *P. falciparum* and *in vitro* stimulation of peripheral blood lymphocytes from various populations with malaria, T_H1 and T_H2 cells are activated during infection (Troye-Blomberg & Perlmann, 1988; Troye-Blomberg *et al.*, 1990; Mshana *et al.*, 1991). Removal of $CD8^+$ cells from the peripheral blood enhances T cell proliferation and the IFN-γ response in normally low-responding immune individuals (Riley *et al.*, 1989a). Although the *in vivo* significance of these findings is not clear, it confirms the importance of $CD4^+$ T cells, but also supports strongly the concept that Ag-specific $CD8^+$ T cells are involved in the suppressive regulation of CMI to malaria (Troye-Blomberg *et al.*, 1983).

Kumaratilake & Ferrante (1992) have shown that IFN-γ and lymphotoxin (LT) enhance neutrophil-mediated killing of *P. falciparum* (Kumaratilake *et al.*, 1991; 1992). These findings support a role for both T_H1 and T_H2 cells in immunity to malaria, since IFN-γ and LT are products of T_H1 cells and parasite killing by neutrophils is antibody-dependent (Kumaratilake *et al.*, 1991; 1992).

Il-4 can depress the macrophage mediated killing of *P. falciparum* (Kumaratilake & Ferrante, 1992). Recent results from other parasite systems demonstrate the ability of Il-4 to inhibit the microbicidal functions of IFN-γ activated macrophages *in vitro* (Liew *et al.*, 1991; Oswald *et al.*, 1992). The role of Il-4, however, in host protection against malaria is far from clear. As yet unpublished results by Von der Weid *et al.* have shown that a *P. c. chabaudi* infection in mice in which the Il-4 gene has been inactivated by gene targetting (Kopf *et al.*, 1993) is resolved with the same kinetics as wild-type littermate controls (Von der Weid & Langhorne, 1993a).

Identifying the roles that a variety of cytokines play in the immunity to *Plasmodium* parasites will contribute to strategies for developing effective vaccines against malaria.

(vi) The role of reactive oxygen intermediates

As discussed above, the release of IFN-γ and other cytokines from $CD4^+$ T cells is an important part of Ab-independent immunity. These stimulate cells of the mononuclear phagocytic cell system to exert anti-parasite effects either directly by phagocytosis or more often through the release of free oxygen radicals, which may in turn give rise to more stable parasiticidal components (Allison & Eugui, 1983; Clark *et al.*, 1987a).

Injection of agents known to generate free oxygen radicals, including alloxan (Clark & Hunt, 1983) and t-butylhydroperoxide (Wood & Clark, 1982; Clark *et al.*, 1983) suppressed parasitaemia in mice. These chemical generators of reactive oxygen species including H_2O_2, superoxide anions (O_2-) and hydroxyl radicals (OH·) may mimic a mechanism of CMI towards blood stage malaria. Indeed, not only have ROI been shown to be toxic to asexual stages of a variety of different *Plasmodium* species, both *in vitro* and *in vivo* (Dockrell & Playfair, 1983), free radical scavengers have accentuated *P. c. adami* infections (Clark *et al.*, 1987a). Macrophage-oxidative capacity was also found to be deficient in susceptible mice compared to resistant mice (Stevenson *et al.*, 1992).

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.*, 1987a; Rockett *et al.*, 1988). The chemicals may then circulate in the blood and effect parasite (and tissue) injury at distant sites.

(vii) The role of toxic nitrogen oxides

Despite the evidence for the involvement of oxidative mechanisms in the control of malaria infections, additional factors appear to be involved. Rockett *et al.* (1988) observed that a non-lipid factor was also involved in the parasiticidal component of tumour necrosis serum (TNS). In addition, in cases of chronic granulomatous disease, in which oxidative metabolism is impaired, macrophages (Ockenhouse *et al.*, 1984) and PMN cells (Kharazmi *et al.*, 1984) were able to inhibit the growth of intraerythrocytic parasites. Cavacini *et al.* (1989) have also reported proficiency of killing in hosts possessing cells deficient in the respiratory burst. These findings suggest the existence of an oxygen-independent parasite killling mechanism. This has recently been elucidated, and involves the cytokine-induced synthesis of toxic nitric oxide (NO) from L-arginine by macrophages, hepatocytes and endothelial cells. NO inhibits iron-sulphur-dependent enzymes involved in cellular respiration and energy production and may react with an oxygen free radical to yield a highly reactive hydroxyl radical and a more stable nitric oxide free radical (James & Hibbs, 1990;

Liew & Cox, 1991).

The role of NO can be demonstrated in *P. c. chabaudi* AS infection (Taylor-Robinson *et al.*, 1993). A sharp peak of NO production, measured as serum nitrate, consistently parallels peak parasitaemia. Treatment with L-NMMA, an inhibitor of NO synthase, completely abolishes NO production and mice suffer an extended primary parasitaemia; thymectomised, T_H1 cell reconstituted mice suffer a chronic patent parasitaemia. Treatment with L-NMMA during recrudescence does not effect the parasitaemia during this stage of infection. NO also inhibits the development of pre-erythrocytic stages of *P. berghei* (Mellouk *et al.*, 1991) and *P. yoelii* (Nussler *et al.*, 1991b) *in vitro*, and NO derivatives kill asexual erthyrocytic stages of *P. falciparum* (Rockett *et al.*, 1991) *in vitro*. The results of Taylor-Robinson *et al.* (1993) show that NO involvement may be mediated by T_H1 secretion of IFN-γ which activates macrophages to produce large amounts of NO (Marletta *et al.*, 1988; Stuehr & Nathan, 1989) to kill the parasites directly. Alternatively, NO may have an indirect effect by causing blood vessel vasodilation (Knowles & Moncada, 1992). This effect would lead to less efficient parasite sequestration in deep tissue capillaries, allowing removal of the parasites by macrophages (Taylor-Robinson *et al.*, 1993). However, an overproduction of NO, causing brain vessel dilation, could be responsible in turn for the increased intracranial pressure seen in cerebral malaria patients (Clark *et al.*, 1991; Newton *et al.*, 1991) or it may be involved in another process at the neuron level (Clark *et al.*, 1992) causing a disruption of the regulation of glutamate-induced neural NO. However, Senaldi *et al.* (1992), Asensio *et al.* (1993) and Kremsner *et al.* (1993) have reported that there is no influence of NO inhibitors on the development of cerebral malaria in the mouse model, *P. berghei* ANKA, even upon intracranial administration.

(viii) The role of the spleen

Resolution of malaria infection is dependent upon the presence of an intact spleen for the majority of host parasite combinations. Following splenectomy, non-lethal challenges may become lethal and latent infections may relapse (Taliaferro, 1929; reviewed by Wyler *et al.*, 1979). However, in some studies splenectomy does not effect the outcome of infection (Dockrell *et al.*, 1980). This paradoxical situation may be reconciled by the finding of Wyler *et al.* (1979) that the spleen plays a beneficial role for the host early in infection, but it may later have a deleterious effect by promoting chronicity in some infections. The effect of splenectomy on the course of a subsequent malaria infection is dependent on the species of the 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.*, 1976a), this quantitative loss is probably not as important as losing access to the normal splenic architecture and filtering ability. In support of this, Phillips (1970) and 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.

It was proposed by Barker & Powers (1971a,b) 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 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.

Why the spleen is so vital in malaria infection has only recently become clear with several claims for a physical role in trapping pRBCs, enabling localised parasite elimination (Conrad & Dennis, 1968; Schnitzer *et al.*, 1972; Wyler *et al.*, 1981). Observations suggest that the spleen efficiently filters out pRBCs as soon as they can be recognised as foreign. Rat *P. berghei* pRBCs are more rapidly removed from the circulation into the spleen than are nRBCs (Quinn & Wyler, 1976b; 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 pRBCs 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 RBCs 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 RBCs passing through it must be pliant. If RBC deformability is reduced, as is the case for pRBCs (Miller *et al.*, 1971b), their passage is delayed and a pool of pRBCs forms within an environment rich in effector cells. This regional concentration of pRBCs was first reported by Taliaferro & Cannon (1936).

During malaria infection, the changes in the spleen depend on 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, 1983a,b) or, for pRBCs, 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 pRBCs from the blood (Taliaferro & Cannon, 1936; Taliaferro & Mulligan, 1937; Taliaferro & Taliaferro, 1944; Quinn & Wyler, 1979b; 1980; Wyler *et al.*, 1979a, 1981; Wyler, 1983a). Crisis fails to occur in the absence of the spleen. The disappearance of circulating pRBCs 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 above, 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, 1979b; Wyler *et al.*, 1981). Furthermore, during *P. berghei* 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 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, 1979b; Wyler *et al.*, 1981).

In studies of lethal and non-lethal *P. yoelii* infections, an activation of reticular cells is at such a rate as to provide a competent blood-spleen barrier (Weiss *et al.*, 1986, Weiss, 1989; 1990). This barrier appears to exclude pRBCs 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 pRBCs 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

The liver is an important organ in the phagocytosis of malaria parasites. In splenectomised animals the liver takes over most of the extra burden of phagocytosis (Taliaferro & Cannon, 1936). In *P. berghei* infections of mice, the liver has a greater macrophage activity than the spleen (Singer, 1954). Partial hepatectomy of rats resulted in a striking enhancement of parasitaemia early in *P. berghei* infections (Cantrell & Moss, 1963).

Hepatomegaly occurs during malaria blood stage infections (Singer, 1954; Russell *et al.*, 1963), the liver becoming extremely friable and dark. During *P. berghei* infection of rats hypertrophy of Kupffer cells 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 cells (Singer, 1954; reviewed by Aikawa *et al.*, 1980).

(d) The immune reponse to gametocytes and gametes

Since the gametocyte is the stage of the malaria life cycle which transfers 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, temperature (Sinden & Smalley, 1976) and pH (Carter & Nijhout, 1977) changes trigger both male and female gametocytes to leave their erythrocytes. The gametes formed are then open to attack from any elements of the vertebrate's host immune response also present in the blood meal (Sinden & Smalley, 1976).

Vaccination studies have revealed that while gametocytes are poor immunogens (Carter *et al.*, 1979a), vaccination with crude gamete mixtures of *P. gallinaceum* (Carter & Chen, 1976), *P. knowlesi* (Gwadz & Green, 1978) and *P. yoelii* (Mendis &

Targett, 1979) limits or totally blocks mosquito transmission of challenge infections.

Serum from vaccinated animals does not affect the ability of gametocytes to initiate exflaggelation *in vitro* (Gwadz & Green, 1978; Mendis & Targett, 1979; Carter *et al.*, 1979b). The male gametes, however, are subsequently immobilised (Carter *et al.*, 1979b; Mendis & Targett, 1981), agglutinated (Carter *et al.*, 1979b; Mendis & Targett, 1982) or fixed to the surface of the slide coverslip on which they are observed (Carter *et al.*, 1979b). Induction of Abs can also 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) but the mechanism is unknown.

Harte *et al.* (1985a,b,c) have reported a form of transmission blocking immunity for *P. yoelii nigeriensis*. Adoptive transfer of immune T cells to naive recipients resulted in a near total reduction in transmission to mosquitoes following subsequent infection of grafted mice. The effect was best achieved with $CD4^+$ 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 response to gametes of *P. falciparum* has also been demonstrated *in vitro* (Good *et al.*, 1987a). 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 stage *P. falciparum* parasites.

Ab-mediated anti-gamete immunity is quite easily induced in man during malaria infection. Studies in Sri Lanka and Papua New Guinea have shown detectable levels of Abs to gamete surface Ags for both *P. falciparum* and *P. vivax* (Mendis *et al.*, 1987; Graves *et al.*, 1988) and these Abs appear to be potent in reducing infectivity of the parasites to the mosquitoes. However, frequent re-exposure to infection is required to maintain Ab levels (Mendis *et al.*, 1987) since long term anamnestic responses are poor (Ranawaka *et al.*, 1988).

1.9 Immunopathology

1.9.1 Non-specific cell activation

Polyclonal B lymphocyte activation occurs in malaria leading to a hyperproduction of IgG (McGregor *et al.*, 1956; Cohen & Butcher, 1969). 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 RBCs (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.*, 1971a), 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.

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 cytokines (Ballet *et al.*, 1987; Kabilan *et al.*, 1987).

1.9.2 Immunosuppression

Ag-specific unresponsiveness is quite often observed at the time of disease manifestation in residents where falciparum malaria is endemic (Ho *et al.*, 1986; Riley *et al.*, 1988b). In addition, malarious children have been shown to be deficient in their ability to mount primary immune reponses to certain, but not all non-plasmodial Ags. Vaccination with tetanus toxoid (McGregor & Barr, 1962); the O Ag of *Salmonella typhi* (Greenwood *et al.*, 1972; Greenwood, 1984) and Group C meningococcal vaccine (Williamson & Greenwood, 1978) resulted in lower Ab levels and lower percentages of seroconversions than in uninfected controls.

Malaria-induced immunosuppression is also instrumental in the association between malaria, Epstein Barr Virus (EBV) and Burkitt's lymphoma (Burkitt, 1969). This hypothesis is supported by *in vitro* findings of Whittle *et al.* (1984), who reported that T-cell control by EBV-infected B cells was lost during *P. falciparum* malaria.

1.9.3 Cerebral Malaria (CM)

CM is the worst manifestation of infection with *P. falciparum.* The pathology of this disease is not directly parasite-related but instead may be due to induction of a detrimental host immune response to the malaria parasite. Vascular congestion and plugging of blood vessels with heavily parasitised RBCs (Polder *et al.*, 1983), brain oedema (Oo *et al.*, 1987), and damage of endothelial cells with altered capillary permeability (Areekul *et al.*, 1984; Depierreux *et al.*, 1987) are all recognised features of CM. The pathogenesis of these changes remains unknown although various hypotheses have been proposed. These include endothelial lesions (Wash, 1979) with attachment of monocytes to the endothelium (Rest, 1982); sequestration of pRBCs in capillaries (Yoeli & Hargreaves, 1974; MacPherson *et al.*, 1985), possibly related to

the particular adhesiveness of pRBCs 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).

Rodent malaria infections have been used to study CM, although none can be labelled as falciparum-like by parasitological, morphological or molecular criteria. However, they 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). In addition they have implicated T cells in the development of experimental CM, since neurological complications are less severe in thymectomised or athymic animals (Wright *et al.*, 1971; Finley *et al.*, 1982). $CD4^+$ T cells render thymectomised mice fully susceptible to neurological complications, while $CD8^+$ cells did not induce pathological changes, although mice died of an overwhelming parasitaemia (Grau *et al.*, 1986).

Multiple factors must determine why some acute *P. falciparum* infections exhibit cerebral symptoms while others 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. Cytoadherence, however, appears to be consistent with all cases of acute CM in man. This is the attachment of pRBCs 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 CM is that of pRBCs with the endothelial cell lining of blood vessels (Trager *et al.*, 1966; Luse & Miller, 1971; MacPherson *et al.*, 1985). Cellular adhesion between pRBCs and other host cells, including nRBCs (Handunnetti *et al.*, 1987;1989) may also be important in sequestration and pathology of acute *P. falciparum* malaria.

Three molecules have been discovered to be involved in the adhesion of pRBCs to the endothelial cell surface. These are: the integral membrane glycoprotein CD36 (Oqendo *et al.*, 1989); thrombospondin (Roberts *et al.*, 1985); and the intercellular adhesion molecule ICAM-1 (Berendt *et al.*, 1989). Howard & Gilladoga (1989) have suggested that *P. falciparum* pRBCs adhere to one or more of the receptors but not all of them. Induction of ICAM-1 expression is regulated by cytokines (Staunton *et al.*, 1988); indeed, TNF-α, Il-1, Il-2 and IFN-γ can all upregulate ICAM-1 levels on diverse cell types (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 high levels of ICAM-1 are also infected with a strain of malaria parasite that has a high affinity for ICAM-1.

In the *P. berghei*/CBA/Ca mouse model, it has been demonstrated that excessive

release of TNF plays a critical role in the pathogenesis of experimental CM (Grau *et al.*, 1987). Elevated serum TNF levels were seen only at the time of neurological syndrome; *in vivo* depletion of $CD4^+$ T cells, which blocks neurological complications, prevented the dramatic rise in serum titres of TNF (Grau *et al.*, 1986). Moreover, treatment with a single injection of anti-TNF Ab exerted a protective effect on the *P. berghei*-induced neurological syndrome (Grau *et al.*, 1987) and prevented all forms of lesions. Administration of recombinant murine TNF to a strain of mouse resistant to CM induced a lethal neurological complication with all the clinical and histopathological features of CM (Grau *et al.*, 1989b). These observations show that TNF has both beneficial and deleterious effects depending on the degree of activation, timing and location, and thus may confer protective immunity, or, alternatively cause immunopathology during malaria infection.

TNF is known to induce NO. This may contribute to malaria associated pathology, particularly the coma accompanying CM (Clark *et al.*, 1992). NO produced by vascular and endothelial cells stimulated by TNF-α might diffuse into the brain and disrupt the normal regulation of glutamate-induced neural NO, resulting in alterations in neurotransmission and possibly coma. However, evidence against this accumulates and has been reported earlier.

In man altered levels of TNF have been reported in the serum of malaria patients (Scuderi *et al.*, 1986; Van der Meed *et al.*, 1988; Grau *et al.*, 1989c) and the levels shown to correlate with disease severity. TNF may, therefore, also be of pathogenic significance in human CM.

1.10 Immune evasion

Malaria infections are characteristically of long duration. In the case of *P. vivax* infections, latent liver stages intermittently complete their development and infect the blood. In the other malarias, however, such as *P. falciparum*, there are no latent forms and the persistent fluctuating blood infection must reflect, therefore, either an incomplete immune response, and/or evasion by the parasite of the full effects of the host's acquired resistance.

1.10.1 Sequestration

RBCs 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 is 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 may cause CM. 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. In other non-human primates schizont withdrawal of this species does not occur to the same extent and the main sites are different (Miller, 1969; David *et al.*, 1983). Therefore, host factors clearly influence sequestration. Sequestration occurs to some degree in *P. vivax* infections (Garnham, 1966) but not in *P. malariae* infections (Howard, 1988).

Relative schizont disappearance has been shown for several primate and rodent species. These include *P. coatneyi, P. fragile* (Desowitz *et al.*, 1969; Fremount & Miller, 1975), *P. knowlesi* (Miller *et al.*, 1971a) and *P. berghei* (Alger, 1963; Weiss, 1983b; Mackey *et al.*, 1980; Rest, 1982; Warrell, 1987). *P. c. chabaudi*, used in this study, also exhibits peripheral withdrawal (McDonald & Phillips, 1978; Gilks *et al.*, 1990) and schizonts have been noted to accumulate markedly in the liver (Cox *et al.*, 1987).

Marked stage-specific peripheral withdrawal of pRBCs 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). The extent, however, 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.

Cytoadherence of pRBCs to endothelial cells during falciparum malaria represents the acquisition of a specific, functional cell surface property on the infected cell. Immature pRBCs, rings and early trophozoites, and nRBCs 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 pRBCs, must retain structural and functional homogeneity regardless of other phenotypic variations and therefore, it could represent an antigenically conserved target for an anti-malaria vaccine (Howard, 1988).

1.10.2 Antigenic variation

Extensive serological and immunochemical data point to the ability of blood stage plasmodia to undergo antigenic variation. Evidence for this phenomenon was presented by Cox (1962) for *P. berghei* and Brown & Brown (1965) in studies on the SICA antigen of *P. knowlesi* in rhesus monkeys. They found that antisera collected from monkeys reacted only with the population of parasites collected prior to the

antisera. Parasites collected from later relapse populations in the same host did not react significantly with the earlier antisera. Howard *et al.* (1983) have confirmed these findings with cloned parasite lines of *P. knowlesi*. Antigenic variation of a cloned line of *P. falciparum* has also been demonstrated in Saimiri monkeys (Hommel *et al.*, 1983) and *in vitro* (Biggs *et al.*, 1991; Roberts *et al.*, 1992). Whilst the mechanism responsible remains unclear, major changes at the genomic level do not appear to be necessary (Hommel *et al.*, 1991).

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 but before any recrudescences had occurred (McLean *et al.*, 1982b). This characteristic was stable through syringe passage and cryopreservation. Reversion to serum-sensitivity was, however, observed after the recrudescent population was passaged through mosquitoes (McLean *et al.*, 1987). Using a live IFAT on schizont-infected RBCs Brannan *et al.*(1993) have also shown that mosquito transmission results in a change of antigenicity of cloned recrudescent parasites. Variant parasites have been detected during the ascending patent parasitaemia with a high rate of switching observed (Brannan *et al.*, 1994), and have also been detected during the period of declining parasitaemia (McLean *et al.*, 1990).

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-antigens, expressed at late trophozoite and schizont stages have been shown to be one group of Ags that is variable. 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 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.

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; Gilks *et al.* (1990), however, have reported the use of *P. c. chabaudi* AS in CBA/Ca mice as such a model. Both clonal antigenic variation and deep vascular schizogony in the liver have been reported, and both these features were modulated by the spleen. Surface Ag expression was crucially involved in the sequestering phenotype.

1.10.3 Antigenic diversity

Antigenic diversity 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 pRBCs taken at various times from a non-cloned parasite isolate cultivated *in vitro*. 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).

Significant antigenic diversity has been demonstrated in *P. falciparum* in humans (eg. Jeffrey, 1966) by challenge with a heterologous strain of parasite from the infecting strain. 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 sequences. Nevertheless, *P. falciparum* is still considered a single species worldwide in which gene exchange can freely occur (Walliker, 1983).

It has been shown that numerous malarial Ags exhibit antigenic diversity in different *P. falciparum* isolates; eg. the major glycoprotein on the surface of mature asexual stage pRBCs (reviewed by McBride *et al.*, 1982) and the S Ag released into plasma during rupture of schizont-infected RBCs (Wilson, 1980).

The great phenotypic diversity achieved by malaria parasites is thought to be of advantage to the parasite as many host immune responses are relatively specific, at least initially. Within the limited information available, it has always been assumed that the antigenic diversity of various pRBCs 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 with human malaria (Walliker *et al.*, 1987).

1.11 Development of a malaria vaccine

Due to the inability of insecticides and chemotherapy/chemoprophylaxis to eliminate the vectors or disease caused by *Plasmodium*, vaccines against the parasite are urgently needed.

Each of the different stages of the malaria life cycle presents 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, pre-erythrocytic forms, asexual and sexual forms. These will be

described later. However, as immunity appears to be stage specific, this presents a problem in that a parasite which escapes immunity at one stage of the life cycle may be unaffected by this immunity during its development in the next stage. Therefore, an effective vaccine may need to be multicomponent, providing protective immunity to all stages of the life cycle.

The features 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 contribute by far the largest populations that would benefit from successful vaccination and they are the major target group for eventual vaccine use. Effective natural immunity to malaria is achieved only after repeated infection, usually over a period of years. At such a time, blood infections may not be suppressed completely and asymptomatic parasitaemias persist. It is possible that an asexual blood stage vaccine would have an effect similar to that produced by natural exposure, merely limiting disease severity whilst not completely preventing parasitaemia. In such a situation, subsequent infections may boost vaccine-induced immunity.

Attenuated (Clyde *et al.*, 1973; Weinbaum *et al.*, 1976b; 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 and animals. Such vaccine preparations are not currently used for disease prevention because of the difficulties of large scale *in vitro* cultivation of plasmodia (Trager & Jenson, 1976) and the associated risks of preparing pRBCs from cultures containing human serum, which is an essential requirement of *in vitro* cultivation. If such problems were overcome, an attenuated live *P. falciparum* vaccine could be developed, one which, through gene deletion, had lost its capacity to adhere to endothelial cells (Pologe & Ravetch, 1986). These pRBCs would not cause organ pathology, notably CM, 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, the goal is 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. It is necessary that these vaccines contain multiple T cell epitopes, to overcome genetic restriction, for natural boosting of the antibody response. Moreover, these vaccines must preferentially induce immune effector mechanisms rather than responses with no protective activity or worse still causing immunopathology.

1.11.1 Sporozoite/pre-erythrocytic stage vaccine

A fully effective sporozoite vaccine is expected to induce immunity which prevents infection by either stopping invasion of liver cells, or if invasion occurs preventing the parasite completing its pre-erythrocytic cycle. Incomplete immunity, through a single sporozoite escaping the response, would not prevent infection of the blood and in the non-immune in particular may not prevent disease. However, a reduction in the number of sporozoites invading or completing the liver cycle may delay the numbers of blood stage parasites increasing to levels which normally might cause pathology.

Attenuated sporozoites, eg. through X-irradiation, in rodents, monkeys (reviewed by Nussenzweig & Nussenzweig, 1986) and in man (Clyde *et al.*, 1975) can induce a sterilising immunity. A mAb to the sporozoite coat protein (circumsporozoite/CS protein) was protective in mice to a sporozoite challenge (Yoshida *et al.*, 1980). The gene coding for the CS protein was subsequently cloned and sequenced for human, simian and murine malarias (Ellis *et al.*, 1983; Dame *et al.*, 1984; Enea *et al.*, 1984; Arnot *et al.*, 1985; Eichinger *et al.*, 1986; Lal *et al.*, 1987). The CS protein is the major sporozoite protein and in each species it has the same general makeup. The central third consists of multiple repeating immunogenic sequences which are unique for each species.

In *P. falciparum* the major repeating sequence (~40 times) is asparagine-alanine-asparagine-proline (NANP). The central repetitive region is the immunodominant B cell epitope and antibodies to the repetitive region block sporozoites in culture (Zavala *et al.*, 1985). It is also conserved in different isolates of *P. falciparum* (Weber & Hockmeyer, 1985). For these reasons the repeat region was an attractive vaccine candidate and two human trials were carried out (Ballou *et al.*, 1987; Herrington *et al.*, 1987). In one trial the immunogen was a synthetic peptide, $(NANP)_3$, conjugated to tetanus toxoid, and in the other a 30 repeat unit of NANP, 2 repeat units of the minor repeat of asparagine-valine-aspartate-proline (NVDP) and a 32 amino acid tail of the tetracycline resistance gene (R32tet32) encoded by the vector : both used aluminium hydroxide as adjuvant. Protection in both studies correlated with antibody levels; most of the vaccinees, however, had poor antibody

responses. From these studies the necessity to include T cell epitopes, preferably from the CS protein itself, was highlighted.

1.11.2 Asexual blood-stage vaccines

Protective immune responses to the asexual blood stages of malaria parasites include both antibody and non-antibody mechanisms and have been described elsewhere.

A large number of Ags of asexual blood-stages of *P. falciparum* have now been described and the genes or gene fragments encoding many of them have been cloned. As yet none of the antigens appears to be especially important in inducing protection alone and it is therefore likely, that an effective vaccine will require a combination of several antigens.

Antigens currently being considered as candidates for inclusion in an anti-*P. falciparum* asexual blood stage vaccine are briefly described below. The major surface proteins of the merozoite of *P. falciparum* are a complex of processed products of a 185-220 kDa precursor glycoprotein and have variously been termed PMMSA, p190, p185, PSA, MSPP, MSA1, MSP1. The protein is synthesised during schizogony and is proteolytically processed around the time of schizont rupture. A number of mAbs to MSP1 inhibit merozoite invasion *in vitro* and homologous proteins in animal models are involved in protective immunity (Holder & Freeman, 1984; Holder, 1988). Immunisation of monkeys with MSP1 purified from *P. falciparum* parasites (Siddiqui *et al.*, 1987) or with an N terminal synthetic peptide gave some protection (Cheung *et al.*, 1986; Holder *et al.*, 1988). Antigenic diversity in this molecule between isolates has been reported The relative importance of conserved and variable regions in inducing specific immune responses is being investigated, and the molecule is being mapped for T and B cell epitopes.

The ring-infected erythrocyte surface antigen (RESA or Pf155) was first detected by a modified immunofluorescence assay on ring-infected *P. falciparum* infected red blood cells (Holder *et al.*, 1988; Foley *et al.*, 1993). This molecule, MW 155 kDa, may facilitate invasion by interfering with membrane or cytoskeleton organisation. The gene coding for the molecule has been cloned and sequenced (Cowman *et al.*, 1984; Favaloro *et al.*, 1986). Immunodominant epitopes are found in two blocks of repetitive sequences. Antibodies against the native molecule or to various repeat sequences block invasion *in vitro*. Vaccine experiments in monkeys with fragments of the molecule gave limited protection (Collins *et al.*, 1986).

The gene for another merozoite surface antigen, MSA2 or MSP2, has been cloned and sequenced from several strains, and contains relatively conserved flanking regions around strain variable regions containing repetitive sequences (Smythe *et al.*,

1988; Smythe *et al.*, 1990). By SDS electrophoresis it has a molecular weight of approximately 45 kDa. Monoclonal antibodies to MSA2 have been found to inhibit merozoite invasion *in vitro*, which has encouraged further work on this molecule.

Rhoptry antigens at the apical end of the merozoite are implicated in the invasion process. Highly membranous material is discharged from rhoptries into a membrane lined vacuole which surrounds the merozoite as it enters the RBC (Bannister *et al.*, 1986; Crewther *et al.*, 1990). Two target antigens have been extensively studied. Rhoptry associated protein-1 (RAP-1) (Ridley *et al.*, 1990) is a non polymorphic Ag which protects Saimiri monkeys against a lethal challenge of *P. falciparum*. AMA-1 (apical membrane antigen-1) described by Crewther *et al.* (1990) shows minimal strain variation. For this reason there is considerable interest in this molecule, as well as its probable functional importance and conserved nature (Phillips, 1992).

Antigens on the surface of infected erythrocytes containing mature asexual forms of *P. falciparum* have been detected by several methods (Hommel & Semoff, 1988). No RBC surface antigen has yet been used in a vaccine trial. Several Ags, however, are of particular interest. PfEMP-1 may mediate the the cytoadherence of the schizont infected cells to vascular endothelium (sequestration). Antigenically it is very diverse and varies in size from 240-350 kDa (Leech *et al.*, 1984; Marsh & Howard, 1986; Anders & Smythe, 1989; van Schravendijk *et al.*, 1991). As yet uncloned and despite considerable polymorphism, PfEMP-1 is an attractive vaccine candidate because induction of immunity would prevent cytoadherence and hence CM. PfEMP-2 (MESA) (Anders & Smythe, 1989); two histine rich proteins PfHRP-1 and PfHRP-2 (Howard *et al.*, 1986; Rock *et al.*, 1987); rosettin (Helmby *et al.*, 1993) and Ag332 (Mattei & Scherf, 1992; Mattei *et al.*, 1992) are other membrane surface Ags with vaccine potential.

Patarroyo and colleagues have recently carried out extensive testing of purified protein preparations from merozoites for protective activity in Aotus monkeys (reviewed by Moreno & Patarroyo, 1989). On the basis of these experiments and information from published work they prepared two hybrid polymers, SPf66 and SPf105. The former contained two merozoite specific proteins of unknown location, a fragment of MSP-1 and CS repeats. SPf105 was made up of a MSP-1 fragment, a repeat of Pf155/RESA, CS repeats and a T cell epitope from the CS protein (Patarroyo *et al.*, 1987). In current vaccine trials the polymers are combined with alum. Initial studies (Patarroyo *et al.*, 1988; Valero *et al.*, 1993) and the recent reports by Sempértegui *et al.* (1994) and Teuscher *et al.* (1994) in vaccine trials of SPf66 show protection against severe clinical disease in a proportion of vaccinees.

pRBC rupture allows merozoite release and invasion of new RBCs. Since

pRBC development *in vivo* is synchronous, large amounts of pRBC-derived lipids, glycolipids and (glyco)proteins, as well as particulate materials, are released into the plasma within a relatively short time. These materials undoubtedly elicit host responses that lead to the periodic symptoms of acute malaria. It has been proposed that vaccine-induced Abs specific for these components could lead to immune-mediated removal of these Ags without elicitation of the cascade of undesired host responses (Playfair *et al.*, 1990; Playfair, 1990; Kwiatkowski, 1991; 1992).

1.11.3 Sexual stage vaccine/Transmission blocking vaccine

A vaccine against the sexual stages of the parasite would not protect the vaccinee but would interrupt transmission by abolishing or reducing the ability of gametocytes from that individual to infect a biting mosquito. Such vaccines would only interrupt malaria transmission at the level of the mosquito, but would not afford any protection in the vaccinated human. The inhibitory immune response would be directed against gametocytes, gametes or zygotes/ookinetes. Animal studies (reviewed by Carter *et al.*, 1988; Targett, 1990; Kaslow *et al.*, 1992) have demonstrated the feasibility of artificially inducing transmission blocking immunity and there is also evidence that transmission blocking immunity develops in human populations to *P. vivax* (Peiris *et al.*, 1988) and to *P. falciparum* (Carter *et al.*, 1988). Abs appear to be the major effector mechanism but T cell mediated Ab-independent mechanisms have a role (Harte *et al.*, 1985).

A number of vaccine candidate Ags have been identified. Three target Ags (230 kDa, 48/45 kDa and 25 kDa) expressed on the surface of the extracellular forms of the sexual stage parasite have received the most attention. Abs to Pfs230 and Pfs48/45 (which are also present in the intracellular gametocyte) disrupt development of the parasite if they are present before fertilisation occurs (Rener *et al.*, 1983; Quakyi *et al.*, 1987) and can be detected in immune sera in some humans resident in malarious areas (Graves *et al.*, 1988; Carter *et al.*, 1989; Quakyi *et al.*, 1989).

The Pfs25 gene has been cloned (Kaslow *et al.*, 1991) and Abs to this Ag can completely block transmission to mosquitoes (Kaslow *et al.*, 1988). Polymorphism in Pfs25 is minimal, the gene has been inserted into both vaccinia virus and yeast (Kaslow *et al.*, 1992), and expression of the recombinant protein found to be correct in both systems. These results suggest that human trials will soon be justified with a yeast secreted analogue of Pfs25, however, immunity to Pfs25 would not be boosted by natural infection.

1.12 History of *Plasmodium chabaudi chabaudi*

Plasmodium chabaudi chabaudi, the parasite used in this study, 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 RBCs (Landau, 1965) although under conditions of anaemia, for example following peak parasitaemia, it can invade reticulocytes (Carter & Walliker, 1975; Jarra & Brown, 1989). Multiple infection of RBCs with *P. c. chabaudi* occurs (Carter & Walliker, 1975).

P. c. chabaudi is an invaluable model in which to carry out many aspects of malaria research. It has become a recognised animal model for *P. falciparum* infection in humans, showing a number of important similarities (Long, 1988; Mons & Sinden, 1990; Gilks *et al.*, 1990). Asexual stage parasites of both species have a synchronous cycle in the peripheral circulation, although for *P. c. chabaudi* the asexual erythrocytic cycle is completed in only 24 hrs. Peripheral withdrawal of schizonts to deep tissue capillaries occurs in both species as schizont maturation takes place (Shungu & Arnold, 1972; McDonald, 1977; McDonald & Phillips, 1978; Gilks *et al.*, 1990).

The CB strain of *P. c. chabaudi* was selected for this project because it is a more virulent strain of parasite than the commonly used AS strain. Cloned, well characterised lines of the CB strain have been established by Carter & Walliker (1975) in laboratory mice from wild-caught infected *Thamnomys* 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 *Eperythrozoon coccoides* and *Haemobartonella muris* (Cox, 1978). Isoenzyme patterns have been established for these CB strain clones, which have, unlike many laboratory strains of rodent malaria, remained close to the original isolate (Beale *et al.*, 1978; D. Walliker, personal communication)

While *P. c. chabaudi* CB is virulent, infected mice usually eliminate infection with this strain. In NIH mice, which show a genetic resistance to *P. c. chabaudi* (Stevenson *et al.*, 1982), it displays a course of infection consisting of a high peak parasitaemia followed by a subpatent period of unpredictable duration, and one or two short lasting recrudescences of low parasitaemia (Figure 1.12.1a). It is sometimes necessary, however, to assist mice through the peak parasitaemia with subcurative chloroquine treatment. Such treatment results in rapid parasite remission but a higher recrudescence than that seen in naturally recovering mice follows (Figure 1.12.1b). In addition, the course of infection is much more predictable following chloroquine intervention.

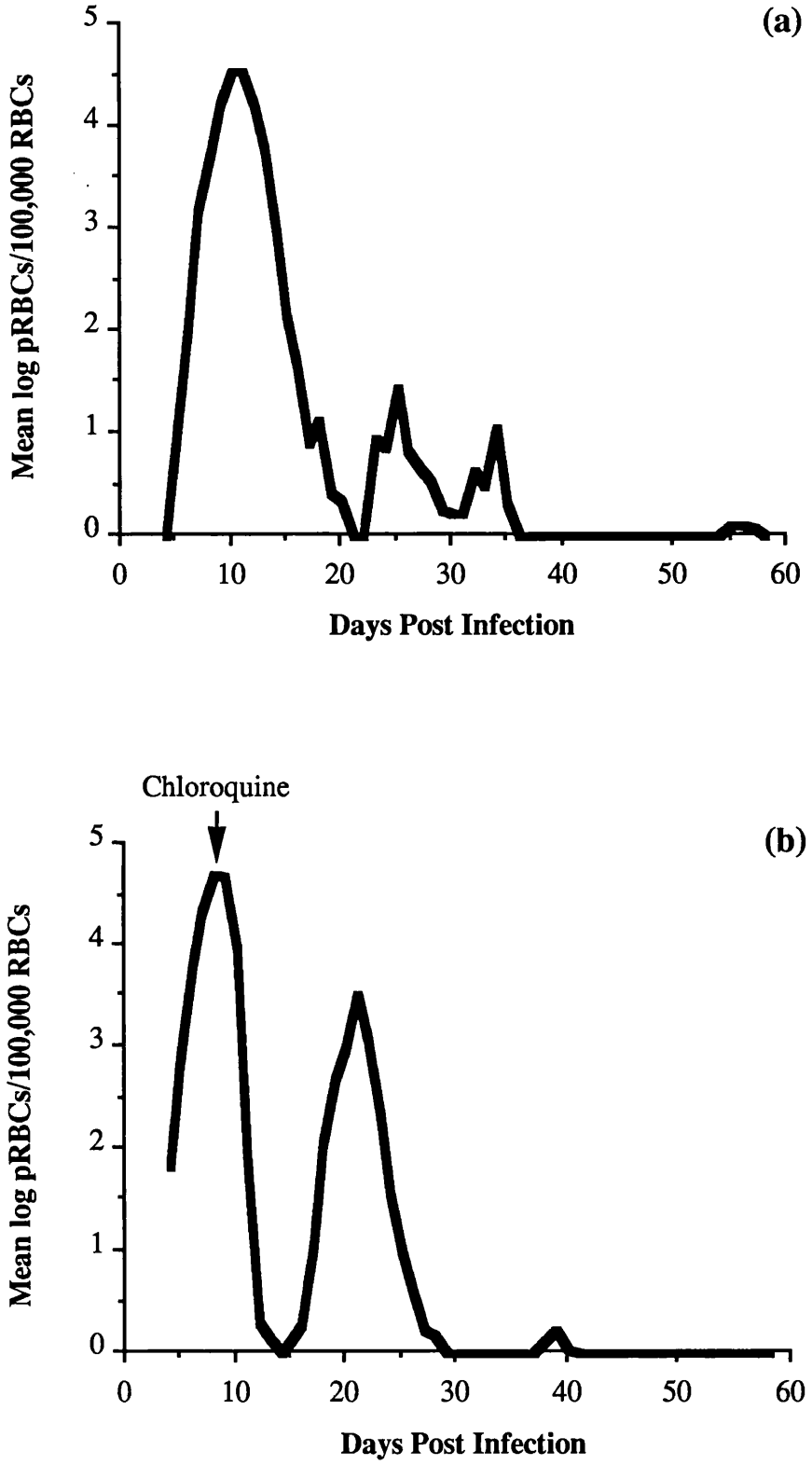


FIGURE 1.12.1 The course of *Plasmodium chabaudi chabaudi* CB strain infection usually observed in naive NIH mice (a) naturally recovering from infection, or (b) treated with chloroquine 10 days post infection.

1.13 Experimental Rationale

Within Professor Phillips' laboratory much work has been carried out on the role T cells play during infection with the AS strain of *P. c. chabaudi.* It is now clear that the major protective immune mechanisms to the erythrocytic stages of this strain of parasite require the presence of $CD4^+$ T cells. Indeed, cloned T cell lines of either the T_H1 or T_H2 phenotype (Taylor-Robinson & Phillips, 1992; 1993; 1994a) can protect immunocompromised mice upon adoptive transfer. This model of infection, however, differs from another subspecies of *P. chabaudi, P. c. adami*, where immunity is mediated solely by Ab-independent mechanisms (Grun & Weidanz, 1981). Only a limited number of studies have been carried out using the more virulent strain, *P. c. chabaudi* CB. One report suggests that this strain is also resolved in a totally Ab-independent manner (Cavacini *et al.*, 1990), thereby differing from the AS strain (Langhorne *et al.*, 1989; Taylor-Robinson & Phillips, 1992; 1993; 1994a). The experiments described in this thesis were performed to define further the role of cellular immunity to rodent malaria infection, using the *P. c. chabaudi* CB/NIH mouse model. In this host parasite combination there is an unpredictable course of infection, and in order that mice survive the acute peak parasitaemia subcurative chloroquine treatment is often required at this time. Such treatment, therefore, also allowed the effect of chemotherapy on the development of an immune response to be investigated.

At the outset of this study, the effect of depletion of specific T cell subsets on the course of *P. c. chabaudi* CB infection by mAb treatment was investigated. This established that T cells of the $CD4^+$ phenotype were critical for resolution of this strain. Adoptive transfer experiments were performed with lymphocyte populations taken from naturally recovered or chloroquine treated donor mice at various times during infection. This was of interest, for all previous reconstitution studies had employed lymphoid populations taken after parasite clearance from recovered animals. Initial findings showed that immunity could be transferred with lymphocytes prepared from all stages of infection investigated. Interestingly, T cells from donor mice 21 days post infection could transfer an immunity that appeared to transcend antigenic variation. To analyse this further, a passive transfer system was employed to dissect the degree of immunity which could be transferred with serum and to look at the ability of the CB strain of *P. c. chabaudi* to undergo antigenic variation, a phenomenon well documented for the AS strain (McLean *et al.*, 1982b; 1986a; Gilks *et al.*, 1990). In addition, the effect of chloroquine treatment on antigenic variation was also considered. The role of nitric oxide during the course of infection of *P. c. chabaudi* CB was also studied and the effect of different chloroquine treatment regimes on the production of NO determined. Finally, to examine further

the role of the CD4+ T lymphocyte subset in the mediation of protection to *P. c. chabaudi* CB infection, an attempt was made to establish spleen cells *in vitro* as homogeneous CD4+ T cell lines. The ability of one successful line to moderate a challenge infection and to respond *in vitro* to *P. c. chabaudi* CB Ags is described.

The results of the experiments outlined above are described and the dynamics of T cell subsets during the evolution of an effective immune response to malaria infection are discussed with regard to the development of effective asexual blood stage malaria vaccines. The implications of subcurative chloroquine treatment on the development of immunity are also considered.

CHAPTER TWO

MATERIALS AND METHODS

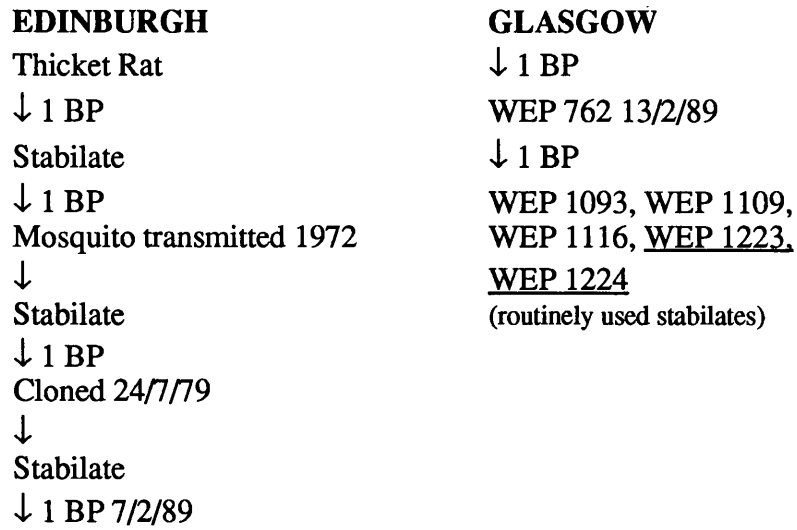
2.1 Mice

For most experimental procedures female inbred NIH mice were used. These were bred in the WLEP animal house breeding facility, inbred to between 26 - 35 generations from an original mating pair purchased from Hacking and Churchill Ltd in 1980. All mice born in the breeding facility were weaned at three weeks of age, and were used for experimental procedures between 6 and 12 weeks old when they weighed approximately 25 g. The mice were kept in the animal house at 22°C ± 2°C and 50-60% relative humidity and maintained in 12 hrs artificial light from 0800 to 2000 hr. They were maintained on pelleted CRM breeder diet (Labsure Ltd) and given both food and water *ad libitum*.

2.2 Parasites

The CB strain of *Plasmodium chabaudi chabaudi* was originally isolated from a thicket rat (*Thamnomys rutilans*) caught in the Central African Republic in 1970 and transported to Edinburgh (Beale *et al.*, 1978). The parasite line was established in laboratory mice and then cloned by limiting dilution (Walliker *et al.*, 1971) in 1979 and the parasites frozen. The parasites were supplied to WLEP by Professor Walliker from the World Health Organisation Registry of Standard Strains of Malaria Parasites in 1989. Since then, CB parasites have been maintained by cryopreservation and subpassage through mice (see 2.3 & 2.4). Figure 2.2.1 shows a history of *P. c. chabaudi* CB since isolation.

FIGURE 2.2.1
History of *Plasmodium chabaudi chabaudi* CB strain populations
Derived from *Thamnomys rutilans* caught in the Central African Republic in September 1970



2.3 Maintenance of Parasites

Suspensions of pRBCs 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 (1984a,b). Each stabilate was defrosted by immersion of the vial in a 37°C waterbath, then diluted with an equal volume of 15% w/v glucose in PBS (pH 7.2) (Appendix) before immediate i.v. or i.p. injection into one or two recipient naive mice.

Parasites were maintained by blood passage in mice every 2-4 days. Mice were bled by cardiac puncture, under terminal ether anaesthesia, into a syringe containing sodium heparin (1000 i.u./ml, Evans Medical Ltd.) in PBS as an anticoagulant, at 10 i.u. heparin per ml of blood. The infected blood was subinoculated into recipient mice either by i.v. or i.p. injection. Experimental mice were infected with blood one passage from stabilate.

2.4 Cryopreservation of infected blood

Parasites were cryopreserved as stabilates using the method of Phillips & Wilson (1978). Blood with a parasitaemia of 5-10%, containing ring stage parasites (rings survive the freezing and subsequent thawing the best), was collected into heparinised PBS (10 i.u./ml) and diluted 1:1 with a solution of sorbitol-glycerol (Appendix), added slowly dropwise with frequent mixing (Gray & Phillips, 1981). 0.2 ml aliquots were dispensed into 1.2 ml cryopreservation vials, each labelled with the WEP code and a number. These were immediately snap frozen by immersing in liquid N_2, and stored in cannisters (Union Carbide or Taylor-Wharton).

2.5 Challenge Infections

Infected blood for challenge infections was obtained by cardiac puncture (as above) and collected into 10 i.u./ml heparinised PBS. The parasitaemia of the donor mouse was determined by examination of a Giemsa's stained thin blood smear (see below). The blood was diluted to the required concentration of pRBCs/ml in complete RPMI 1640 medium (Gibco) (Moore *et al.*, 1967) (Appendix). For all infections, naive mice were challenged with 5×10^4 pRBCs administered as a 0.2 ml i.v. inoculum, using a 1 ml syringe fitted with a 26 G needle (both Becton Dickinson).

2.6 Inoculation

Prior to injection mice were warmed gently under a heat lamp to cause vasodilation. I.v. infection was then performed via one of the lateral tail veins.

2.7 Chloroquine treatment

Where necessary experimental mice were treated with subcurative doses of chloroquine. Each mouse received approximately 50 mg/kg chloroquine diphosphate (Sigma) in PBS (Appendix) administered i.p. as a 0.25 ml inoculum on one or sometimes two occasions.

2.8 Determination of parasitaemias

Infections with *P. c. chabaudi* CB were monitored by determining parasitaemias from thin blood smears made directly from infected mouse tail blood. The smears were taken between 0900 - 1100 hr each day.

Blood smears were made on microscope slides with ground glass edges (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 between each mouse and group of mice in order to minimise the possibility of transfer of pRBCs between animals. The smears were air dried 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 Giemsa's phosphate buffer (pH 7.4) (Appendix) for 30 min. 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.

Parasitaemias were obtained by calculating the number of pRBCs from a total number of RBCs. If the parasitaemia was >2-3% (determined by observing more than 3-4 parasites per field of view), direct counts of the number of parasites per 500 RBCs 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 250-300 RBCs. Infections were considered subpatent when no parasites were seen in 50 fields.

For experimental procedures, the day of infection was termed d 0 and smears were taken from d 4, when most mice began to carry a patent infection, to d 60, when all mice had cleared infection.

2.9 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 pRBCs/10^5 RBC) against time (expressed in days). Vertical bars showing one S.D. are included where necessary. However, for clarity, S.D.s are only put in if differences in the courses of parasitaemia 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 the peak parasitaemia; (iii) any extension of the time taken for the primary parasitaemia to be resolved relative to 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 controls.

The minimum number of mice in each experimental group was 5-6 for all *in vivo* studies. 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 was encountered in the counts, especially after crisis and resolution of the primary parasitaemia. The data were transformed using Cricketgraph version 1.3.2 (Cricket Software) on an Apple Macintosh Classic microcomputer (Scotsys Computer Systems). Nonparametric statistical analysis was performed on parasitaemia data using the Mann-Whitney or Kruskal-Wallis tests. Test results and degrees of freedom have been inappropriately omitted from the text; only p values are presented.

2.10 Production of mAbs for T cell subset depletion

Rat IgG_{2b} mAbs to murine T lymphocyte subsets were prepared by propagation of Ab-secreting hybridoma cells as ascites tumours in (LOU x DA) F_1 rats. The hybridomas were obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology and 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 CD4 and CD8 the differential expression of which separates all T cells into two subsets. YTS 169.4 binds to mouse suppressor/cytotoxic T cells ($CD8^+$) and their precursors *in vitro*, but not to helper T cells. Conversely, YTS 191.1 is specific for CD4-bearing mouse helper/inducer and delayed hypersensitivity T cells, but not for cytotoxic T cells or their precursors *in vitro* (Aqel *et al.*, 1984).

mAb production in high concentration was conveniently achieved by growing the Ab-secreting hybridoma cells *in vivo* as ascites tumours in (LOU x DA) F_1 rats primed with mineral oil (Potter *et al.*, 1972). 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 complement mediated cytolysis (Bruce *et al.*, 1981) and immunofluorescence (Cobbold *et al.*,

1986) respectively.

2.10.1 *In vitro* culture of hybridoma cells

The hybridomas were received as two frozen ampoules packed in dry ice. The contents of each cryopreservation tube were thawed rapidly in a 37°C water bath, and the cells washed in excess RPMI 1640 medium containing 10% FCS (complete medium) (250 g for 5 min). They were then cultured at 37°C in an atmosphere of 5% CO_2 at 2 x 10^5 cells/ml. When the cells had reached confluency they were subcultured every 2-3 days in 20ml volumes at 2 x 10^5 cells /ml. Stocks of the two cell lines were cryopreserved in liquid N_2 for longterm storage as outlined later (2.29-Cryopreservation of T cell lines).

2.10.2 Ascites production

Adult female LOU and male DA rats were purchased from Harlan Olac Ltd and were mated in the WLEP animal house. At 12 weeks old ten male (LOU x DA) F_1 hybrids were used. Rats were primed with an i.p. injection of 0.5 ml of pristane, 2,6,10,14-tetramethylpentadecane, a component of mineral oil (Hoogenraad *et al.*, 1983; Brodeur *et al.*, 1984) (Aldrich Chemical Co.), 7 d prior to inoculation with 5 x 10^6 cells of each hybridoma 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 Universals. Animals were tapped subjectively based on the degree of swelling from 12-19 d after hybridoma implantation. On each day, the ascites collected were centrifuged at 500 g for 10 min, the S/N harvested and stored at -20°C.

2.10.3 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. Aliquots of 20 ml of ascitic fluid were thawed in a 25°C waterbath and 5.4 g ammonium sulphate (BDH Ltd) added to each to make a 45% w/v solution. Each ascites sample was then incubated for 30 min at 25°C after thorough stirring. The large 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.

The redissolved precipitate was dialysed overnight against PBS (pH 7.2) to remove any traces of ammonium sulphate and to concentrate the IgG present in

solution. 35 cm lengths of cellulose acetate dialysis tubing (nominal MW cut-off 12000, flat width 35 mm) (Sigma) were presoaked in PBS and then sealed at one end with a Pierce dialysis clip. 20 ml of either mAb solution was then pipetted into the tubing. After sealing the top end, each tube was placed in a 5 l glass beaker filled with PBS and agitated gently with a magnetic bar and stirrer motor. The dialysis tubes were left overnight at 4°C to reach equilibrium. When dialysis was complete the volume in each tube had approximately doubled due to the high osmolarity of the salted out S/N. The partially purified IgG anti-Ly-2 and anti-Ly-4 mAb samples were pooled, filter sterilised (0.22 μm, Millipore), and the IgG concentration determined by a modification of the Bradford dye-binding assay. For each of the mAbs the protein concentration was approximately 16 mg/ml. The mAb preparations were stored as either 10 ml or 1 ml aliquots at -20°C and repeated freeze-thawing avoided.

2.11 Determination of total protein concentration

A quantitative estimation of protein concentration in the prepared rat mAbs (and pRBC and nRBC lysates - see later) was determined by spectrophotometric measurement at 595 nm. The procedure used was the BCA standard assay (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 adapted from the original technique of Bradford (1976), and is based on the absorbance shift from 465 to 595 nm which occurs when Coomassie Blue binds to proteins in an acidic solution. The standard assay procedure can be used to determine protein concentrations in the range of 150 μg/ml to 1500 μg/ml, where the colour response is relatively linear, allowing quick, accurate determinations.

A known protein concentration series was prepared by diluting a 2 mg/ml stock BSA standard (Pierce Chemical Co.) in deionised water. Convenient standard data points were used to cover the range 150-1500 μg/ml. 5 ml protein assay reagent was added to 100 μl of each of the diluted standards or unknown protein samples in 16 x 100 mm clear, clean test tubes. The samples were mixed well. Absorbance was read versus deionised water at 595 nm on a u.v. spectrophotometer (Pye Unicam PU 8600). The net absorbance of each standard or unknown protein sample was obtained by subtracting the absorbance of the deionised water from each sample. A standard curve was constructed from which the protein concentration for each unknown sample was determined.

2.12 *In vivo* depletion of T cell subsets

The mAbs raised as outlined above were used to deplete substantially *in vivo* either or both of the two subsets of T cells recognised. The methodology followed was essentially similar to that described by Süss *et al.* (1988). Mice were injected i.v.

with purified mAbs diluted to the appropriate concentration in PBS (pH 7.2) and delivered as a 0.25 ml inoculum. Animals received a total of three pretreatments before infection , 500 μg of purified Ab 5 d prior to challenge, followed by 250 μg Ab 4 d and 1 d before infection. At 14 DPI experimental mice were given a further dose of 250 μg Ab. A control group of mice that received normal rat serum in place of the specific mAb was set up in parallel.

At various time points throughout the course of an infection, individual mice were sacrificed and splenic T cells were examined to monitor the efficiency of depletion of the relevant T cell subset(s), as determined by immunofluorescence (see later).

2.13 Complement-mediated cytotoxicity assay

The specificity and cytotoxic potential of the anti-Ly-2 and anti-Ly-4 mAbs was ascertained by use of the technique of complement-mediated cytolysis (Bruce *et al.*, 1981). The mAbs were used to kill enriched splenic T cells carrying these Ags, with the aid of complement, and cell death was assayed by exclusion of the vital dye trypan blue (see later).

T cells prepared from the spleens of naive mice by incubation on nylon wool columns (see later) were washed twice (300 g for 5min) in 10% FCS RPMI 1640 medium and the proportion of viable cells assayed by the trypan blue dye exclusion. The concentration of cells was adjusted to 1 x 10^7 cells/ml.

Six serial dilutions of each of the mAbs were made in 1ml 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 the 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. After incubation the microcentifuge tubes were placed on ice to prevent any further complement fixation and cell lysis. The cells were washed again 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 an equal volume 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 phase contrast microscope. Assuming approximately equal proportions of both major T cell subsets in a suspension of naive spleen T cells, the efficacy of the mAbs was determined.

2.14 Determination of cell viability

The viability of cell preparations was measured by the trypan blue dye exclusion test (Naysmith & James, 1968; recommended by Jerne *et al.*, 1974). An appropriate dilution of cells in PBS (pH 7.2) 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 eyepieces) on a light microscope. Dead cells were unable to exclude the vital dye and stained blue, whereas viable cells remained clear. The proportion of live to dead cells was expressed as a percentage viability, and adjustments to total cell numbers made accordingly.
At least 100 cells were counted.

2.15 Live Immunofluorescent Antibody Test (live IFAT)

As a confirmatory test to determine the specificity of the rat mAbs raised against the **CD4** and **CD8** cell surface glycoproteins, splenic T cells were assessed for the presence of these two markers using live IFAT.

Serial 1:2 dilutions of the primary mAbs and normal rat serum (as a negative control) starting at an initial dilution of 1:10 were prepared in PBS (pH 7.2). 50 µl of each mAb dilution was mixed with 50 µl of test suspension (1 x 10^8 cells/ml) and incubated for 1 hr in a 37°C waterbath. The cells were then washed twice in PBS (300 g for 3 min in an MSE microcentaur microcentrifuge), resuspended in 100 µl of a 1:200 dilution of FITC-conjugated goat anti-rat IgG (Sigma) and incubated for 30 min at 37°C. Cells were washed twice as before to remove any unbound conjugate, resuspended in 100 µl of cold PBS and kept on ice until ready to view.

The % of positive cells was determined by fluorescence microscopy with the use of a Leitz incident light u.v. microscope equipped with a mercury lamp with an appropriate excitation filter for fluorescein. Positively stained cells were visible as green rings, since the FITC-conjugated antiserum is unable to cross the intact cell membrane of viable lymphocytes, while dead cells showed a bright homogeneous intracytoplasmic fluorescence. The % of live fluorescing lymphocytes was determined for each specimen tested from a minimum of 200 cells counted.

2.16 Irradiation of mice

Mice were irradiated with 400 rads (4 Gy) whole body gamma irradiation from a ^{60}Co source chamber (Nuclear Engineering) in the Department of Veterinary Physiology, University of Glasgow. To receive doses of 4 Gy mice were exposed for appropriate lengths of time depending on the dose output of the source. During the course of the study, the source emitted between 3.35 and 2.49 Gy/min. Mice were lowered in pairs, in a restraining container, into the source chamber and irradiated in an upright position. Preceding treatment mice were starved for 24 hours. Irradiation

of recipient mice took place either 24 hours prior to transfer or on the day of transfer. Subsequently, mice received terramycin (3g/l)(Pfizer) in their drinking water for one week to prevent superinfection-associated deaths (McDonald & Phillips, 1978).

2.17 Preparation of splenic lymphocytes for adoptive transfer i.v.

Suspensions of splenic lymphocytes suitable for adoptive transfer were prepared as follows. Donor mice were killed by ether (Rhône-Poulenc Ltd.) overdose, the spleens dissected out aseptically and placed in a 9 cm Petri dish (Sterilin) containing incomplete RPMI 1640 medium. The spleens were dissociated by pushing through a stainless steel sieve (mesh size 0.025 cm^2) using the inside plunger of a syringe to push the cells through, and collected into the medium. Using a sterile Pasteur Pipette the cells were disaggregated by aspiration, and the supernatant collected after connective tissue debris and clumps of cells had sedimented. The spleen cells were washed twice in fresh medium (250 g for 5 min), then resuspended in 1 ml of 10% FCS RPMI 1640 medium. Contaminating red blood cells were removed by haemolysis. 1 ml of spleen cells was incubated in 9 ml cold 0.83% Tris-ammonium chloride (pH 7.4) (Appendix) for 5 min at RT (Boyle, 1968) Most macrophages and other adherent cell populations were removed by filtration through glass wool (Julius *et al.*, 1973; Trizio & Cudkowicz, 1974). Glass wool columns were prepared by packing 10 ml plastic syringe barrels to the 8 ml mark with glass wool (Travenol Laboratories). Before the removal of phagocytic cells, the column was clamped in a vertical position and 30 ml incomplete RPMI 1640 medium followed by 10 ml 10% FCS complete medium washed through. The spleen cell suspension was loaded onto the soaked glass wool column under gravity, incubated for 15 min at RT and washed out dropwise with 10% FCS complete medium.

2.17.1 Enrichment of spleen T and B cell populations by nylon wool separation

Spleen cell suspensions were fractionated on the basis of their differential adherence to nylon fibres. Enriched T cell populations were 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).

Sterile nylon wool columns were prepared by packing tightly, to the 7 ml mark, the barrels of 10 ml plastic syringes with scrubbed nylon wool (Leuko-pak, Fenwal Laboratories) followed by autoclaving. These were stored wrapped in aluminium foil. Before use, the 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. The wet columns, sealed at the top with nescofilm (Bando Chemical Ind. Ltd) and at the bottom with a 25 G needle stuck

in a rubber bung, were kept in a humidified incubator at 37°C for at least 90 min prior to use. They were then washed through with 10 ml of warm, fresh medium before 2-3 x 10^8 cells in 2 ml volumes were added dropwise to each column. The cells were washed into the columns with 1 ml of medium, the columns resealed and incubated at 37°C for 45-60 min. After incubation, the non-adherent cells - considered as the enriched T cell population - were run out dropwise from the syringe and washed out with 20 ml of prewarmed medium. Nylon wool adherent cells were eluted from the columns by physically disrupting the nylon wool with flamed forceps and by forcing fresh medium through the column using a 10 ml syringe plunger. The released adherent cells were collected in 10 ml and thereafter referred to as the enriched B cell subpopulation. Both cell populations were washed and resuspended in 10% FCS RPMI 1640 medium and kept on ice until either injected into recipient mice or manipulated *in vitro*. Spleen cell numbers were counted in a haemacytometer (Improved Neubauer, Weber) and counts adjusted after cell viability was determined by the trypan blue exclusion method (2.14).

The proportions of cells in each enriched subpopulation was determined by immunofluorescence (for methodology see 2.15), using a rat IgG_{2b} mAb against Thy-1.2 (Sera-Lab) as a T cell marker, and a rat IgG_1, IgM cocktail mAb against mouse IgG heavy chain (Serotec) as a B cell marker. The enriched T cell populations typically contained >85% T cells with <1-2% contaminating B cells, while the B cell enriched populations contained >55% B cells with < 7% contaminating T cells.

2.18 Collection of serum

Large volumes of sera were collected by exsanguination by cardiac puncture under ether anaesthesia. Where appropriate the blood was pooled and allowed to clot. The clot was loosened from the edges of the container and left to contract for 1 hr at 37°C. The overlying serum was collected by pipetting and any contaminating RBCs removed by centrifugation (300 g for 5 min). The serum was then aliquoted and stored frozen at -20°C until required. Subsequent freeze-thawing was kept to a minimum. Large volumes of hyperimmune and normal mouse sera were collected using this methodology. A stock of hyperimmune serum for use as a positive control in the anti-*P. chabaudi* slide IFAT was raised from donors which were challenged on three occasions successively with 5 x 10^4, 1 x 10^7 and 1 x 10^8 pRBCs at two monthly intervals. The mice were bled one week after the last challenge. Normal serum was obtained from uninfected animals.

Smaller volumes of serum (up to 100 µl) were collected from tail blood by the method of Gray (1979) using hard glass capillary tubes (BDH Ltd). Mice were prewarmed under a heat lamp, then the tip of the tail snipped off using a pair of clean, sharp scissors. Later in the study, blood was obtained from mice by venepuncture

alone, using a lancet (Monoject Scientific). Blood was collected into the capillary tubes and was 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 RBCs and transferred to a microcentrifuge tube and spun (300 g, 5 min) (MSE Microcentaur, Fisons) to pellet any remaining RBCs. The samples were then stored at -20°C.

For the collection of immune serum from infected mice to show the levels of specific anti-malarial antibodies or levels of nitric oxide during the course of infection, serum was collected from individual mice in different experimental groups at 2-3 day intervals from d 0-60. Within each 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 mouse, and also that the circulating leukocyte count was maintained in a steady state (Sluiter *et al.*, 1985).

2.19 Determination of anti-malarial antibody titres

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

2.19.1 Preparation of malarial antigen slides

Trophozoite/schizont stage parasites were collected from infected mice and used as the source of antigen. Donor mice with an ascending parasitaemia of 5-15% were bled into 10 i.u./ml heparinised PBS. pRBCs 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 cells were resuspended to less than the original blood volume and used to make thin blood smears covering the microscope slide. The slides were dehydrated overnight in a desiccator, wrapped in batches of five in tissue, packed with silica gel and stored at -20°C until required (Manawadu & Voller, 1978).

2.19.2 Assay

Slides were brought up to RT in a desiccator for 1-2 hr before use in the IFAT to avoid condensation. The slides were fixed in absolute acetone (Rhône-Poulenc Ltd) for 5 min and air dried. Reaction zones were marked on the antigen slides using an H series texpen (Deacon Laboratories). The slides were washed in two successive Coplin jars of PBS, drained and rehydrated in a third jar of PBS for 15 min. From this point onwards it was important that the slides remained hydrated, preventing non-specific fluorescence (A.W. Taylor-Robinson, S.A. McLean, personal

communication). The area around each reaction zone was dried and serial 1:2 dilutions of test and control sera applied to the reaction zones in 20 µl volumes. For each slide, the first reaction zone contained PBS in place of serum, to act as a control for non-specific fluorescence. The slides were incubated for 15 min at RT in a humidified chamber, then washed and rehydrated (as above). The slides were recovered from PBS and the edges dried. 1 ml of FITC-conjugated sheep anti-mouse IgG (SAPU) diluted 1:200 in PBS containing Evans Blue (1:10,000 w/v) (Merck) was applied to each of the slides which were then incubated for a further 15 min. 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 any unbound conjugate, and rehydrated in fresh PBS before mounting under a long coverslip in a 1:1 solution of non-fluorescent PBS:glycerol (Merck).

2.19.3 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. The overhead u.v. 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 slides were examined using a X50 water immersion objective and a X12 binocular eyepiece. The titre (endpoint) of the serum was considered to be the last dilution of serum at which specific parasite fluorescence was observed. The control zones of hyperimmune or normal serum, as well as that of PBS alone, were examined for comparison for each slide.

2.20 Passive transfer of serum

Sera collected for passive transfer was stored at -20°C. After thawing, the sera was dialysed overnight against PBS to remove any residual chloroquine that may have been present in the serum. Benzoylated dialysis tubing (for separating compounds with a MW of 1200 or less, flat width 32 mm) (Sigma) was cut into 20 cm lengths and presoaked in PBS. Each serum sample was poured into the tubing, sealed with a Pierce dialysis clip and placed in a 5 l beaker filled with PBS which was agitated gently with a magnetic bar and stirrer motor. The dialysis bags were left overnight at 4°C to reach equilibrium.

Mice challenged with 5 x 10^4 pRBCs were injected with 0.65 ml serum i.v. within 1 hr of infection. Their subsequent course of infection was followed by examination of Giemsa's stained thin blood smears (see earlier). Results of passive serum transfers were usually presented as histograms showing the average day of 2% parasitaemia. The day on which each mouse reached 2% was estimated by drawing a graph of log no.pRBC/10^5 RBCs against time expressed in days, and estimating the

time at which the line passed the 3.303 level (2%). The arimethic mean and standard deviation were calculated for each group and displayed on histograms. Significance of differences between control (receiving NMS) and experimental (receiving immune serum) groups was obtained using the Student's *t* test (Minitab Release 8.2, Minitab Inc.). Groups were considered to be significantly different if $p<0.05$.

2.21 Live IFAT on schizont-infected RBCs

In order to detect variant recrudescent populations in mice receiving subcurative chloroquine treatment or mice naturally recovering from infection, IFATs were carried out on viable, *P. c. chabaudi* schizont-infected erythrocytes as described by McLean *et al.* (1986b).

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 pRBCs.

Infected mice 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 2300-0100 hr, an unreasonable time to perform an experiment!) (Jarra & Brown, 1985).

Cardiac blood was collected from donor mice with a parasitaemia >20%, 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 sequestra tion). After washing twice in 5% FCS RPMI 1640 medium, RBCs were resuspended to a 10% haematocrit in the same medium and cultured using the candle jar method of Trager & Jensen (1976). 1.5 ml of the 10% w/v suspension of the infected blood in medium were dispensed in 35 mm 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% O_2, ie. 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, cultured parasites were washed with 25 ml filtered (0.22 μm pore size, Millipore) PBS (pH 7.2), before use in the immunofluorescence test.

For the live IFAT all washes and dilutions were made with filtered PBS, supplemented with 5% v/v FCS (PBS/FCS). Between each incubation with serum or

reagent, the cells were washed twice in 1 ml PBS/FCS and centrifuged for 1 min at low speed in a MSE microfuge. Incubations were for 30 min in a 37°C waterbath: 2 x 10^7 pRBCs were incubated with, successively, 100 µl of the appropriate dilution of immune serum; 100 µl (12.5 µg/ml) biotinylated anti-mouse IgG (Sera-Lab); then 100 µl of a 1:200 dilution of phycoerythrin-conjugated streptavidin (Sera-Lab). After a further two washes with 1 ml PBS/FCS the cells were resuspended in 20 µl PBS/FCS and fluorescence observed as for section 2.19.

The test was performed on the infecting parasite population and each of the collected recrudescent populations from chloroquine treated or naturally recovering mice, using subpatent sera from both models of infection. The test was scored qualitatively on a positive/negative basis. Samples were scored negative when no positive fluorescence was observed on >3000 pRBCs. Hyperimmune and normal serum (2.18) acted as positive and negative controls, respectively.

2.22 Measurement of serum nitrate

Nitrate levels in the sera of infected mice were used to reflect the serum NO (see text) (Moncada *et al.*, 1991). This was assayed by chemiluminescence as described by Palmer *et al.* (1987) and Downes *et al.* (1976). Initial measurements were carried out by Dr Alison Severn (Department of Immunology, University of Glasgow) and then later with assistance from Dr Kate O'Donnell and Ms Jackie Doyle (Department of Immunology).

The nitrate in the serum was first converted to nitrite using the nitrate conversion assay. This was set up in 96 well v-bottomed microtitre plates (Sterilin). Standards of both NO_2^- and NO_3^- were prepared in the range of 0 - 300 µM by diluting a 10^{-2} mM stock of $NaNO_2$ and $NaNO_3$ respectively in distilled water. 30 µl of the standards in duplicate and the samples, diluted if necessary, were plated out and then 30 µl of conversion buffer (Appendix) containing the enzyme nitrate reductase and the cofactors NADPH and FAD added. The plate(s) were then incubated at 37°C for 2 hours, after which time they were assayed for NO_2^- or frozen at -20°C.

A chemiluminescence method was used for the determination of NO_2^-. Briefly, 20 µl of serum sample or standard was injected, using a Hamilton syringe, into a reaction vessel containing 75 ml 1.0% sodium iodide in glacial acetic acid under reflux. NO was removed from the refluxing mixture under reduced pressure in a stream of N_2, mixed with ozone and the chemiluminescent product measured with a photomultiplier (Model 2107 Chemiluminescent Nitric Oxide analyser (Dasibi Environmental Corporation)) and quantified by reference to 1min infusions of authentic NO into the reflux vessel.

Nitrate levels given represent the mean ± 1S.D. of individual values (nM/ml) from two or three mice within each group of mice in an experiment, and these were

compared by the Student's *t* test, significance achieved if $p<0.05$.

2.23 Measurement of nitrite in culture supernatant

J774 macrophages were stimulated to produce NO with 10 ng/ml LPS (Sigma) and 75 U/ml recombinant IFN-γ (Sigma). Cells were maintained in culture in 20% FCS RPMI 1640 medium and plated out in 1 ml volumes at 1 x 10^6 cells/ml in 24-well tissue culture plates (Greiner) and the cells allowed to adhere. The medium was replaced with fresh medium or medium containing LPS and IFN-γ. After 48 hours incubation, the supernatants were collected and stored at -20°C until tested for NO_2^-.

The Greiss reaction for nitrite analysis was carried out in 96-well flat bottomed plates. 50 µl of samples and standards (10 µg/ml $NaNO_2$ (217 nmol/ml) diluted in medium in two-fold dilutions) in duplicate were plated out and 50 µl of the Greiss reagent (Appendix) added to each well. After 10 mins at RT the plate was read in an ELISA reader (Dynatech, MR5000) at 540 nm. Nitrite levels were compared by the Student's *t* test, significance achieved if $p<0.05$.

2.24 IFN-γ ELISA

To quantify IFN-γ in spleen cell supernatants stimulated with Con A, a capture ELISA was performed.

Spleen cell suspensions were prepared as described in section 2.17. Erythrocytes were lysed with cold NH_4Cl, the cells washed twice in fresh medium and the viability of the resulting suspension determined by trypan blue exclusion. The cells were adjusted to a final concentration of 3 x 10^6 cells/ml in RPMI 1640 10% FCS complete medium. Aliquots of 1 ml in triplicate were incubated in 24 well flat-bottomed tissue culture plates (Greiner) with Con A (5 µg/ml; Sigma). Control cultures were unstimulated. Forty-eight hours later, supernatants were collected, centrifuged at 350 g for 10 min and stored at -70°C until they were assayed for IFN-γ.

For the ELISA flat-bottomed 96-well plates (Corning Easy-Wash, Corning) were coated with 50 µl/well of the capture antibody (Pharmingen) in PBS, and the plates incubated overnight at 4°C. The wells were blocked with 150 µl of 10% FCS in PBS and incubated for 45 mins at 37°C. The plates were then washed three times with PBS/0.05%Tween 20 and this was then repeated twice for 3 mins . The samples and recombinant IFN-γ standards prepared in 10% FCS RPMI, 50 µl/well, were added to the plates and incubated at RT for 2 hrs. The plates were then washed as above and 50 µl biotinylated antibody in 1% BSA/PBS-Tween 20 added and incubated for a further hr at RT. After washing the plates, 75 µl of streptavidin peroxidase (Serotec), 1:1000 in 1% BSA/PBS-Tween 20, was added to each well, incubated for 1 hr at RT and washed three times. 100 µl of the substrate, TMB peroxidase (KPL) was added per well and after 15 mins the plates were read in an

ELISA reader (Dynatech, MR5000) at a wavelength of 630 nm. Units of IFN-γ were calculated from a standard curve constructed for the recombinant cytokine standard.

Differences between experimental groups were analysed by the Student's *t* test, a probability of less than 0.05% was considered significant.

2.25 Preparation of pRBC lysate

For use in the stimulation of T-cell lines, soluble *P. c. chabaudi* CB strain antigens were prepared from whole blood cells enriched for mature trophozoite/schizont-infected RBCs using a modification of the method described by McDonald & Sherman (1980).

Mice to be used as the source of Ag were kept under reversed light conditions, and were bled when most parasites were at the late ring stage. The parasites were cultured to trophozoite/schizont stages as described in section 2.21. 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 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 it in a 37°C waterbath. The rapid temperature transition brought about by this process acted to fracture the RBC, so releasing parasitised material from those lysed infected RBCs 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 50 µl aliquots at -20°C until required. Erythrocytes from normal uninfected mice (nRBC lysate) treated in the same way were used as a control for testing Ag specificity (Dodge *et al.*, 1963).

Since all procedures used were carried out in sterile conditions, the lysate samples were suitable for direct *in vitro* use without prior filtration. The total protein concentration of the lysates was determined by the standard BCA assay (2.11).

The optimal concentration of pRBC lysate for cell stimulation was determined by the ability of the lysate to cause immune spleen cell proliferation, as measured by incorporation of tritiated thymidine (see 2.31, Helper T cell proliferation assay).

2.26 Antigen presenting cells

Irradiated syngeneic spleen cells were used as the most convenient source of large numbers of APCs for routine maintenance of T cell lines (Schwartz *et al.*, 1978). A spleen cell suspension was prepared from the spleens of syngeneic naive NIH mice

(haplotype, H-2^q), in 10% FCS RPMI 1640 medium and placed on ice. The cells were then irradiated to 30 Gy (3000 rads) by exposure to a ^{60}Co γ-emitting source (thereby inhibiting accessory lymphocyte proliferation *in vitro*). The cells were then washed (300 g for 5 min) once and resuspended in complete medium. After taking a total cell count, the suspension of APCs was added to the cultured T cells at an appropriate final concentration.

2.27 *In vivo* priming of T_H cells to *P. c. chabaudi*

Prior to generating a helper T cell line by culturing spleen cell suspensions with pRBCs or the pRBC lysate as sources of plasmodial antigens, and irradiated splenic APCs, the donor mice were primed *in vivo* to the relevant antigenic stimulation, ie., CB strain parasites of *P. c. chabaudi.*

Mice were primed *in vivo* to varying degrees to the full range of plasmodial asexual erythrocytic stages. At different times following infection or reinfection donor mice were killed and the spleens dissected out aseptically. Both naturally recovering and chloroquine treated mice were used as spleen cell donors.

2.28 Initiation and maintenance of Ag-specific helper T cell lines.

The protocol followed for generation of T_H cell lines *in vitro* was that first described by Kimoto & Fathman (1980) and Fathman & Kimoto (1981), as modified by Taylor *et al.* (1987). Conditions were optimised from Taylor-Robinson (1991) and in consultation with Dr A.W. Taylor-Robinson (W.L.E.P).

Spleen cell suspensions were prepared and an enriched T cell population obtained by nylon wool incubation. Cultures were established at 4 x 10^6 cells/ml in 10% FCS RPMI 1640 medium containing either 250 μg/ml pRBC lysate (previously determined optimal concentration of lysate shown to induce T cell proliferation) or 5 x 10^5 pRBCs, and freshly prepared APCs at a final concentration that ranged between 5 x 10^5 and 2 x 10^6/ml. 40 ml aliquots were dispensed into 75 ml tissue culture flasks and these incubated in a humidified 5% CO_2 atmosphere for 4 d. The cells were then recultured every 4 d, the number of responder cells reduced at each subculture to 2.5 x 10^4 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 sufficiently to outgrow the fixed APCs (1 x 10^6/ml) in culture.

2.29 Cryopreservation of T_H cell lines

T_H cell lines were stored for an indefinite period in a frozen state under liquid N_2. A cryoprotective solution was prepared by mixing 10% v/v DMSO (Sigma) and 90% v/v FCS (Gibco), usually as a 10 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 resuspended 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.*, 1986a). A cooling rate of 1°C/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°C for permanent storage.

Cells were recovered from liquid N_2 storage by thawing rapidly by incubating at 37°C; a warming rate of 12000°C/min could be achieved by removing the cryopreservation tubes from frozen storage and dropping them directly into a waterbath at 37°C (Mutetwa & James, 1984b). 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 a viable cell count performed. The suspension was adjusted to a suitable cell concentration immediately prior to culturing *in vitro*.

2.30 Surface phenotyping of T cell lines

In vitro-propagated T cell lines were evaluated for the presence of cell membrane Ags by indirect immunofluorescence, necessary to characterise at the cellular level the T cell subset of the T cells derived by *in vitro* culturing.

Cultures of T cell lines were washed twice (300 g for 5 min) in chilled 10% FCS RPMI 1640 medium, cleansed of dead cells on Metrizamide (see 2.30.1) and washed again. Cells were resuspended in 1 ml of complete medium, and their concentration adjusted to 2 x 10^7/ml. 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 (see 2.15 for method) or coated onto glass slides for slide IFAT (modified from McLean *et al.*, 1982a). For the latter, thin smears of each lymphocyte suspension were aliquoted onto individual zones of teflon-coated 12 well multitest slides (Flow) and air-dried. These Ag slides could then be stored dessicated at -20°C for testing at a later date.

2.30.1 Removing dead cells from culture

Dead responder cells and APCs were removed from suspension prior to surface phenotyping. The methodology used was first described by Kurnick *et al.* (1979a, b)

using the centrifugation gradient medium Metrizamide (Nycomed (UK) Ltd).

A stock solution of 35.3% w/v centifugation grade Metrizamide was prepared in distilled water, filter-sterilised and kept at 4°C. 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 solution was dispensed into a 12 x 75 mm plastic centrifugation tube (Nunc), carefully overlayed with 1 x 10^7 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.

2.30.2 IFAT on fixed T cells

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

Each sample to be analysed, using either the live or fixed method, was tested against a panel of primary mAbs of rat origin, specific for different surface Ags. Anti-mouse IgG_{2b} mAbs specific for CD4 (Dialynas *et al.*, 1983) and CD8 (Ledbetter & Herzenberg, 1979) surface Ags were purified from ascitic fluid as outlined in section 2.10. An IgG_{2b} mAb to the lymphocyte marker Thy-1.2 (pan T cell) (Chayen & Parkhouse, 1982) (Sera-Lab) was also used throughout. Normal rat serum was used 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.

As for the live IFAT the % of positive cells was determined by fluorescence microscopy (see 2.15).

2.31 Helper T cell proliferation assay

This assay measured the ability of cultured T_H cells to respond to specific antigenic stimulation and was a necessary inclusion in all *in vitro* studies to demonstrate that the cell lines employed experimentally were primed to the asexual erythrocytic stages of *P. c. chabaudi* CB to which they were raised. In principal, the assay measured, at the microtitre level (ie. within a 96 well tissue culture plate), the same cellular proliferation after restimulation with Ag and APCs that accounted for cell growth after freshly feeding flask cultures.

Cell lines were deprived of Ag for 6-8 d before testing for cellular proliferation by incorporation of tritiated thymidine. This treatment avoided high background counts which would occur in the presence of residual stimulatory Ag.

For the assay, APCs were prepared at 4 x 10^6/ml in 10% FCS RPMI 1640

medium. The T_H cells to be tested were washed twice in complete medium (300 g for 5 min) to remove residual Ag, resuspended in the APC suspension at 2 x 10^5/ml, and the complete preparation then plated out at 100 µl/well in 96 well flat-bottomed microtitre plates (Nunc).

A range of Ag dilutions in complete medium was prepared from standard stocks of pRBC and nRBC lysates as well as from infected pRBCs and nRBCs. For each concentration, 100 µl of diluted Ag was added to four successive test wells containing T cells and APCs.

The 96 well plates were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 72 hr, at which time 1 µCi (37 kBq) of [^{3}H-methyl]-thymidine (20 µCi/ml, specific activity 5 Ci/mmol; Amersham International) was added to each well. 18 hr later, the wells were harvested with a semi-automatic cell harvester (Titertek, Flow) onto glass fibre filter paper (FG/A, Whatman). When dry, each filter disc was transferred to a plastic beta vial (LKB) and 4 ml non-aqueous scintillation fluid (Optiscint 'safe', LKB) added using an automated dispenser (Jencons (Scientific) Ltd). All the insert tubes were lidded 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) and quantified on a programmed computer (Olivetti DM282 100).

For individual wells, c.p.m. were measured, from which the arithmetic mean c.p.m. for quadruplicate wells could be calculated. Dose-response curves were constructed where necessary, from which optimum Ag concentrations could be determined.

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

CHAPTER THREE

SELECTIVE DEPLETION OF T CELL SUBSETS *IN VIVO*

3.1 Introduction

For many years antibody-mediated effector mechanisms have been considered to be a major component of the protective immune response against the erythrocytic stages of malaria parasites in humans and in experimental animal models (Cohen *et al.*, 1961; Diggs & Osler, 1969). It has become increasingly evident in recent years that cellular and antibody-independent effector pathways operate, at least in rodent malaria models. Early adoptive transfer experiments in a model of *P. yoelii* or *P. chabaudi* in the mouse and of *P. berghei* in the rat demonstrated that T cells alone could transfer immunity - see also Chapter 4 - (eg. Stechschulte, 1969; Phillips, 1970; Brown *et al.*, 1976a, b; Gravely & Kreier, 1976; McDonald & Phillips, 1978; Jayawardena *et al.*, 1982; Brinkmann *et al.*, 1985). This can be explained by the fact that T helper cells are contained within the transferred population and these cells provide help for the production of antibody; however, partial protection can be achieved by transfer of CD8-bearing T cells (Mogil *et al.*, 1987). Further evidence for T cell-dependent non-antibody mechanisms of immunity has been obtained from experiments utilizing B cell-deficient animals and a number of species of plasmodia (Roberts & Weidanz, 1979; Grun & Weidanz, 1983). In particular, Grun & Weidanz (1981) demonstrated that an acute infection with *P. chabaudi adami* can be cleared in the absence of B cells, highlighting a role for antibody-independent immune mechanisms. Since then, B cell-deficient mice have been shown to resolve acute infections with *P. vinckei petteri, P. chabaudi chabaudi* CB, and *Babesia microti* (Cavacini *et al.*, 1990). In the same study, athymic nude mice died when infected with these parasites.

Protection against the sporozoite stage of *P. berghei* in the mouse is absolutely dependent on the presence of CD8-bearing T cells, elucidated by T cell depletion *in vivo* and adoptive transfer experiments (Schofield *et al.*, 1987; Weiss *et al.*, 1988). Moreover, IFN-γ appears to be a necessary component of such protection (Ferreira *et al.*, 1986; Schofield *et al.*, 1987). Although $CD8^+$ T lymphocytes are clearly involved in protecting mice during the pre-erythrocytic stage of infection, their role during the erythrocytic stage of malaria is less clear. In the model of blood-stage *P. c. adami* infection, protection was transferred most efficiently to recipient nude mice by $CD4^+$-enriched immune spleen cells; $CD8^+$-enriched T cells were also found to transfer protection, albeit to a lesser extent (Cavacini *et al.*, 1986). Mogil *et al.* (1987) demonstrated that adoptive transfer of $CD8^+$ T cells from *P. yoelii*-immune animals into naive mice accelerated recovery, whereas Vinetz *et al.* (1990) were unable to demonstrate a protective effect of $CD8^+$ T cells. Recent experiments by Süss *et al.* (1988), Podoba & Stevenson (1991) and Taylor-Robinson (1991) have demonstrated and confirmed the importance of $CD4^+$ T cells in the development of immunity to blood stage *P. c. chabaudi* AS. Süss et al. (1988) and Taylor-Robinson (1991)

suggested that $CD8^+$ T cells did not play a role while Podoba & Stevenson (1991) demonstrated a role for these cells in the resolution of infection.

In the experiments described in this chapter, the contribution of CD4- and CD8-bearing T lymphocytes to acquired immunity to blood stage *P. c. chabaudi* CB infection in NIH mice is examined. Mice treated with mAbs to deplete specific T cell subsets *in vivo* were observed for aberrations in the course of infection with *P. c. chabaudi* CB. The use of these mAbs *in vivo* allows manipulation of the immune response without the concomitant disturbance of the experimental system by whole-body irradiation (Cobbold *et al.*, 1984; Leist *et al.*, 1987). Through a course of repeated treatment, it is possible to reduce to minimal levels the host complement of CD4 and/or CD8 T cells during infection. *In vivo* depletion of one T cell subset enables the analysis of the role of the remaining subset in relative isolation. This method represents the negative corollary of adoptive transfer experiments mentioned above and those that will be described in Chapter 4.

3.2 The effect of anti-CD4 and anti-CD8 mAb treatment on host immunity to *P. c. chabaudi* CB

Five groups of 10 age-matched female NIH naive mice were used for this study. Individual mice were immunologically modified by a series of injections of a specific rat anti-mouse mAb prepared from ascitic fluid (2.10). The methodology was essentially that described by Süss *et al.* (1988). Experimental groups were as follows: mice treated with either anti-CD4 or anti-CD8 mAbs, both mAbs, NRS, or no treatment/mAb diluent only. Treatment with mAbs began four days prior to infection, with a total of three pretreatments before parasite challenge. One further treatment with mAbs was administered 14 DPI. Each mouse within a group was challenged with 5×10^4 pRBCs *P. c. chabaudi* CB on day 0. From each group of mice, 2 mice were sacrificed on days 0 and 23 post infection to determine the efficacy of depletion of the relevant T cell subset; assessed by immunofluorescence (2.15). The remaining mice were retained to determine the parasitaemia throughout infection. In a preliminary experiment, the resulting parasitaemias were very high in all groups of mice. To assist mice through the peak of infection in this experiment, therefore, all animals were treated with 50 mg/kg chloroquine 8 and 9 DPI.

The phenotypic characterisation of spleen cells taken from individual immunodepleted mice (0 and 23 DPI) is shown in Table 3.2.1. These data indicate that the regimen of mAb treatment was effective at *in vivo* immunodepletion of naive mice. The appropriate subset of T lymphocyte was depleted accordingly. For both mAb treatments, by 23 DPI a greater than 90% depletion was achieved. The level of depletion achieved at the time of parasite challenge was, however, somewhat less.

The % depletion attained by 0 DPI was 50.1%, 44.1% and 74.3% for anti-CD4, anti-CD8 and both mAbs respectively. From this it is assumed that the mAb treatment took some time to take effect. Without any analysis between the two time points it is difficult to determine when the maximal depletion was achieved; although only one further injection of mAbs was administered after day 0 (14 DPI) and significant differences in the course of infection had been observed before then (see below). Depletion never reached 100% indicating that there were a few remaining cells of the respective subset present throughout the experiment. Immunofluorescent staining for phenotypic analysis was carried out using the same mAbs as those used to deplete the mice. Masking of the molecules by bound Abs *in vivo* may have affected the level of staining with the anti-CD4 and anti-CD8 mAbs. For all treatments, the appearance of $CD4^-$ and/or $CD8^-$ T cells was not tested for.

DEPLETION [a]	STAIN	DAY	
		0	23
CD4	anti-CD4	50.1	94.7
	anti-CD8	2.6	3.3
	anti-Thy1.2	35.7	63.0
CD8	anti-CD4	1.8	3.4
	anti-CD8	44.1	97.1
	anti-Thy1.2	18.5	31.1
CD4/CD8	anti-CD4	44.2	92.1
	anti-CD8	34.4	93.6
	anti-Thy1.2	74.3	89.9

[a] % depletion compared to control mice given NRS treatment (0% depletion).

TABLE 3.2.1 - Phenotypic analysis of T cell subset depletion *in vivo* by continuous mAb treatment on the day of infection and 23 DPI.

For individual T cell subset depletion, the proportion of the total T cells (Thy 1.2 bearing) lost differed. In those animals treated with anti-CD4 mAb, a 63% depletion of total T cells occurred. In contrast, although 97% of CD8 bearing T

lymphocytes had been removed from the spleen with anti-CD8 therapy, the proportion of Thy 1.2 staining cells lost represented 31% (Table 3.2.1). This probably reflects the balance of CD4 and CD8 cells at this stage of infection (Goronzy *et al.*, 1986).

No functional assays, such as cytotoxic responses to alloantigens, helper T cell function, or proliferation studies, for confirmation of specific functional depletion, were carried out.

The effect of the different T cell subset depletions on the outcome of *P. c. chabaudi* CB infection is shown in Figure 3.2.1. There was a marked difference in the patterns of parasitaemia of mice receiving varying T cell subset depletion treatments following subsequent pRBC challenge. The courses of infection in the diluent only treated group and NRS treated group were essentially the same. Mice receiving anti-CD8 mAbs also demonstrated the same course of infection. Parasites were observed in the peripheral blood 4 days after infection, and the peak parasitaemia (52%, 52% and 47% respectively) occurred 8 or 9 DPI. This parasitaemia was reduced to subpatent levels 14 DPI with the aid of subcurative chloroquine treatment. A recrudescence occurred for each of these groups peaking at approximately 3% for each group. After clearance of this recrudescence by 23 DPI, no further parasites were detected in the peripheral circulation by blood smear examination. At no stage of infection were any significant differences observed between these groups of animals. In contrast, the courses of infection in mice treated with anti-CD4 or both mAbs differed from those described above. Although the peak parasitaemia of the anti-CD4 treated group of mice did not differ significantly from those of the other groups (49%), the peak parasitaemia in the double depleted animals was 60%, significantly different from the other groups ($p<0.05$). Following chloroquine treatment the parasitaemias were reduced to 2% and <1% by 13 DPI but this decrease in parasitaemia was not maintained and the infection peaked again with a mean parasitaemia of 51% for the anti-CD4 treated mice and 49% for double depleted mice; highly significant compared to control mice and anti-CD8 treated mice ($p<0.001$). This parasitaemia, for both groups, was subsequently reduced and remained at between 3 and 14% until the experiment was terminated 30 DPI.

These results suggest that lymphocytes of the $CD8^+$ phenotype are only of marginal importance to host acquired resistance to infection, since in their absence the pattern of parasitaemia is very similar to undepleted control animals, with only a slightly higher parasitaemia noted in animals that had been depleted of both CD4 and CD8 lymphocytes compared to CD4 depletion alone. Elimination of $CD4^+$ lymphocytes, however, in the presence or absence of CD8 cells, had a profound effect on the outcome of infection. Despite subcurative chloroquine treatment, animals with only $CD8^+$ cells (CD4 depleted) or those essentially devoid of functional T cells were

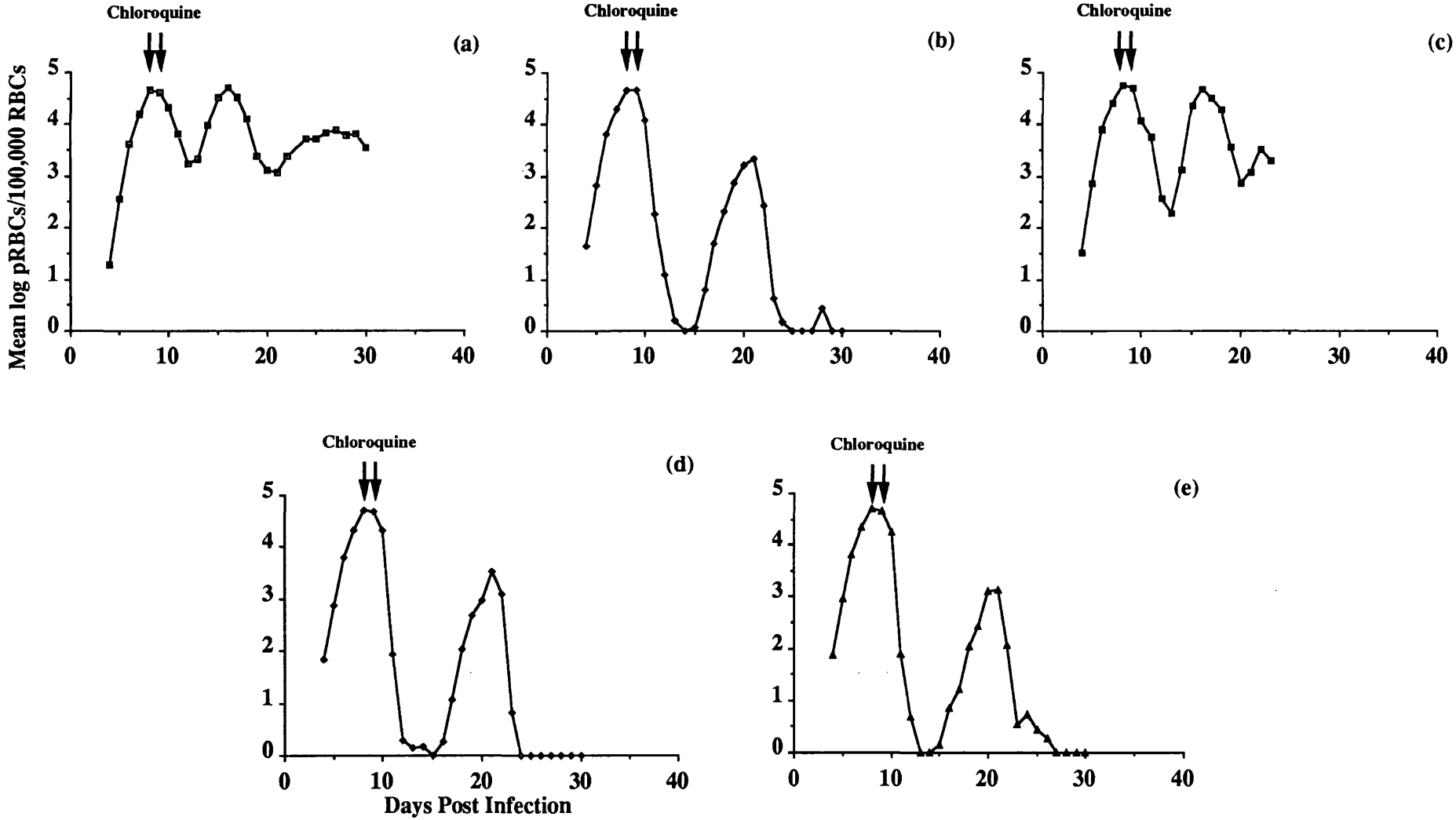


FIGURE 3.2.1
Courses of infection in mice selectively depleted of T cell subsets by treatment with specific monoclonal antibodies. (a) anti-CD4 mAb, (b) anti-CD8 mAb, (c) both mAbs, (d) NRS, (e) no serotherapy.
Mice were infected with 5 x 10^4pRBCs *P. c. chabaudi* CB. 8 & 9 days post infection mice were treated with 50mg/kg chloroquine.

never able to reduce their parasitaemias below 3%. The experiment was terminated 30 DPI. This was due to a high proportion of mice (~75%) that had been treated with anti-CD4 mAb alone, or in combination with anti-CD8 mAb, developing unusual complications of infection. Mice developed ascites and subcutaneous oedema, an apparently fatal condition in some mice, with *post mortem* pathology revealing many signs of a typical chronic malaria infection, including hepatosplenomegaly. Liver and kidney, haematoxylin and eosin stained, sections of a not seriously affected mouse revealed no obvious severe hepatic or renal pathology but demonstrated very nicely the presence of malaria pigment in kupffer cells and glomeruli respectively. The ascites did not show any specificity for *P. c. chabaudi* CB pRBCs by slide IFAT.

3.3 Discussion

The experiment described herein was designed to examine and evaluate the roles of the major T lymphocyte subsets in the immune response of an unprimed host to primary infection with *P. c. chabaudi* CB.

Selective depletion of the T cell subsets was effectively achieved with rat IgG_{2b} anti-CD4 or anti-CD8 mAbs. A greater than 90% depletion (Table 3.2.1) was achieved with serotherapy with either Ab. This degree of elimination compares favourably with that recorded originally using mAbs prepared in an identical manner (Cobbold *et al.*, 1984). Total elimination of each T cell subset did not occur in this study but this is in agreement with previous investigations (Cobbold *et al.*, 1984; Leist *et al.*, 1987; Schofield *et al.*, 1987; Süss *et al.*, 1988; Weiss *et al.*, 1988; Howard *et al.*, 1989; Podoba & Stevenson, 1991; Taylor-Robinson, 1991). However, it has been shown in similar studies that functional depletion is achieved using the same regime as described here (Süss *et al.*, 1988; Podoba & Stevenson, 1991). Efficacy of depletion was examined by immunofluorescence of spleen cells. It was considered important to use splenic lymphocytes due to essential role of the spleen in malaria infections and the relative ease with which they can be prepared. IgG_{2b} mAbs 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). *In vivo* administration of the mAbs resulted in approximately 25% of the double-depleted animals dying immediately after the last inoculation of Ab 14 DPI. Although it may have been a consequence of high parasitaemia, it is possible that the mice produced an Ab response to the injected mAbs. This is a feature of *in vivo* depletion of primates and calves (Howard *et al.*, 1989) but it has not been observed with murine recipients. It has previously been reported, however, that mice inoculated with rat IgG_{2b} mAbs directed against either CD4 or CD8 surface determinants become tolerant to the injected Abs (Benjamin & Waldmann, 1986; Gutstein *et al.*, 1986). The best regime that has been demonstrated

to result in the most effective immunosuppression, leading to the induction of tolerance, is obtained with Abs that can both deplete T cells and exert functional inhibition (Cobbold *et al.*, 1985). The same workers have shown that the best suited Ig isotype to perform this task is the IgG_{2b} subclass, used in this study.

Although the importance of T cells in immunity to the CB strain of *P. c. chabaudi* has been demonstrated by use of B-cell deficient mice (Cavacini *et al.*, 1990) the relative contributions of $CD4^+$ and $CD8^+$ T cells has not previously been dissected for this strain of parasite. Herein, it has been shown that $CD4^+$ T cells are critical for the development of an effective immune response to *P. c. chabaudi* CB; the depletion of these cells resulted in mice being unable to clear their infections. More than one wave of high parasitaemia occurred, despite subcurative chloroquine treatment, and until the experiment was terminated 30 DPI the parasitaemia was never reduced below 3%. These results are very similar to those of Süss *et al.* (1988), Podoba & Stevenson (1991) and Taylor-Robinson (1991) working with *P. c. chabaudi* AS. In these studies the strain of mouse used was either C57Bl/6 ($H\text{-}2^b$) or NIH ($H\text{-}2^q$) (used in this study). For both of these strains of mice infection with the AS strain of parasite is usually non-lethal (Stevenson *et al.*, 1982). The data described here show that CD4 cells are critical for the resolution of infection with the more virulent strain of parasite, even with chloroquine intervention.

One difference observed between the results of the studies cited above and those presented in this chapter is the % parasitaemia during the chronic phase of infection observed in CD4 depleted mice. The studies of Süss *et al.* (1988), Podoba & Stevenson (1991) and Taylor-Robinson (1991) demonstrated that for the AS strain of *P. c. chabaudi*, following peak parasitaemia, the parasitaemia was reduced to approximately 20%, at which it remained with small variations until the termination of the experiment in each case. Here mice were treated with 50 mg/kg chloroquine at the peak of infection, a subcurative dose, and this treatment resulted in a rapid reduction of the parasitaemia in all the experimental groups. However, CD4 depleted and double-depleted mice experienced another wave of high parasitaemia, the peak of which was as high as that observed before chloroquine treatment. This was subsequently reduced to 3%, without further chloroquine intervention, with only minor fluctuations until 30 DPI when the experiment was terminated. It therefore appears that, even in mice depleted of both T cell subsets, their parasitaemias were controlled at around 3% for the duration of the experiment. These mice were demonstrated to be substantially depleted of T cells (Table 3.2.1). It is possible that the few remaining CD4- or CD8-bearing cells are responsible for this reduction, although 89.9% of $Thy1.2^+$ cells were depleted; in other studies severe impairment of the immune responsiveness of these cells in all lymphoid organs studied was demonstrated using functional assays (Süss *et al.*, 1988). In addition, adult

thymectomised and irradiated mice, infected with *P. c. chabaudi* AS, control their parasitaemias, maintaining a chronic parasitaemia, but not to the same extent as observed in this experiment. Some other mechanism, T cell independent, must be operative to partially control parasitaemia. Süss *et al.* (1988) attributed partial limitation of parasitaemia to the presence of low titres of transient malaria-specific IgM Abs in the peripheral blood of CD4 depleted mice. To rule this out in the study described here, the experiment would have to be repeated with measurement of Ab levels and Ig subclasses and also in mice without circulating Igs. This would be of interest since it has been reported that B cell-deficient mice can completely resolve an infection with *P. c. chabaudi* CB (Cavacini *et al.*, 1990).

Another mechanism for the partial control of parasitaemia demonstrated could be due to $CD4^-CD8^-$ T cells having some immune function. Recent evidence indicates that the γδ T cell subset responds in both human and murine malaria. The splenic γδ T cell number significantly increases in the spleens of both mice and humans with malaria (Minoprio *et al.*, 1989; Ho *et al.*, 1990; Roussilhon *et al.*, 1990; van der Heyde *et al.*, 1993b) and specific proliferative responses have been demonstrated *in vitro* with human blood-stage parasites (Goerlich *et al.*, 1991; Behr & Dubois, 1992; Goodier *et al.*, 1992). Although the γδ T cell subset is not developmentally regulated by αβ T cells (Philpott *et al.*, 1992; Mombaerts *et al.*, 1992) van der Heyde *et al.* (1993b) have shown that $CD4^+$ αβ T cells are required for the γδ T cell response to occur during murine *P. c. adami* malaria. It, therefore, seems unlikely, that in the context of this experiment, γδ T cells have contributed to the limitation of parasitaemia.

Süss *et al.* (1988) and Podoba & Stevenson (1991) observed at the same time as the reduction in parasitaemia, a sudden influx of reticulocytes followed by a persistent reticulocytosis. This was also noted in this experiment, but is also a feature of *P. c. chabaudi* CB infection following peak parasitaemia. It has been suggested therefore, that the rapid reduction in the number of mature erythrocytes, the host cell preferred by *P. c. chabaudi*, as a result of lysis of infected cells and the influx of reticulocytes may account for the sharp drop in parasitaemia (Süss *et al.*, 1988; Podoba & Stevenson, 1991). A similar conclusion was reached by Ott (1968) on the basis of the observation that stimulation of reticulocytosis by injection of phenylhydrazine resulted in a diminished level of parasitaemia during infection with *P. c. chabaudi.* It appears that the chronicity of infection is maintained by a balance between reticulocytosis and parasite invasion of immature red cells. Podoba & Stevenson (1991) proposed that CD4 depleted mice may compensate for the lack of immune function by mounting a marked and sustained erythropoietic response, with the spleen becoming the major site of erythropoiesis. In the present study the spleens of CD4 depleted mice on day 29 post infection were twice the size of those from animals that

received either NRS or no mAb treatment (Dr H. Thompson, personal communication). However, Yap & Stevenson (1994) have recently shown that inhibition of *in vitro* erythropoiesis is not dependent on T cell derived cytokines.

The CD4+ T cell can be characterised functionally as a cell which can mediate delayed-type hypersensitivity and which can act as a helper cell for Ab production. Mosmann & Coffman (1987) indicated that these functions may be carried out by separate subsets of CD4 cells (T_H1 and T_H2, respectively). This study, however, did not allow delineation of the functions of the CD4-bearing lymphocytes *in vivo* as the anti-CD4 mAb used could not discriminate between T_H1 and T_H2 cells. Recent evidence, which will be discussed at greater length in Chapter 8, indicates that CD4 cells of both phenotypes are activated during the course of *P. c. chabaudi* AS infection (Langhorne *et al.*, 1989a, b). Moreover, Taylor-Robinson (1991) and Taylor-Robinson & Phillips (1992; 1993; 1994a) have produced T cell clones of both phenotypes which are protective upon adoptive transfer to naive and immunocompromised mice. In addition, the protective mechanisms of T_H1 and T_H2 clones have been elucidated by Taylor-Robinson *et al.* (1993). T_H1 cells protect by a nitric oxide dependent mechanism, whereas T_H2 cells protect by the enhancement and accelerated production of specific IgG_1 Ab.

Studies of *P. yoelii* in the mouse have suggested that CD8 cells can partially protect against challenge infection (Mogil *et al.*, 1987). This ability was correlated with an increase of expression of class I MHC molecules on infected erythrocytes and led to the postulation that CD8+ cells may be directly cytotoxic for intraerythrocytic parasites (Jayawardena *et al.*, 1982; Jayawardena *et al.*, 1983). CD8+ T cells may also exert an antiplasmodial effect indirectly via the production of IFN-γ. The results presented here, and those of Süss *et al.* (1988) and Taylor-Robinson (1991) show little indication that CD8+ T cells have any significant role in an erythrocytic infection with *P. c. chabaudi*. In their absence, parasitaemias did not deviate from those of control animals, although in double-depleted mice the peak parasitaemia was significantly higher than in all other groups. Süss *et al.* expressed the opinion that, under experimental conditions, a role for CD8 cells could not be discounted, since it is possible that in the absence of CD4 cells and their soluble mediators CD8 effector cells could not be activated appropriately. Although it is not known whether CD8+ T cells *in vivo* require an exogenous Il-2 source (supplied at least *in vitro* by CD4+ cells), in a second experiment, recombinant Il-2 was given to CD4-depleted mice. This did not affect parasitaemia significantly, challenged recipients still being incapable of clearing infection (Süss *et al.*, 1988). In contrast, however, Podoba & Stevenson demonstrated a role for CD8 cells in immunity to the blood stages of *P. c. chabaudi* AS. Depletion of CD8+ cells had no effect on the course and level of parasitaemia during the prepatent or peak parasitaemia. However, compared to

control animals, CD8 depleted mice had two recrudescences. These workers, therefore, concluded that both T cell subsets play a role in acquired immunity to *P. c. chabaudi* AS. These data supported the adoptive transfer studies of Cavacini *et al.* (1986) and Mogil *et al.* (1987) in *P. c. adami* and *P. yoelii* infections, respectively. These conflicting reports led van der Heyde *et al.* (1993a) to examine the courses of *P. yoelii yoelii* 17X, *P. c. adami* and *P. c. chabaudi* blood stage infection in β_2-microglobulin-deficient (β_2-$m^{0/0}$) mice. These mice are completely devoid of functional $CD8^+$ T cells, since the β_2-$m^{0/0}$ mouse lacks surface expression of both MHC class I glycoproteins on nucleated cells, this being required for positive selection to occur in the thymus (Koller *et al.*, 1990; Zijlstra *et al.*, 1990). The resolution of each of the three subspecies of *Plasmodium* in β_2-$m^{0/0}$ mice was similar to intact mice, supporting the lack of a requirement for $CD8^+$ T cells in the suppression of malarial parasitaemia. This is in direct contrast to the crucial role played by $CD8^+$ cells in the protective response against the pre-erythrocytic stages of *P. berghei* (Schofield et al., 1987; Weiss et al., 1988).

In conclusion, the results described in this chapter demonstrate that $CD4^+$ but not $CD8^+$ T cells are essential for a successful primary immune response to the erythrocytic stages *P. c. chabaudi* CB. These findings justified the generation of stable $CD4^+$ T cell lines specific for this strain of parasite, as described in Chapter 7.

CHAPTER FOUR

ADOPTIVE TRANSFER OF IMMUNITY WITH SPLENIC LYMPHOCYTES

4.1 Introduction

In order to study the contribution of lymphocytes to the immune response against malaria parasites, various investigators have performed experiments in which immune spleen cells or selected spleen cell populations were transferred to non-immune recipient animals. The basic model for cell transfer studies in rodent malaria was developed by Stechschulte (1969), who showed that immunity to *P. berghei* could be transferred from recovered rats to syngeneic susceptible rats by the injection of splenic lymphocytes. Since this study, a wealth of evidence has shown that protection can be conferred by adoptive transfer with immune spleen and lymph node cells (Roberts & Tracey-Patte, 1969; Phillips, 1970; Kasper & Alger, 1973; Brown *et al.*, 1976a, b; Gravely & Kreier, 1976; Zuckerman & Jacobsen, 1976; McDonald & Phillips, 1978, 1980; Jayawardena *et al.*, 1978; Fahey & Spitalny, 1986; Cavacini *et al.*, 1986; Mogil *et al.*, 1987; Favila-Castillo *et al.*, 1990; Legorreta-Herrera *et al.*, 1993). In most of these studies unfractionated cells or enriched T and B cell populations were used from mice that had recovered from at least one previous infection. Only in two instances have spleen cells been taken from donor animals at a range of time points during the course of a primary infection; *P. berghei* in rats (Brown *et al.*, 1976a) and *P. yoelii* 17X in mice (Fahey & Spitalny, 1986).

Until the present study, no adoptive transfer experiments have been carried out for the *P. c. chabaudi* CB system, although transfer of immunity with splenic lymphocytes has been shown in NIH (McDonald & Phillips, 1978, 1980), CBA/Ca (Favila-Castillo *et al.*, 1990) and (BALB/c x C57Bl/6) F_1 (Legorreta-Herrera *et al.*, 1993) mice infected with *P. c. chabaudi* AS. However, in these investigations, only spleen cells taken from recovered or post-infective mice were used. A study was therefore undertaken to determine whether lymphocytes from *P. c. chabaudi* CB strain infected mice at different stages of infection were capable of transferring immunity to sublethally irradiated recipient mice. In addition, similar experiments were carried out using spleen cells from mice that had received subcurative chloroquine treatment, to investigate if such treatment would affect the ability of splenic cells to confer protection.

In all the adoptive transfer experiments described in this thesis the lymphocytes transferred were of splenic origin, as the spleen represents a large pool of readily available T and B cells. In addition, all recipient mice were exposed to γ-irradiation shortly before cell transfers and subsequent parasite challenge. It is widely accepted that lethal or sublethal irradiation of an animal results in a depression of the recipient's own immune response to an antigenic stimulus administered after such treatment (Taliaferro & Taliaferro, 1951). Immunologically suppressing the host immune response of recipient animals prior to challenge has been used for the *P. c. chabaudi*

AS/NIH mouse system to study the adoptive transfer of splenic cells (McDonald & Phillips, 1978;1980). The reason for using irradiation as a tool in these studies is that in immunologically competent recipients, it is often difficult to differentiate between the protective activity provided by the transferred cells alone and that resulting from the cooperation between the repopulating inoculum and the host's fully operational immune system. In sublethally irradiated recipients, the contribution of the recipient's own immune response is reduced considerably. Within the present study, this depression of background immunity allowed differences in the primary parasitaemias of mice given normal or primed lymphocytes to be amplified, and thus enabled a clearer examination of the effect of the transferred populations on the course of *P. c. chabaudi* CB infection.

4.2 Adoptive transfer of splenic lymphocytes from donor mice 75 DPI

A group of 10 NIH female mice was infected with 5 x 10^4 *P. c. chabaudi* CB pRBCs and the course of infection followed. At the same time, another group of mice was sham-infected. These mice were used as the donor animals for the transfer. 75 DPI, when the infected animals had completely cleared their infections, the donor mice were killed, their spleens removed and the cell populations prepared.

Recipient NIH female mice were exposed to 4 Gy γ-irradiation on the morning of the cell transfer. At the time of challenge with 5 x 10^4 pRBCs, each mouse was also inoculated with an aliquot of 2 x 10^7 lymphocytes. Recipients of day 75 lymphocytes were separated into groups of 6 mice each, and received either immune or naive unfractionated splenic lymphocytes, or immune or naive enriched T cell populations. In addition, a further group of 6 mice received only an inoculation of medium and served to demonstrate the course of infection in irradiated hosts without any influence from a transferred cell population. The identity of the transferred cell populations is shown in Table 4.2.1. The degree of enrichment of the T cell populations was assessed by live IFAT. For the immune T cell fraction (75 DPI cells), prepared by nylon wool incubation, 86.7% of the cells were Thy-1.2 staining, with 2.3% contaminating B cells. In the case of the naive cell fractions a similar proportion of cells was achieved with 86.2% T cells and 1.7% contaminating B cells. The unfractionated cell suspensions contained roughly equal numbers of T and B cells. In all cases, no staining for other contaminating cells, such as macrophages was carried out.

TRANSFERRED CELL POPULATION	PERCENTAGE OF CELLS STAINING FOR:	
	THY-1.2 (T cells)	IgG(Heavy chain) (B cells)
Unfractionated 75DPI cells	46.3	39.9
Unfractionated naive cells	37.3	36.8
Enriched 75 DPI T cells	86.7	2.3
Enriched naive T cells	86.2	1.7

TABLE 4.2.1 Phenotypic analysis of the identity of the transferred cells (75 DPI) by live IFAT.

The courses of infection in recipient mice are shown in Figure 4.2.1. Mice receiving immune T cells taken from donor mice at 75 DPI expressed a depression and shortening of the primary parasitaemia with no recrudescences of infection (Figure 4.2.1a). In comparison, mice receiving the same number of naive T cells did not control the primary parasitaemia until 17 DPI, with a recrudescence occurring 2 days later. Statistical comparison of the peak parasitaemia revealed that there was no significant difference between these two groups, but a significant difference ($p<0.01$, $p<0.001$ and $p<0.01$) was detected during the remission, the values quoted being those for days 11, 12 and 13 respectively. The transfer of a population of unfractionated cells resulted in a similar pattern of protection (Figure 4.2.1b). The mean peak parasitaemia of mice receiving immune 75 DPI unfractionated cells was 8.5% compared to 25.8% observed in mice receiving naive cells and this was a significant difference ($p<0.05$). Again there was rapid parasite clearance, with no parasites detected in the peripheral blood of the recipients from 12 DPI onwards. Recipients of naive unfractionated cells experienced two recrudescences. Figure 4.2.1c shows the course of infection in mice that did not receive any cells. Although there were no significant differences between the peak parasitaemia of these animals and mice that received naive cell suspensions, the mice did experience an extended primary parasitaemia, and the recrudescence that followed was significantly higher ($p<0.05$) than that observed in any other of the groups. This demonstrates that a degree of protection was achieved by the transfer of naive cell populations.

Splenic T cells, prepared 75 DPI, were, therefore, very effective upon adoptive transfer to immunocompromised hosts. Although the pattern of protection was similar in recipients of unfractionated cells, these cells conferred a better level of protection than the T cells alone, indicated by the significant reduction in peak parasitaemia. The enhanced ability of these cells to reduce the peak of infection was probably due to a synergistic activity of the T and B cells and unidentified cells within this population.

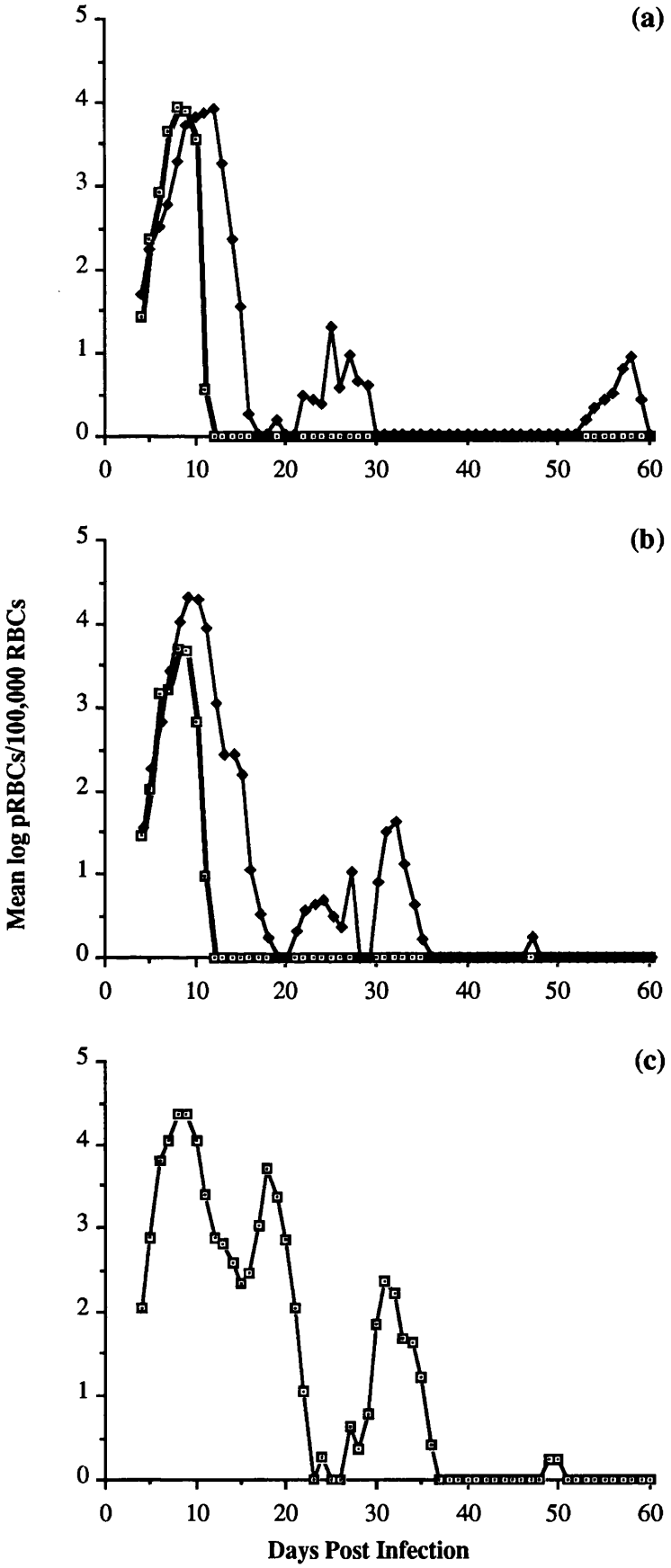


FIGURE 4.2.1 The course of infection in 4 Gy irradiated recipients of 2 x 10^7 spleen cells: a) enriched naive (◆——◆) or 75 DPI (▫——▫) T cells, b) unfractionated naive (◆——◆) or 75 DPI (▫——▫) cells, or c) no cells. Mice were challenged with 5 x 10^4 pRBCs *P. c. chabaudi* CB.

4.3 Adoptive transfer of splenic lymphocytes 21 DPI

In the experiment described here, spleen cell suspensions were prepared from donor mice on day 21 post infection. This stage of infection represented the point at which the parasitaemias of all mice were subpatent. This experiment was performed to determine the degree of immunity that could be transferred with cells prepared from subpatent mice, and to determine whether either or both T and B lymphocyte subsets were responsible for the protection conferred by immunologically primed lymphocytes.

Six groups of six NIH female mice were irradiated with 4 Gy on the day of transfer and parasite challenge. As soon as possible after parasite challenge with 5 x 10^4 pRBCs of *P. c. chabaudi* CB mice received an inoculum of either 2 x 10^7 enriched 'semi-immune' T or B cells (21 DPI), 2 x 10^7 enriched naive T or B cells, or 2 x 10^7 semi-immune or naive unfractionated splenic cells. In addition, another group of irradiated mice received 2 x 10^7 unfractionated cells prepared from 21 DPI donor animals, with no parasite challenge, as a control of pRBC contamination in the prepared spleen cell suspensions. A further group of mice were given only the parasite challenge. To provide sufficient numbers of cells for the adoptive transfer of primed or naive splenic lymphocytes, 10 infected mice were sacrificed 21 DPI and 12 naive animals were killed at the same time.

The degree of enrichment of the spleen cell populations was assessed by live IFAT and is shown in Table 4.3.1.

TRANSFERRED CELL POPULATION	PERCENTAGE OF CELLS STAINING FOR:	
	THY-1.2 (T cells)	IgG (Heavy chain) (B cells)
Unfractionated 21 DPI cells	51.1	43.9
Unfractionated naive cells	45.8	44.2
Enriched 21 DPI T cells	85.1	1.8
Enriched naive T cells	83.9	2.2
Enriched 21 DPI B cells	5.5	62.1
Enriched naive B cells	6.1	57.5

TABLE 4.3.1. Phenotypic analysis of the transferred cell populations (21 DPI) by live IFAT.

For both the naive and 21 DPI enriched T cell preparations more than 80% of the cells were Thy-1.2 staining, with 2.2% and 1.8% contaminating B cells respectively. The enrichment of B cells was somewhat less. The 21 DPI suspension contained 62.1% B cells with 5.5% contaminating T cells; the naive B cell preparation contained 57.5% B cells with 6.1% contaminating T cells. As for the 75 DPI adoptive

transfer, the unfractionated cell suspensions contained approximately equal numbers of T and B cells.

The effect of the transferred 21 DPI cell populations on the course of *P. c. chabaudi* CB infection is shown in Figure 4.3.1. The transfer of a population of semi-immune 21 DPI enriched T cells resulted in a reduction of the peak parasitaemia, rapid parasite clearance and no recrudescences of infection compared to recipients of the same number of naive T cells (Figure 4.3.1a). The peak parasitaemia of 21 DPI T cells was 25.8% compared to 33.7% in naive recipients ($p<0.05$). Mice receiving naive T cells experienced two recrudescences. The transfer of a population of B cells 21 DPI resulted in a reduction in the peak parasitaemia, with a more rapid parasite remission compared to naive B cell recipients (Figure 4.3.1b) Although a recrudescence did occur in these animals, it followed an extended subpatent period compared to recipients of naive B cells. The parasitaemia in the 21 DPI B cell recipients reached a peak of 23.8% 10 DPI which was significantly lower ($p<0.05$) than in naive B cell recipients (41.7%). Remission of the peak parasitaemia was complete by 15 DPI, significantly different from the parasitaemia of naive B cell recipients ($p<0.01$ on days 13 and 14 post infection, and $p<0.001$ on days 15-19 post infection). Naive B cell recipients did not control their primary parasitaemias to subpatent levels until 23 DPI. A recrudescence occurred in these mice 2 days later, while semi-immune B cell recipients did not recrudesce until 27 DPI. There was no significant difference in the level of the recrudescence. Recipients of day 21 unfractionated cells demonstrated a course of infection shown in Figure 4.3.1c. This shows that the transfer of an unfractionated population of spleen cells results in a similar pattern of protection as the transfer of enriched T cells from the same stage of infection. Compared to the transfer of a population of naive unfractionated cells, there was a reduction in the peak parasitaemia (28.2% compared to 47.4%, $p<0.05$) and a much faster parasite remission ($p<0.01$, days 12, 13, 14, 15 post infection; $p<0.001$ days 16-22). No recrudescences were observed in 21 DPI unfractionated cell recipients. Naive cell recipients either died at the peak of infection or were killed due to anaemia and the development of ascites. Parasitaemias did not develop in the unchallenged recipients of unfractionated 21 DPI cells. The course of infection is not shown in the mice that did not receive any cells. These mice either died or were killed at peak parasitaemia (37.8%).

There were no significant differences ($p>0.05$) between the peak parasitaemias of any of the recipients of the 21 DPI cell preparations or those of the recipients of naive cell populations. Similarly there were no significant differences during the remission phase between 21 DPI cell recipients.

Cell populations prepared from donor mice at 21 DPI transferred a considerable degree of protection. Enriched T cells and unfractionated cells, upon adoptive

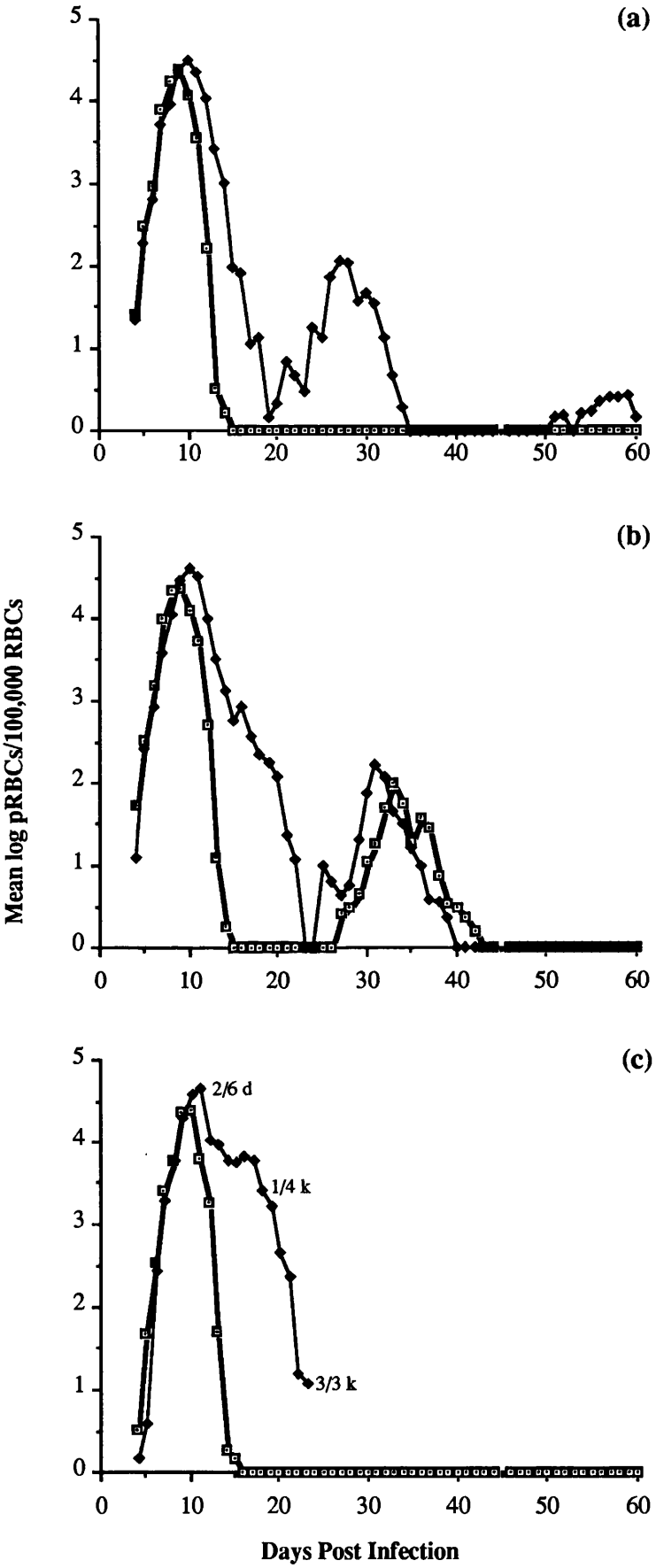


FIGURE 4.3.1 The course of infection in 4 Gy irradiated recipients of 2 x 10^7 spleen cells: a) enriched naive (◆——◆) or 21 DPI (■——■) T cells, b) enriched naive (◆——◆) or 21 DPI (■——■) B cells, or c) unfractionated naive (◆——◆) or 21 DPI (■——■) cells. Mice were challenged with 5 x 10^4pRBCs *P. c. chabaudi* CB.
d = dead, k = killed

transfer, resulted in what appeared to be a sterilising immunity (see section 4.4). With no recrudescences of infection detected by monitoring of daily blood smears it appeared that T cells could transfer an immunity that transcended antigenic variation, since the donor mice at 21 DPI would still have experienced one or more recrudescences. A difference in the pattern of protection was observed between recipients of enriched day 21 T or B cells. B cells, upon transfer, controlled the primary peak parasitaemia in the same manner as T cells (no significant differences, $p>0.05$), the only difference between the two groups of recipients was the appearance of a recrudescence in the B cell recipients.

4.4 Titration of effective lymphocyte numbers, 21 DPI, for the transfer of protection against *P. c. chabaudi* CB challenge

As a result of the efficiency with which enriched T cells from donor mice during subpatency (21 DPI) could transfer immunity, a study was carried out to determine the number of T lymphocytes that had to be transferred to an immunocompromised mouse to confer the level of resistance observed in section 4.3.

Four groups of recipient female NIH mice were exposed to 4 Gy of γ-irradiation 24 hours prior to adoptive transfer and challenge with 5×10^4 pRBCs *P. c. chabaudi* CB. The groups of mice were as follows: recipients of 2×10^7 enriched T cells from donor mice 21 DPI (32 mice), recipients of 10^7 T cells (6 mice), recipients of 10^6 T cells (6 mice) and recipients of 2×10^7 naive T cells (25 mice). A further group of 3 mice were not challenged but received 2×10^7 T cells as controls for pRBC contamination in the enriched spleen cell population.

The course of infection in recipient mice was followed by monitoring daily blood smears. In addition, to explore the function of the transferred T cells, serum was taken from recipient mice, either daily or every two to three days, for determination of Ab levels by IFAT, and nitrate levels as a measurement of NO production. On days 8, 9, 10, 11 and 13, four mice from the recipients of 2×10^7 naive or day 21 T cells were sacrificed and spleen cells prepared from the individual mice. These were stimulated with Con A and the supernatants analysed for IFN-γ by ELISA.

The proportion of cells staining by live IFAT for T and B cell markers is shown in Table 4.4.1. The percentage of T cells in the naive and 21 DPI cell preparations was 83.8% and 84.9% respectively, with less than 1.5% contaminating B cells in each case. Thus, the degree of enrichment was very similar to that in the previous 21 DPI transfer (4.3).

TRANSFERRED CELL POPULATION	PERCENTAGE OF CELLS STAINING FOR:	
	THY-1.2 (T cells)	IgG (Heavy chain) (B cells)
Enriched 21 DPI T cells	84.5	1.4
Enriched naive T cells	83.8	1.3

TABLE 4.4.1 Phenotypic analysis of 21 DPI T cell populations for titration of lymphocyte numbers.

The courses of infection in recipient mice are shown in Figure 4.4.1. The course of infection in mice receiving 2 x 10^7 day 21 T cells (Figure 4.4.1**a**) showed the same pattern of protection as that described earlier (4.3). There was a significant reduction in the peak parasitaemia compared to naive T cell recipients (11.2% compared to 28.1%, $p<0.01$) and there was a very rapid parasite remission; parasites were not detected in the peripheral blood of recipient mice from 15 DPI onwards. 15, 18, 21, and 30 DPI one mouse was killed, exsanguinated and the blood sub-inoculated into a naive recipient mouse. The recipients were then monitored for the presence of parasites in the peripheral blood by examination of daily blood smears. After 14 days none of the recipients showed signs of a patent parasitaemia, indicating that the immunity transferred by 2 x 10^7 T cells prepared from donor mice 21 DPI was a sterilising one, demonstrable as early as 15 DPI. On day 56 post infection, the remaining mice in this group were challenged with 1 x 10^7 pRBCs *P. c. chabaudi* CB. No patent parasitaemia developed. In appropriate controls, however, a parasitaemia was detected two days later.

Recipients of 10^7 day 21 enriched splenic T cells displayed a course of infection consisting of a reduction in peak parasitaemia, rapid parasite remission and extension of the subpatent period compared to naive T cell recipients (Figure 4.4.1**b**). The peak parasitaemia occurred 9 DPI, at 10.6% compared to 28.1% in naive cell recipients ($p<0.02$). During the remission phase, significant differences were detected in the parasitaemia from day 10 onwards compared to naive cell recipients ($p<0.001$). Although there were no differences between the peak parasitaemias of the recipients of 2 x 10^7 and recipients of 10^7 day 21 T cells ($p>0.05$), or between the parasitaemias during the remission of primary parasitaemia, the recipients of 10^7 cells showed a recrudescence 29 DPI, 5 out of 6 recipients recrudescing by 33 DPI. The peak of this recrudescence was lower than in naive recipients but was not statistically significant. Elimination of this recrudescence represented complete parasite clearance; sub-inoculation of blood into naive mice 43 DPI did not result in the development of a patent parasitaemia.

The adoptive transfer of 10^6 T cells from day 21 donors resulted in a course of infection shown in Figure 4.4.1**c**. Again there appeared to be a reduction in the peak

parasitaemia compared to naive cell recipients but this was not significant (14.3% compared to 28.1%, $p>0.05$). There was, however, no significant difference between the peak parasitaemia of this group of mice and the 2 x 10^7 and 10^7 T cell recipients. Parasite remission was more rapid in these recipients compared to naive cell recipients ($p<0.05$ 11, 12 and 14 DPI) but a recrudescence occurred the day after the parasitaemia reached subpatent levels. There was no difference in the level or day of the peak of this recrudescence.

The pattern of protection, therefore, changed as the number of transferred T cells decreased. A sterilising immunity was the result of the transfer of 2 x 10^7 day 21 T cells, complete parasite clearance having occurred by 15 DPI. The transfer of a smaller number of T cells conferred a level of immunity, but in each case a recrudescence did occur. The timing of this recrudescence was affected by the number of transferred cells, such that 10^7 cells delayed the onset of a recrudescence by 9 days whereas 10^6 transferred cells did not effect the timing or level of recrudescence compared to the naive cell transfer.

The antibody levels of the cell recipients, as measured by slide IFAT, are illustrated in Figure 4.4.2. The maximal Ab titres differed between the groups of cell recipients. In the recipients of 2 x 10^7 and 10^7 T cells, the peak Ab titre occurred just after the peak of parasitaemia while 10^6 T cell or naive T cell recipients showed maximal Ab titres following the recrudescence. The mean peak Ab titre of 2 x 10^7 T cell recipients was significantly higher ($p<0.05$) than in the other groups. It appears, therefore, that a more rapid antibody response occurs in mice receiving 2 x 10^7 T cells. The transfer of a smaller number of cells did not significantly affect the level or the pattern of antibody production.

Figure 4.4.3 shows the levels of nitrate in the serum of recipient mice. In every case a peak of NO production occurred on the day of peak parasitaemia, with no significant differences between any of the groups.

IFN-γ production by spleen cells from 2 x 10^7 day 21 T cell or naive T cell recipients is shown in Figure 4.4.4. Uninfected mice were used as controls and in response to stimulation with Con A produced approximately 20 U/ml IFN-γ within this experiment. On days 8 and 9 post infection, 21 DPI cell recipients produced significantly higher levels of IFN-γ ($p<0.01$) compared to either naive cell recipients or uninfected mice. Naive cell recipients produced a high level of IFN-γ 10 DPI, the day preceding peak parasitaemia, significantly higher ($p<0.01$) than on any other day when levels were measured. There was no significant difference ($p>0.05$) between the maximum levels in naive or day 21 cell recipients. These results indicate that in both naive and 21 DPI T cell recipients IFN-γ is produced maximally before the peak parasitaemia, but that in the latter case this occurs earlier and is more prolonged.

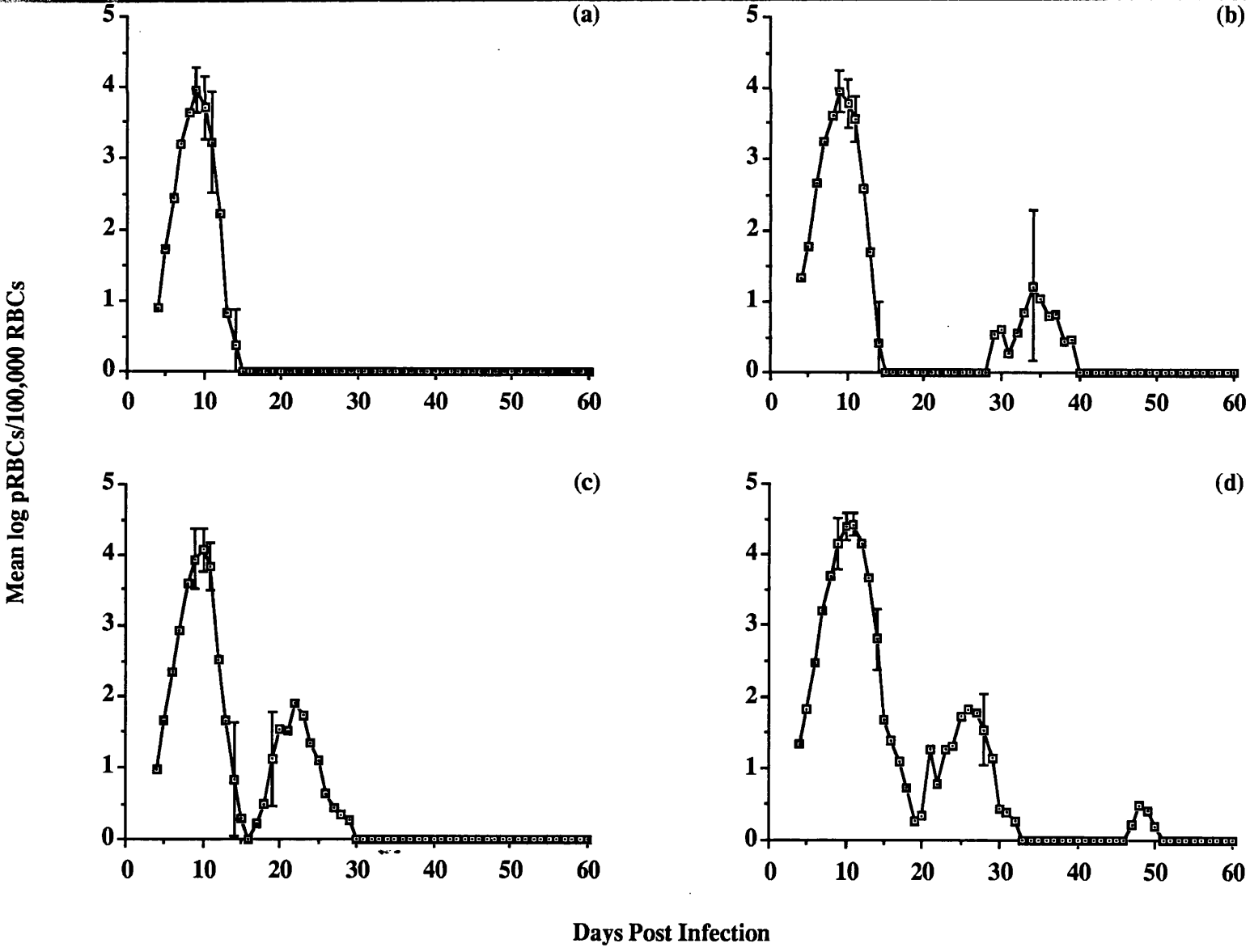


FIGURE 4.4.1 The course of infection in 4 Gy irradiated recipients of a) 2 x 10^7, b) 10^7, c) 10^6 splenic T cells from 21 DPI donors, and d) 2 x 10^7 naive T cells. Mice were challenged with 5 x 10^4pRBCs *P. c. chabaudi* CB.

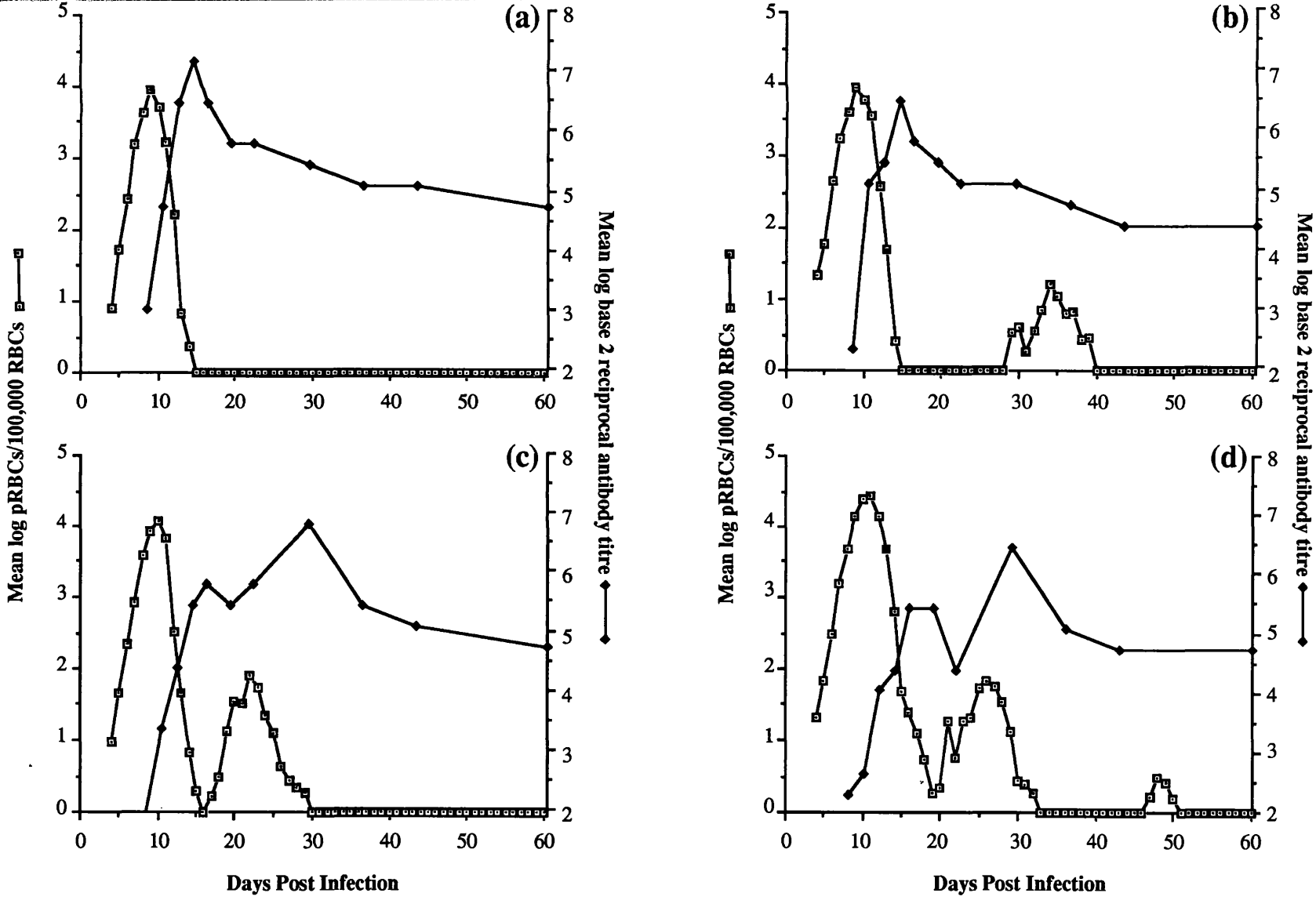


FIGURE 4.4.2 The serum antibody titre during the course of infection in 4 Gy irradiated recipients of a) 2×10^7, b) 10^7, c) 10^6 21 DPI T cells, and d) naive T cell recipients.

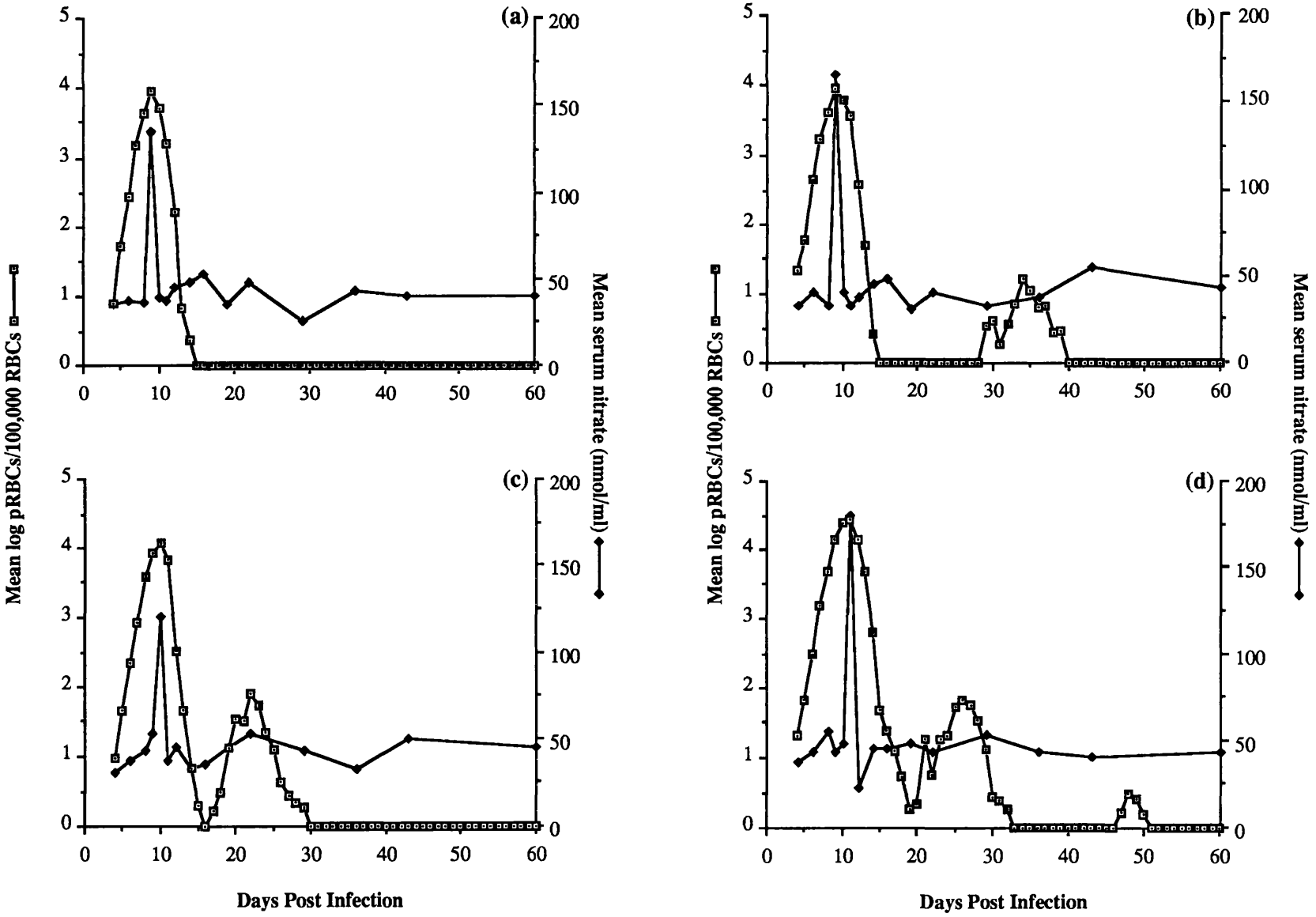


FIGURE 4.4.3 The serum nitrate levels during the course of infection in 4 Gy irradiated recipients of a) 2 x 10^7, b) 10^7, c) 10^6 21 DPI T cells, and d) naive T cells.

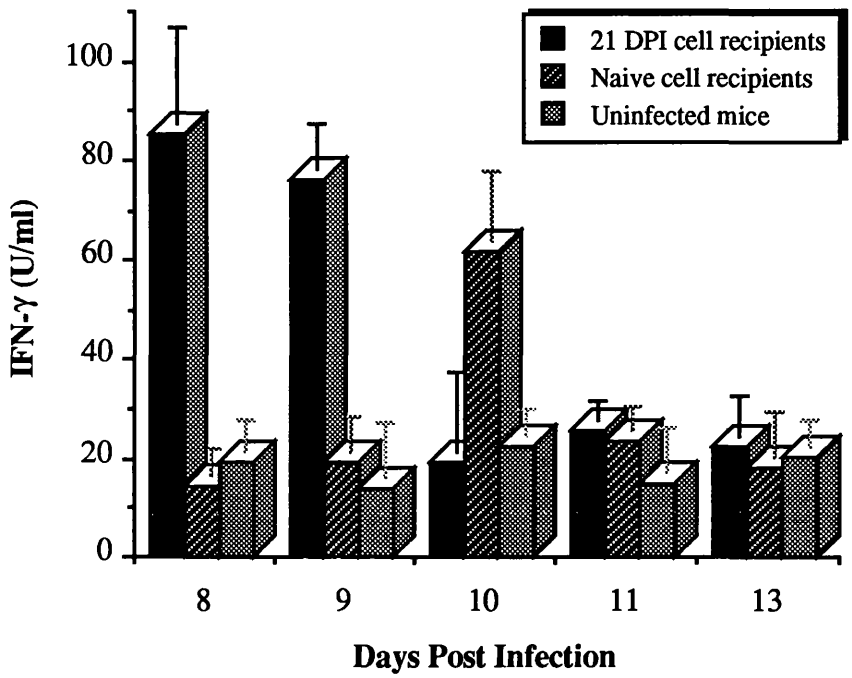


FIGURE 4.4.4 Interferon-gamma production by spleen cells from 4 Gy irradiated recipients of 2 x 10^7 21 DPI or naive T cells.

4.5 Adoptive transfer of splenic lymphocytes from chloroquine treated donor mice 75 DPI

This experiment was carried out to determine if subcurative chloroquine treatment (50mg/kg) of donor mice 10 DPI would affect the level of immunity transferred with T cells 75 DPI compared to that observed with T cells from naturally recovered mice at the same stage of infection. In addition, fluorescence Ab levels, to fixed parasite material, were measured every 3 days in the serum of recipient mice. In the transfer carried out 75 DPI from naturally recovered mice, only T cells were transferred (see 4.2); in this experiment mice receiving enriched B cells were also included.

Eight groups of six mice were irradiated to 4 Gy 24 hours before the transfer. The groups of mice received 2 x 10^7 aliquots of: enriched T or B cells from 75 DPI donors, enriched naive T or B cells, unfractionated spleen cells from 75 DPI or naive mice, no cells (challenge inoculum only), or 75 DPI unfractionated cells and no challenge (as a control for pRBC contamination in the spleen cell preparation). Mice were challenged with 5 x 10^4 pRBCs *P. c. chabaudi* CB immediately prior to adoptive transfer.

The identity of the transferred spleen cell populations was analysed by IFAT following the transfer, and this is shown in Table 4.5.1.

TRANSFERRED CELL POPULATION	PERCENTAGE OF CELLS STAINING FOR:	
	THY-1.2 (T cells)	IgG(Heavy chain) (B cells)
Unfractionated 75DPI cells	47.6	41.0
Unfractionated naive cells	31.1	34.9
Enriched 75 DPI T cells	86.4	0.4
Naive enriched T cells	86.0	0.7
Enriched 75 DPI B cells	5.3	60.9
Naive enriched B cells	5.0	51.5

TABLE 4.5.1 Phenotypic analysis of the transferred cell populations prepared from chloroquine treated (10 DPI) mice 75 DPI, measured by live IFAT.

The degree of enrichment achieved was good. In each of the enriched T cell populations, greater than 85% of the transferred population consisted of T cells, with less than 1% contaminating B cells. The enriched B cell populations consisted of more than 50% B cells, with less than 6% contaminating T cells.

The courses of infection in the recipient mice are shown in Figure 4.5.1. Recipients of 75 DPI unfractionated cells, without challenge, did not develop a patent parasitaemia. The course of infection in the mice that did not receive any cells is not

shown but was similar to that shown in Figure 4.2.1c.

The transfer of a population of T cells from chloroquine treated donor mice 75 DPI, resulted in a course of infection consisting of a reduced peak parasitaemia compared to the transfer of a naive T cell population (Figure 4.5.1a). There was a significant difference between the levels of the peak parasitaemia (10.4% compared to 28.7%, $p<0.05$). A faster parasite remission occurred in the 75 DPI T cell recipients compared to naive T cell recipients ($p<0.01$, 10 and 11 DPI; $p<0.001$, 12 and 13 DPI), no parasites were detected by blood smear from day 13 post infection through to 60 DPI when the experiment was terminated. Naive T cell recipients did not all become subpatent until 34 DPI and a recrudescence followed 4 days later in 4 out of 6 mice. There were no recrudescences of infection in the 75 DPI T cell recipients.

Mice that received enriched 75 DPI B cells displayed a course of infection similar to T cell recipients (Figure 4.5.1b). There was, however, no significant difference between the peak parasitaemia in 75 DPI B cell recipients compared to naive B cell recipients ($p>0.05$) although the peak occurred a day earlier in the former. Protection was manifest as a more rapid parasite remission compared to naive B cell recipients, $p<0.01$ (10, 11 and 12 DPI) and $p<0.001$ (13 and 14 DPI). There were no recrudescences of infection; two occurred in naive B cell recipients.

The transfer of an unfractionated population of splenic cells from day 75 chloroquine treated donors significantly reduced ($p<0.01$) the peak parasitaemia (5.6%) compared to naive unfractionated cell recipients (17.8%) (Figure 4.5.1c). There was rapid parasite remission and no recrudescences of infection. Naive unfractionated cell recipients experienced a prolonged primary parasitaemia, with a very short subpatent period before a recrudescence appeared. A further recrudescence occurred in 2 out of 6 mice 53 DPI.

Subcurative chloroquine treatment of donor mice 10 DPI did not affect the ability of selected lymphocyte populations to transfer immunity. Protection was manifest in the same manner as the adoptive transfer of splenic lymphocytes from naturally recovered mice 75 DPI (4.2). In this experiment, however, a population of B cells was also transferred. The course of infection in these recipients followed the same pattern as that of T cell recipients. The peak parasitaemia, however, was not significantly affected by the transfer of B cells. The transfer of a population of unfractionated cells resulted in a better degree of protection than the transfer of either T or B cell enriched populations alone. There was a significant reduction ($p<0.05$) in the peak parasitaemia (5.6%) of unfractionated cell recipients compared to that (10.4%) of T cell recipients. This indicates that a level of synergy between the T and B cell populations within an unfractionated cell population occurred.

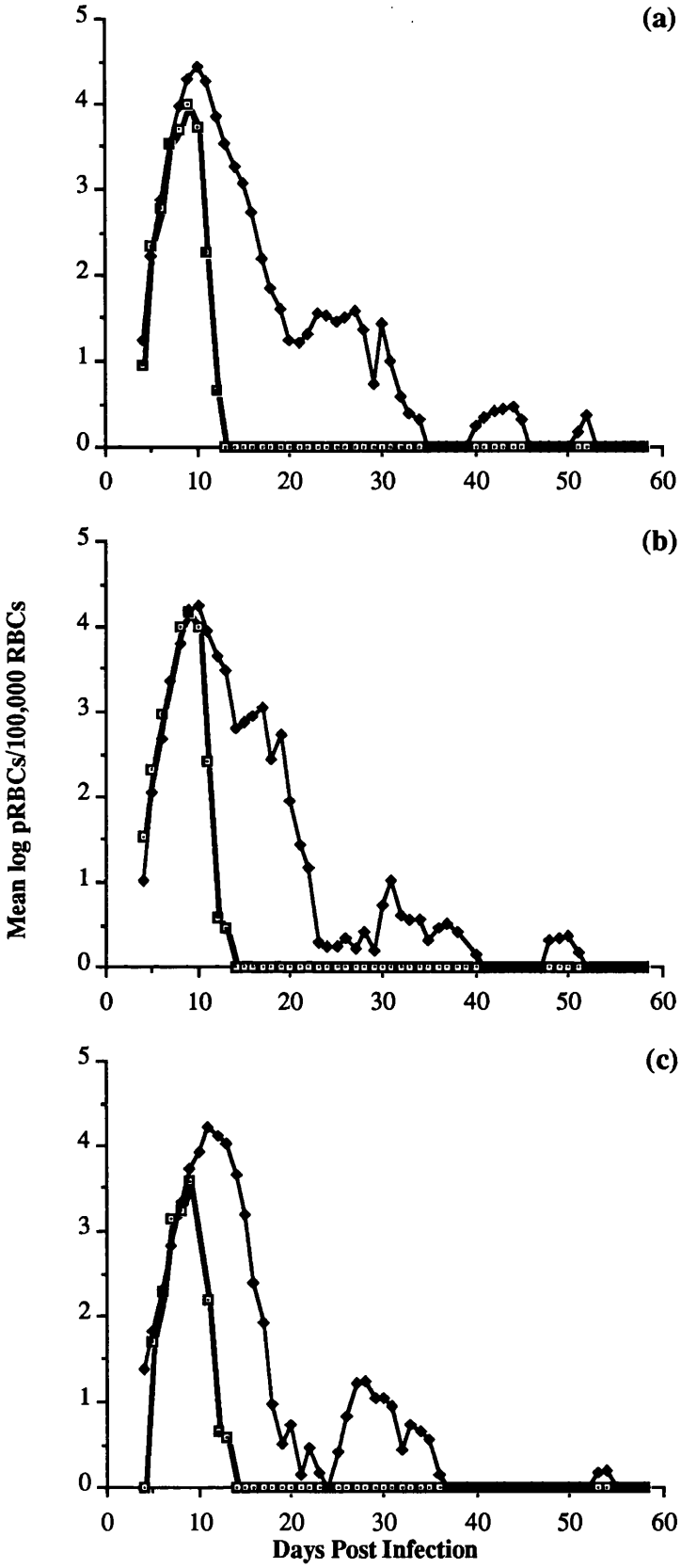


FIGURE 4.5.1 The course of infection in 4 Gy irradiated recipients of 2 x 10^7spleen cells: a) enriched naive (◆——◆) or 75 DPI (▫——▫) T cells, b) enriched naive (◆——◆) or 75 DPI (▫——▫) B cells, or c) unfractionated naive(◆——◆) or 75 DPI (▫——▫) cells. Day 75 cell populations were prepared from donor mice that had been treatedwith chloroquine 10 DPI. Mice were challenged with 5 x 10^4pRBCs *P. c. chabaudi* CB.

The Ab levels in the recipient mice is illustrated in two different ways. Figure 4.5.2 illustrates the Ab levels, as measured by slide IFAT, associated with the course of infection of recipient mice. Figure 4.5.3 shows the Ab titres of the recipients of each lymphocyte population from 75 DPI compared to the recipients of the corresponding naive population.

In each group of recipient mice, the maximum Ab titre occurred after the peak parasitaemia (Figure 4.5.2). There then followed a decline in the Ab level, although in the recipients that experienced a recrudescence, the Ab titre increased following the recrudescence.

Recipients of cell populations from 75 DPI donors developed specific anti-*P. c. chabaudi* CB Abs before recipients of naive cell populations, these in turn making an Ab response before irradiated mice that did not receive any cells (Figure 4.5.3). In each case there was a significant difference ($p<0.05$) between the maximum Ab titres in 75 DPI recipients compared to those in naive recipients. There were no significant differences ($p>0.05$) at any stage throughout the course of infection in the Ab titres of any of the 75 DPI cell recipients.

The 75 DPI transferred cell populations, therefore, all caused an increase in the production of anti-malarial Abs compared to the transfer of naive cell populations. There was, however, no difference between the different cell populations in the kinetics of Ab production. The titres illustrated in Figures 4.5.2 and 4.5.3 are measured by slide IFAT and do not give a measure of protective activity or Ig isotype conferring immunity in each of the recipients.

4.6 Adoptive transfer of splenic lymphocytes from chloroquine treated mice 14 DPI

In mice that are treated with 50 mg/kg of chloroquine at peak parasitaemia (usually 10 DPI) there is a rapid parasite remission to subpatent levels by 14 DPI. This is followed by a recrudescence which is higher than that observed in untreated mice. The time point of 14 DPI was chosen for this adoptive transfer because it represented the first point during the course of infection in chloroquine treated mice where the parasitaemia was subpatent. It, therefore, was used as a comparison of the 21 DPI adoptive transfers from naturally recovering mice; subpatency in these mice.

Ten donor mice were infected with 5×10^4 pRBCs *P. c. chabaudi* CB and the course of infection monitored. 10 DPI, at the peak parasitaemia (35.4%), the mice were treated with 50 mg/kg chloroquine. Four days later (14 DPI) when the parasitaemias of these mice were subpatent, the mice were killed and spleen cell populations prepared. Ten naive, sham-infected and chloroquine treated mice were used as the naive cell donors.

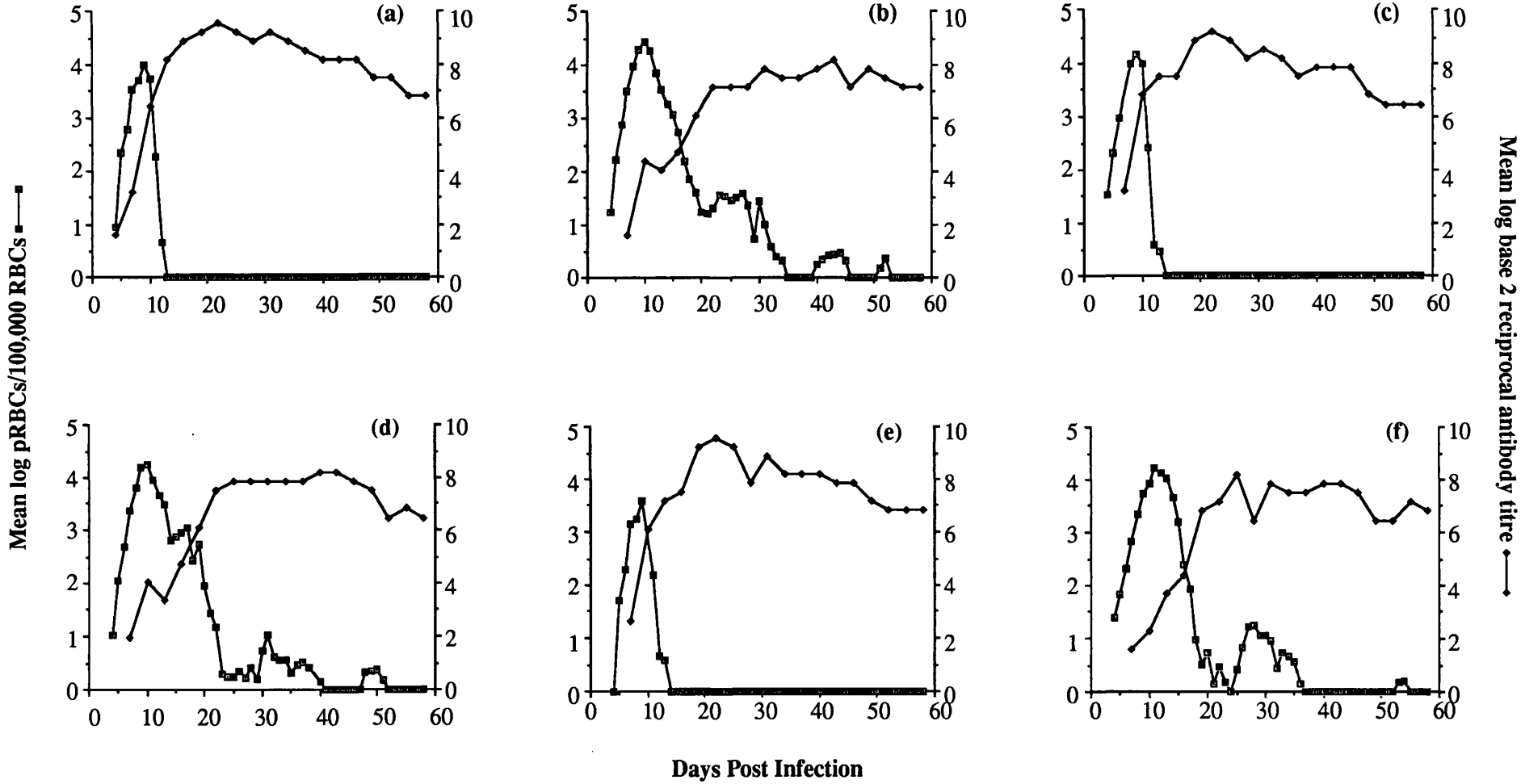


FIGURE 4.5.2 Serum antibody titres during the course of infection in 4 Gy irradiated recipients of 2 x 10^7 a) 75 DPI enriched T cells, b) naive T cells, c) 75 DPI enriched B cells, d) naive B cells, e) unfractionated 75 DPI cells, and f) naive unfractionated cells. 75 DPI donor mice were treated with chloroquine 10 DPI.

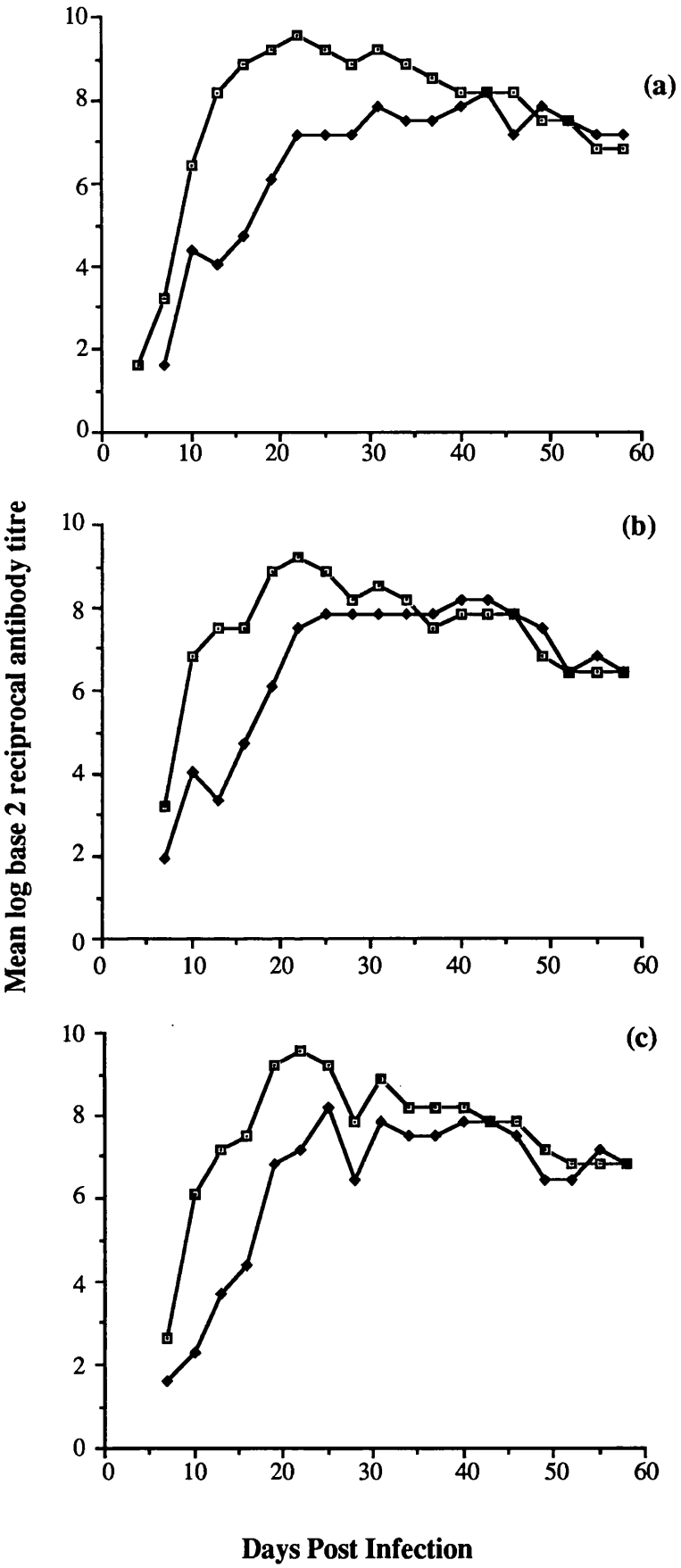


FIGURE 4.5.3 The serum antibody titres of 4 Gy irradiated recipients of a) T cells, b) B cells, and c) unfractionated cells prepared from chloroquine treated donor mice 75 DPI (□——□) or from naive mice (◆——◆).

Six groups of six mice were exposed to 4 Gy γ-irradiation 24 hours before adoptive transfer and parasite challenge. The groups of mice receiving aliquots of 2 x 10^7 spleen cells were as follows: recipients of enriched T or B cells from 14 DPI donors, recipients of naive enriched T or B cells and recipients of 14 DPI or naive unfractionated cells. In addition three mice received unfractionated cells without parasite challenge as a control for pRBC contamination. Mice were challenged with 5 x 10^4 pRBCs prior to adoptive transfer.

The degree of enrichment of the prepared spleen cell populations was analysed by live IFAT and is shown in Table 4.6.1. The percentage of T cells in the enriched 14 DPI and naive spleen cell populations was 83.8% and 81.0% respectively with less than 2% contaminating B cells in each case. The enriched B cell populations contained greater than 45% IgG (heavy chain) staining cells with less than 8% contaminating T cells. The unfractionated populations contained approximately equal numbers of T and B cells, but less than 40% of each in both prepared populations.

TRANSFERRED CELL POPULATION	PERCENTAGE OF CELLS STAINING FOR:	
	THY-1.2 (T cells)	IgG(Heavy chain) (B cells)
Unfractionated 14 DPI cells	39.0	31.2
Unfractionated naive cells	36.0	33.1
Enriched 14 DPI T cells	83.8	1.6
Naive enriched T cells	81.1	1.2
Enriched 14 DPI B cells	7.8	48.8
Naive enriched B cells	7.5	45.3

TABLE 4.6.1 Phenotypic analysis of transferred cell populations from chloroquine treated donor mice 14 DPI.

The courses of infection in recipient mice following adoptive transfer are shown in Figure 4.6.1. Recipients of 14 DPI enriched T cells demonstrated a course of infection consisting of a slight reduction in peak parasitaemia, although this was not significant compared to the peak parasitaemia in naive T cell recipients ($p>0.05$) (Figure 4.6.1a). Protection, however, was manifest as a more rapid parasite remission ($p<0.01$, 10 and 11 DPI; $p<0.001$, 12-15 DPI). All mice had subpatent parasitaemias by 17 DPI although this was not long lasting. By 19 DPI 4 out of the 6 recipients had shown recrudescences and there were no further significant differences throughout the rest of the infection.

The course of infection in recipients of 14 DPI B cells is shown in Figure 4.6.1b. Compared to naive B cell recipients there was a reduction in the peak parasitaemia

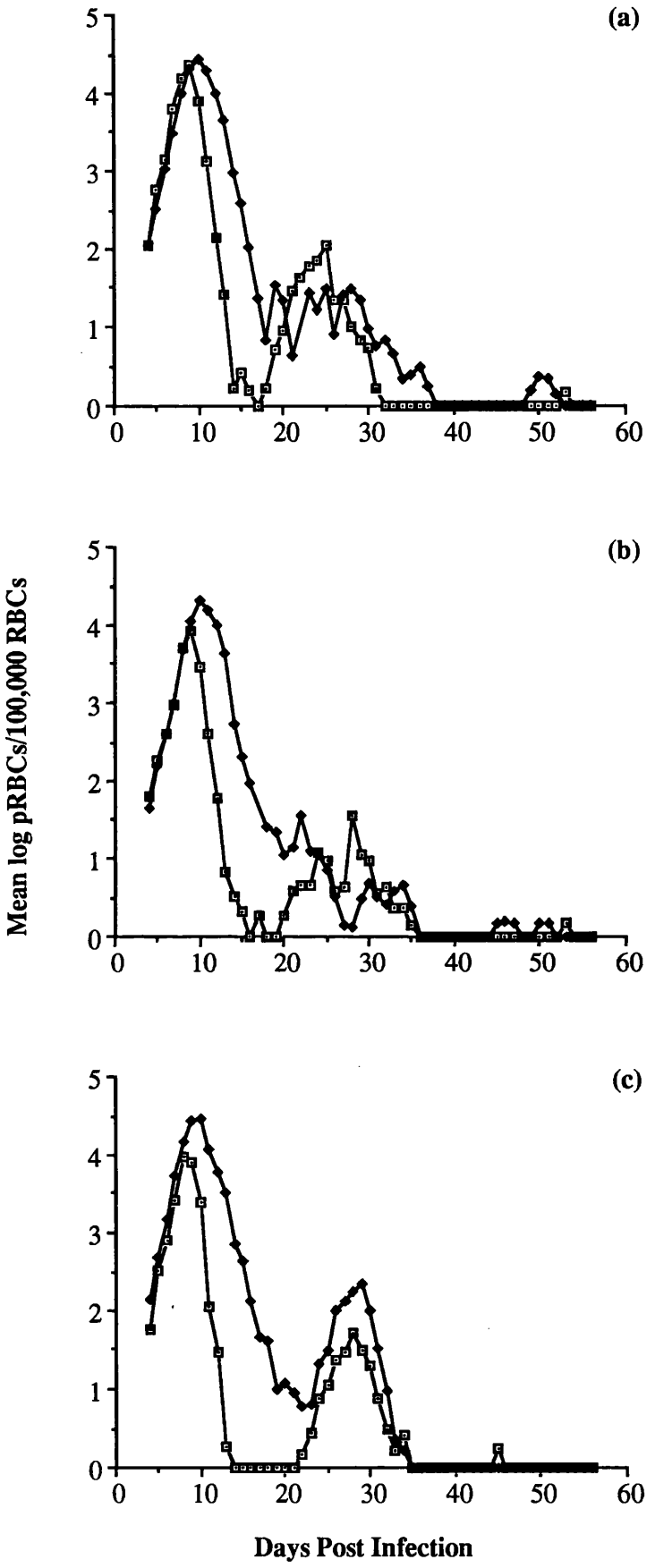


FIGURE 4.6.1 The course of infection in 4 Gy irradiated recipients of 2 x 10^7 spleen cells: a) enriched naive (◆——◆) or 14 DPI (▫——▫) T cells, b) enriched naive (◆——◆) or 14 DPI (▫——▫) B cells, and d) unfractionated naive (◆——◆) or 14 DPI (▫——▫) cells. 14 DPI cell populations were prepared from donor mice that had been treated with chloroquine 10 DPI. All mice were challenged with 5 x 10^4 pRBCs *P. c. chabaudi* CB.

(11.8% compared to 22.1%, $p<0.05$) and a faster parasite remission ($p<0.001$, 12-15 DPI), subpatency reached 15 DPI. A recrudescence occurred a few days later, from which point no further significant differences were observed between these two groups of cell recipients.

The adoptive transfer of 14 DPI unfractionated spleen cells engendered the greatest level of protection. Compared to the transfer of a naive unfractionated cell population (Figure 4.6.1c) there was a significant depression of the peak parasitaemia (13.6% compared to 30.8%, $p<0.02$) and more rapid parasite remission ($p<0.01$, 10 DPI; $p<0.001$, 11-15 DPI). Parasitaemias of all recipient mice were subpatent by 14 DPI and remained so until 22 DPI when one of the recipients showed a patent parasitaemia. A defined recrudescence then followed significantly lower than that observed in naive cell recipients ($p<0.05$).

Cell populations from chloroquine treated mice 14 DPI were, therefore, clearly effective at transferring a degree of immunity. While recipients of naive/unstimulated cell populations ultimately cleared their infections they never achieved a period of subpatency. Primed T and B cells both conferred a degree of immunity, but a sterilising immunity was not achieved as was demonstrated with the other adoptive transfer experiments. Interestingly, an enhanced protection was conferred by B cells over the T cell population, seen as a more profound reduction of primary parasitaemia and day at which subpatency was reached. Unfractionated cells clearly gave the most protection, with significant reduction of peak parasitaemia and extension of the subpatent period.

Most *in vitro* investigations have shown that chloroquine suppresses the proliferation of lymphocytes in a dose-dependent manner. To examine whether the dose of 50 mg/kg of chloroquine used at peak parasitaemia may have affected the proliferative response of lymphocytes from donor animals 14 DPI *in vivo*, naive mice were treated with chloroquine prior to *in vitro* stimulation of splenic lymphocytes. Five groups of two mice were used for this experiment, receiving chloroquine treatment four days, three days, two days or one day prior to Con A stimulation *in vitro* of spleen cells. Another group of mice was untreated. Figure 4.6.2 shows the result. There was no difference in the proliferative response to Con A of naive spleen cells whether they were treated with chloroquine or not. It appears, that at least to a T cell mitogen, naive cells are not affected by the addition of chloroquine at doses used *in vivo*. The response of primed lymphocytes to either Con A or pRBCs was not tested for.

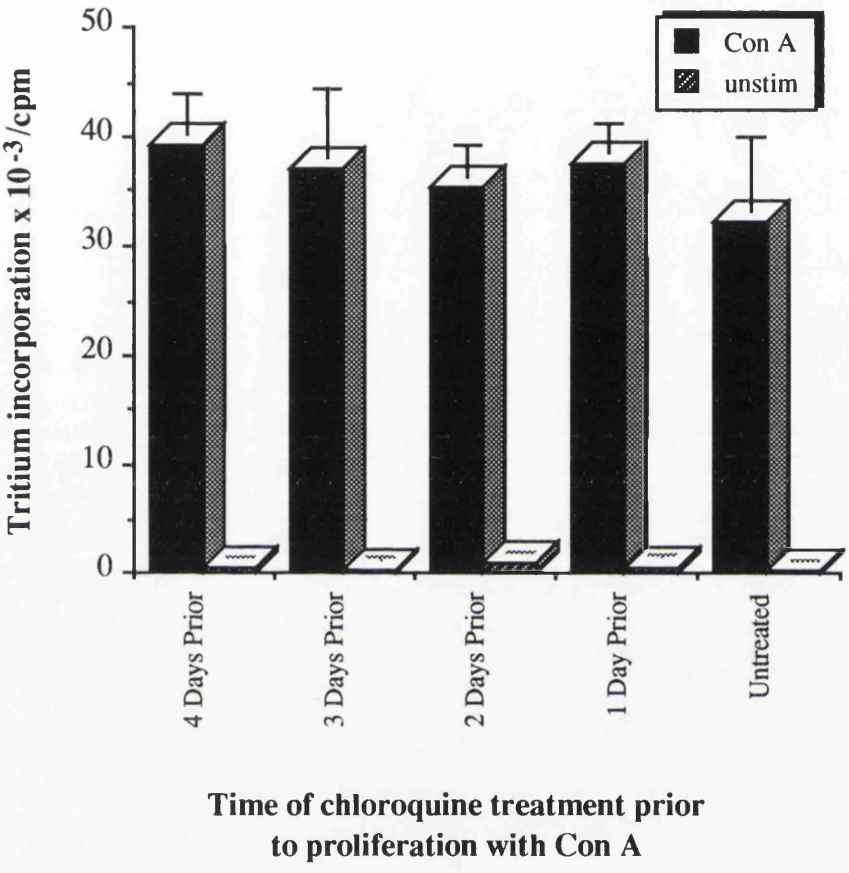


FIGURE 4.6.2 The effect of *in vivo* chloroquine treatment (50 mg/kg) on the proliferation of naive spleen cells with 5 μg/ml Con A.

4.7 Discussion

The spleen represented a readily available source of lymphocytes for the adoptive transfers throughout the course of *P. c. chabaudi* CB infection. Kumararatne *et al.* (1987), however, showed that by day 11 post *P. c. chabaudi* AS infection the spleen shows partial lymphocyte depletion, coinciding with a massive absolute lymphocytosis in the peripheral blood, due to a redistribution of lymphocytes. Shortly after peak 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 & DeSouza, 1982). Sequestration of malaria parasites occurs in the liver (Shungu & Arnold, 1972; Cox & Hommel, 1984) and this may be the reason for lymphocytes accumulating in this organ. This suggests that the liver is important in the development of protective immunity to malaria. A study, therefore, to investigate the ability of liver, lung and peripheral blood lymphocytes to transfer protection would also be of interest.

A consistent proportion of enriched splenic cell subpopulations was achieved by nylon-wool incubation. For each of the adoptive transfers the degree of enrichment was such that an enriched effluent T cell population always contained >80% T cells and less than 2% contaminating B cells. Eluted B cell populations contained more than 50% B cells and always less than 7% contaminating T cells. These are similar proportions as those obtained by Julius *et al.* (1973) who first used this method.

Subpopulations of lymphocytes derived from the spleens of infected donor mice were effective at transferring immunity from all the stages of infection from which cells were prepared. In general, both enriched T cells and enriched B cells upon adoptive transfer were able to confer a level of protection to immunocompromised mice, but unfractionated spleen cells conferred the greatest level of protection. These observations with the *P. c. chabaudi* CB/NIH mouse 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 DPI, peaked at 14 DPI and was no longer detectable by 21 DPI, 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 report of Favila-Castillo *et al.* (1990). These investigators used the AS strain of *P. c. chabaudi*, but in CBA/Ca mice, and reported 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. A recent study by the same workers (Legorreta-Herrera *et al.*, 1993) showed that spleen cells from recently recovered *P. c. chabaudi*

AS infected (BALB/c X C57Bl/6) F_1 mice could transfer protection into irradiated syngeneic recipients. In addition, Taylor-Robinson (1991) using the same strain of parasite demonstrated that protection could be achieved as early as 12 DPI with unfractionated peripheral blood lymphocytes. This points to the possibility of a genetic factor involved in host immunity to *P. c. chabaudi*, as suggested by Stevenson *et al.* (1982), although neither CBA/Ca or NIH mice were employed in this study. Other reasons for the lack of immunity transferred with lymphocytes other than those from superinfected donors, as described by Favila-Castillo (1990) are difficult to explain considering the ease with which immunity could be transferred in this study and those reported above.

In none of the experiments described in this thesis 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 agreement with previous studies of adoptive immunity to rodent malarias (Brown *et al.*, 1976a, b; Gravely & Kreier, 1976; McDonald & Phillips, 1978; 1980; Brinkmann *et al.*, 1985; Cavacini *et al.*, 1986) which showed that in various levels of immunosuppression, infected mice and rats inoculated with immune lymphocytes exhibited a protective response demonstrable by a reduction in the peak parasitaemia, quickened pRBC elimination and/or host survival.

Regarding the adoptive transfer experiments described in this chapter, in general, the protective effect of unfractionated splenic cells was greater than that of either enriched T or enriched B lymphocytes. This was manifest as a significantly reduced peak parasitaemia compared to all other cell recipients. In some cases, however, the immunity conferred by T cells and unfractionated cells could not be distinguished. The maximal protection of unfractionated cells was suggestive of a synergistic effect between the cells of mixed phenotypes within the population. McDonald & Phillips (1978), working with *P. c. chabaudi* AS in the NIH mouse, showed that for the most efficient protection of irradiated mice against 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. They failed, however, to show unequivocally synergistic activity between primed lymphocyte subsets in irradiated mice. The same pattern of protection was conferred by T and B lymphocytes from donor mice 75 DPI, with or without chloroquine treament, indicating that both lymphocyte subsets are required for the elaboration of immunity to *P. c. chabaudi* CB. This is not in agreement with the findings of Cavacini *et al.* (1990) who showed that B-cell deficient mice could resolve infection with the CB strain of *P. c. chabaudi* with the same kinetics as normal mice. In each of the 75 DPI cell transfers, the greatest level of protection was achieved by the

transfer of an unfractionated population of spleen cells. This was manifest as a significant reduction in the peak parasitaemia. To investigate the synergistic activity of the cells within the unfractionated cell population, the Ab levels of the day 75 cell recipient mice were measured by IFAT. Recipients of immune day 75 lymphocytes all showed higher antibody titres compared to the recipients of naive cells, but there was no difference in the titres of unfractionated spleen cell recipients compared to recipients of either T cells or B cells. Synergy, therefore, was not indicated by the IFAT Ab levels of the recipient mice.

McDonald & Phillips (1978) showed that most but not all of the protective activity on adoptive transfer of *P. c. chabaudi* AS immune spleen cells lay in the B cell-enriched fraction; Ferraroni & Speer (1982) showed a similar result with *P. berghei*-primed mouse splenic lymphocytes. Brown *et al.* (1976a) demonstrated protective activity of T-enriched populations from the spleen of *P. berghei* infected rats upon adoptive transfer of naive syngeneic recipients, but there was increased protection on the addition of B cells. 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 and B cells. They considered, therefore, that the primed T cells were acting as helper cells in the production of specific anti-*P. berghei* protective Ab as first proposed by Brown (1974).

The results of the 21 DPI cell transfers are in direct contrast to the above studies. The transfer of enriched T cells conferred a sterilising immunity, while the transfer of the same number of B cells, while conferring protection, did not prevent the development of a patent recrudescence. It appeared, therefore, that T cells from mice during the subpatent period of infection were capable of imparting an immunity that transcended antigenic variation. Brown (1974) suggested that T cells might be important as helpers in establishing variant-transcending immunity. He suggested that, during malarial infection T cells become primed to a determinant common to all plasmodial variants but characteristic of the strain producing the infection. 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 variants developed later in infection. This could be feasible as the transfer of B cells alone, 75 DPI, could transfer immunity without the development of recrudescences while those from 21 DPI could not. 75 DPI B cells were presumably primed to all variant parasite Ags while those from 21 DPI donors had not seen the variants arising during the recrudescent parasitaemias that would have developed in donor mice. The transfer of B cells from 21 DPI donors (immunologically immature) to immunocompromised hosts would result in the recipients having only the immunity conferred by the

differentiated B cells that they received. Because insufficient T cells were present in the transferred population these animals could not respond rapidly to antigenic variants and thus a recrudescence arose. If this is the case, T cells act as helpers for antibody production, and immunity to *P. c. chabaudi* CB is mediated by humoral mechanisms. This is a direct contradiction of the results of Cavacini *et al.* (1990) who demonstrated that immunity to the CB strain is Ab-independent.

Non-humoral mechanisms appear to resolve infection in some rodent models of malaria (Jayawardena *et al.*, 1982; Brinkmann *et al.*, 1985; Cavacini *et al.*, 1986). Brinkmann *et al.* (1985) demonstrated that the reconstitution of nude mice with naive T cells, but not with naive B cells, restored a protective immune response in recipient animals. In the case of *P. c. adami*, infected athymic mice develop a high-grade, fulminating infection; T cell but not B cell enriched naive or immune spleen cells suppress challenge infection in otherwise susceptible nude mice (Cavacini *et al.* (1986). In this study, T cells from 21 DPI were more effective at transferring immunity to *P. c. chabaudi* CB than the same number of B cells. Titration of the number of T lymphocytes necessary to confer a sterilising immunity showed that while no difference in the reduction or remission of the primary parasitaemia occurred as the number of T cells decreased, a smaller number of T cells did not prevent a recrudescence from arising. It is possible, therefore, that the immunity conferred by enriched B cells was in fact due to the presence of a proportion of contaminating T cells. However, 5% contaminating T cells would be the equivalent of 10^6 T cells and the protection achieved by such a number of T cells follows a different pattern than that produced upon transfer of B cells.

An attempt at analysing the function of the 21 DPI T cells was made. This was done by measuring Ab titres and nitrate levels during the course of infection, as well as looking at the production of IFN-γ from spleen cells around the peak of infection. The antibody levels in the recipient mice were significantly higher than those of naive T cell recipients, although the transfer of a smaller number of cells did not significantly affect the level or pattern of antibody production. It appears, therefore, that a more rapid antibody response occurs in recipients of 21 DPI T cells, but following this there is no further difference in the Ab levels. NO and IFN-γ production are indicators of a T_H1 response. The levels of nitrate in the serum of infected mice did not differ significantly in the recipients of 21 DPI T cells or naive T cells. However, there was a more prolonged and earlier production of IFN-γ in the 21 DPI T cell recipients than in the naive cell recipients. The results, therefore, indicate that a mix of T_H1 and T_H2 cells was probably contained within the transferred 21 DPI T cell population.

Legorreta-Herrera *et al.* (1993) have described the successful transfer of protection aginst *P. c. chabaudi* AS with anti-Thy-1 treated, irradiated spleen cells.

This not only suggests that the protective activity of immune spleen cells lies outwith the T cell fraction, but that a radioresistant cell population confers immunity. It is also possible, therefore, that within the recipients used in this study protection may also have been conferred by radioresistant cells. The different pattern of protection conferred by T and B cells may have been due to a difference in cooperation with radioresistant cells. Although both T and B cells are lost after irradiation, a proportion of T cells (Nossal & Pike, 1973), and B cells (Pilarski & Cunningham, 1974) are relatively radioresistant. Macrophages are relatively radioresistant as far as some macrophage functions are concerned and sensitive with regard to others (Geiger & Gallily, 1974).

Faster parasite remission occurs in mice treated with chloroquine at peak parasitaemia. Consequently, subpatency occurs 14 DPI and a recrudescence follows quickly. Because this stage of infection represented the first point of parasite remission in these animals, an adoptive transfer was carried out to compare the level of immunity transferred from this point of infection with that transferred from subpatency in naturally recovering mice (21 DPI). Significant reduction of the peak parasitaemia was observed in the recipients of the different 14 DPI cell populations. Compared to the transfer of naive cells, transfer of 14 DPI cells resulted in a faster remission of the parasitaemia. Recrudescences, however, followed in all the groups of recipients. A difference, therefore, was demonstrated in the level of immunity conferred by splenic lymphocytes from the subpatent period of naturally recovering mice and those from the subpatent period of mice treated with chloroquine. One reason for this may not be due to a specific defect in the T cell repertoire but simply that the lymphocytes from chloroquine treated mice 14 DPI were not as immunologically mature as those from naturally recovered mice 21 DPI. Another reason may have been that chloroquine treatment *in vivo* resulted in the suppression of lymphocyte activation and proliferation. *In vitro* studies have demonstrated that chloroquine suppresses the proliferation of lymphocytes in a dose-dependent manner (Hurvitz & Hirschhorn, 1965; Panush, 1975; Gery & Eidinger, 1977; Trist & Weatherall, 1981; Salmeron & Lipsky, 1983). To exclude this in the present study the proliferative response of naive spleen cells following *in vivo* chloroquine treatment was investigated. No difference in the response of spleen cells to Con A was observed following several regimes of chloroquine treatment. It would appear that the dose of chloroquine used at peak parasitaemia was not sufficient to cause immunosuppression. Indeed, Bygbjerg & Flachs (1986) demonstrated that the immunosuppressive effects by chloroquine were dose-related as only high concentrations of chloroquine profoundly suppressed the proliferation of mitogen- and antigen-stimulated lymphocytes.

That no recrudescences were observed in the recipients of 21 DPI T cells was somewhat surprising. The patent parasitaemias of donor mice from this stage of infection are expected to recrudesce and, therefore, assuming antigenic variation is a feature of *P. c. chabaudi* CB infection, mice would not be primed to all the variant-specific Ags during a course of infection. The subpatent period is poorly defined for the CB strain of *P. c. chabaudi* so individual donor mice may have experienced different antigenic variants such that sufficient cells reactive to the different variants were included in the transferred T cell population. Alternatively, if recrudescences arise as a result of a decline in the effector arm of the immune response during remission of the primary parasitaemia then it is also surprising that T cells from subpatency transferred a sterilising immunity.

The experiments described in the next chapter examine the phenomenon of antigenic variation in *P. c. chabaudi* CB in both naturally recovering and chloroquine treated mice, as a direct result of the different patterns of protection described here.

CHAPTER FIVE

PASSIVE TRANSFER OF IMMUNITY AND ANALYSIS OF RECRUDESCENT PARASITE POPULATIONS

5.1 Introduction

Chapter four demonstrated that effective immunity could be transferred with enriched populations of spleen cells. The aims of the experiments described in this chapter were twofold: firstly, to determine if passive transfer of serum from donor mice 21 DPI or 14 DPI could confer protection upon parasite challenge, and secondly, to investigate antigenic variation by serum sensitivity, as described for *P. c. chabaudi* AS (McLean *et al.*, 1982b), in recrudescent parasite populations collected from naturally recovering and chloroquine treated mice. This method tests the ability of serum collected from mice during the subpatent period of infection to protect against a challenge infection with either the infecting parasite population or recrudescent parasites. It was considered important to investigate the ability of serum, and presumably Ab, to transfer protection since one report (Cavacini *et al.*, 1990) suggests that immunity to *P. c. chabaudi* CB is independent of Ab.

Numerous investigators have used the method of passive Ab transfer to define more specifically the nature of protective Ab production, to evaluate ways for increasing the level of protective Ab formation, to establish the isotype of protective Abs, and to characterise further protective humoral immunity (Stechschulte *et al.*, 1969; Zuckerman & Golenzer, 1970; Phillips & Jones, 1972; Lourie & Dunn, 1972; Brown & Phillips, 1974; Golenzer *et al.*, 1975; Wells & Diggs, 1976). The majority of these studies examined blood-induced *P. berghei* infection in rats or mice. In general, the results showed that the passive transfer of serum at the same time as infection resulted in a delayed onset in detectable parasitaemia, reduced peak parasitaemias and enhanced the survival of challenged animals (Zuckerman & Golenzer, 1970; Phillips, 1970; Phillips & Jones, 1972; Brown & Phillips, 1974; Golenzer *et al.*, 1975; Lourie & Dunn, 1972). The transfer of hyperimmune sera to *P. berghei*-infected mice resulted in delayed parasitaemia but the animals generally succumbed to the disease (Stechschulte *et al.*, 1969; Brown & Phillips, 1972; Wells & Diggs, 1976). The transfer of hyperimmune sera was considerably better than sera from animals with an ongoing or chronic infection (Stechschulte *et al.*, 1969; Phillips & Jones, 1972) or sera from an animal that had recovered from a single infection (Diggs & Oster, 1969; Zuckerman & Golenzer, 1970). Levels of protective activity change rapidly during the course of infection, therefore, with regard to serum protection, the timing of serum collection is crucial (R.S. Phillips, personal communication).

Antigenic variation in the asexual erythrocytic stages of malaria parasites has been well documented. This phenomenon was first described in *P. berghei* by Cox (1962), and subsequently in *P. knowlesi* (Brown & Brown, 1965; Brown, 1973; Howard *et al.*, 1984), *P. cynomolgi bastianelli* (Voller & Rossan, 1969), *P. c. chabaudi* AS (McLean *et al.*, 1982b; 1986a; Gilks *et al.*, 1990), *P. falciparum in vivo*

(Hommel *et al.*, 1983) and *in vitro* (Biggs *et al.*, 1991; Roberts *et al.*, 1992), and *P. fragile* (Handunnetti *et al.*, 1987). It is now considered likely that antigenic variation is a feature of most, if not all, malaria parasites. Using a passive transfer protection system, recrudescences of *P. c. chabaudi* AS have been shown to be antigenically different from the cloned infecting parental population (McLean *et al.*, 1982b), and appear to contain a mix of variant antigenic types (McLean *et al.*, 1986a).

This chapter describes the results of a passive transfer experiment and demonstrates that protection can be transferred with serum collected during the subpatent period of infection (herein referred to as 'immune subpatent serum') from both naturally recovering and chloroquine treated mice. In addition, it describes the difference in serum sensitivity of recrudescent parasites collected from both these groups of mice. Using a triple-layered indirect fluorescent antibody test on live, schizont-infected erythrocytes as described by McLean *et al.* (1986b) these recrudescent parasite populations were evaluated further for the occurrence of antigenic variation.

5.2 Serum antibody titres during the course of infection

Two groups of six age-matched female mice were infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB and the course of infection followed. One group of mice was treated with 50 mg/kg chloroquine at the peak of infection (10 DPI). Small volumes of serum were collected from the tails of two mice in each group at two day intervals during the course of infection. The antibody levels in the sera of these mice were determined using a slide IFAT on fixed parasite material.

The mean Ab titres in sera collected during the course of infection (Figure 5.2.1) rose during the first wave of patent parasitaemia in both groups of mice, a low titre of Ab being detected as early as 4 DPI. Following chloroquine treatment (Figure 5.2.1b) the Ab levels in these mice continued to rise with no apparent effect on the pattern of Ab production induced by subcurative doses of chloroquine. Maximal Ab was detected immediately following the recrudescence in both naturally recovering and chloroquine treated mice (Figure 5.2.1a and Figure 5.2.1b) respectively. At subpatency, the mean Ab titres for these groups of mice were 1: 960 and 1: 640, respectively but there was no significant difference ($p>0.05$) between these two values.

5.3 Passive transfer of immunity

Two groups of twenty five mice were infected with 5 x 10^4 *P. c. chabaudi* CB pRBCs and the course of infection followed in ten mice chosen at random. 10 DPI one group of mice was treated with chloroquine. When the patent parasitaemia had declined to 0.01% or less in both groups, 14 DPI for the chloroquine treated mice and

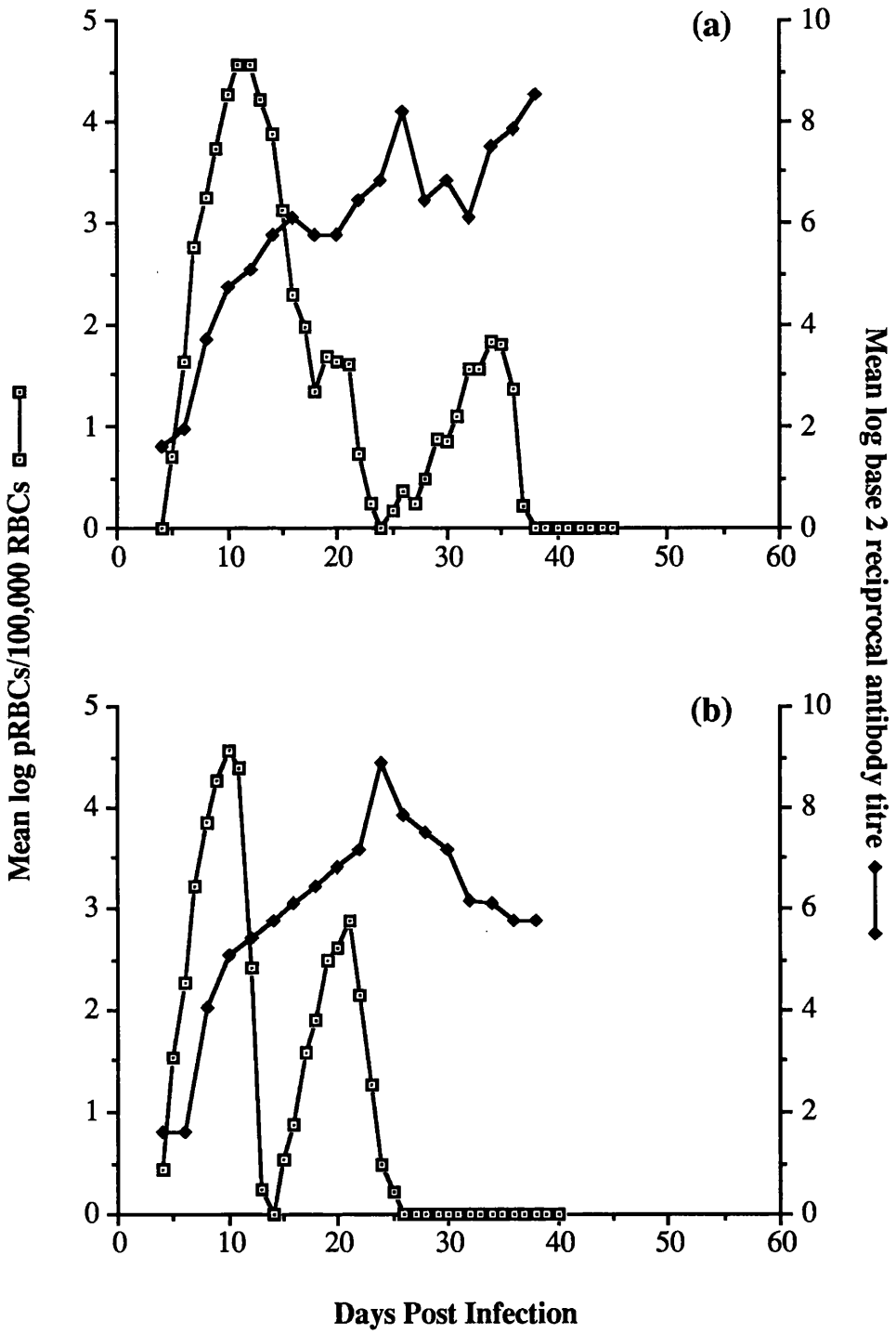


FIGURE 5.2.1 Serum antibody levels during the course of *P. c. chabaudi* CB infection in: (a) naturally recovering mice, and (b) chloroquine treated mice.

21 DPI for the naturally recovering mice, all but five mice in each group were killed and bled by cardiac puncture. The blood was pooled within each group and the serum collected and stored at -20°C. The parasitaemias in the remaining mice were followed until patent recrudescences appeared. For chloroquine treated mice this was 21 DPI and for naturally recovering mice this was between 26 and 32 DPI. Recrudescent parasites from the individual mice were collected and subpassaged, and stabilates prepared when the parasitaemia in individual recipient mice was between 5 and 10%. The stabilates were designated a WEP number and are referred to as such in the text.

Four groups of twelve age-matched female mice were infected with 5×10^4 pRBCs i.v. of either the infecting parasite population (WEP 1224), recrudescent parasites from naturally recovering mice (WEP 1265) or recrudescent parasites from chloroquine treated mice (WEP 1278). Within 1 hr of parasite challenge six of the mice in each group received 0.65 ml NMS i.v. or 0.65 ml of the respective immune subpatent serum. Before passive transfer each serum pool was dialysed overnight against large volumes of PBS to remove any remaining chloroquine. The course of infection was monitored in serum recipients. Protection was measured as a delay in the onset of a patent parasitaemia compared with the controls; this delay was quantified as the increased average time for the mean parasitaemia of the mice receiving subpatent serum to reach a parasitaemia of 2% compared to the recipients of NMS. Two groups of mice provided the NMS, one was chloroquine treated and the other left untreated. In each case, however, the serum pools were dialysed against PBS.

Figures 5.3.1 and 5.3.2 show the results of the passive transfers for naturally recovering and chloroquine treated mice respectively. Mice receiving day 21 subpatent serum and infected with the infecting population (referred to as parent) of *P. c. chabaudi* CB showed a significant ($p<0.01$) delay in reaching 2% parasitaemia (6.7 DPI) compared to mice receiving NMS (5.1 DPI) (Figure 5.3.1a). Similarly, Figure 5.3.2a shows that mice that received subpatent 14 DPI serum from chloroquine treated mice which were infected with the parent parasites showed a significant ($p<0.05$) delay in reaching 2% parasitaemia (5.9 DPI) compared to NMS recipients (5.3 DPI). Figure 5.3.1b shows that 21 DPI serum was also effective at delaying the 2% parasitaemia in mice infected with recrudescent parasites collected from naturally recovering mice compared to mice receiving NMS. 2% parasitaemia was delayed by one day (7.2 DPI compared to 6.2 DPI, $p<0.01$). Conversely, 14 DPI serum from chloroquine treated mice could not protect mice infected with recrudescent parasites compared to mice receiving NMS (Figure 5.3.2b). The mean day of 2% parasitaemia for recipients of 14 DPI serum or NMS was 7.5 or 7.4 DPI, respectively; $p>0.05$.

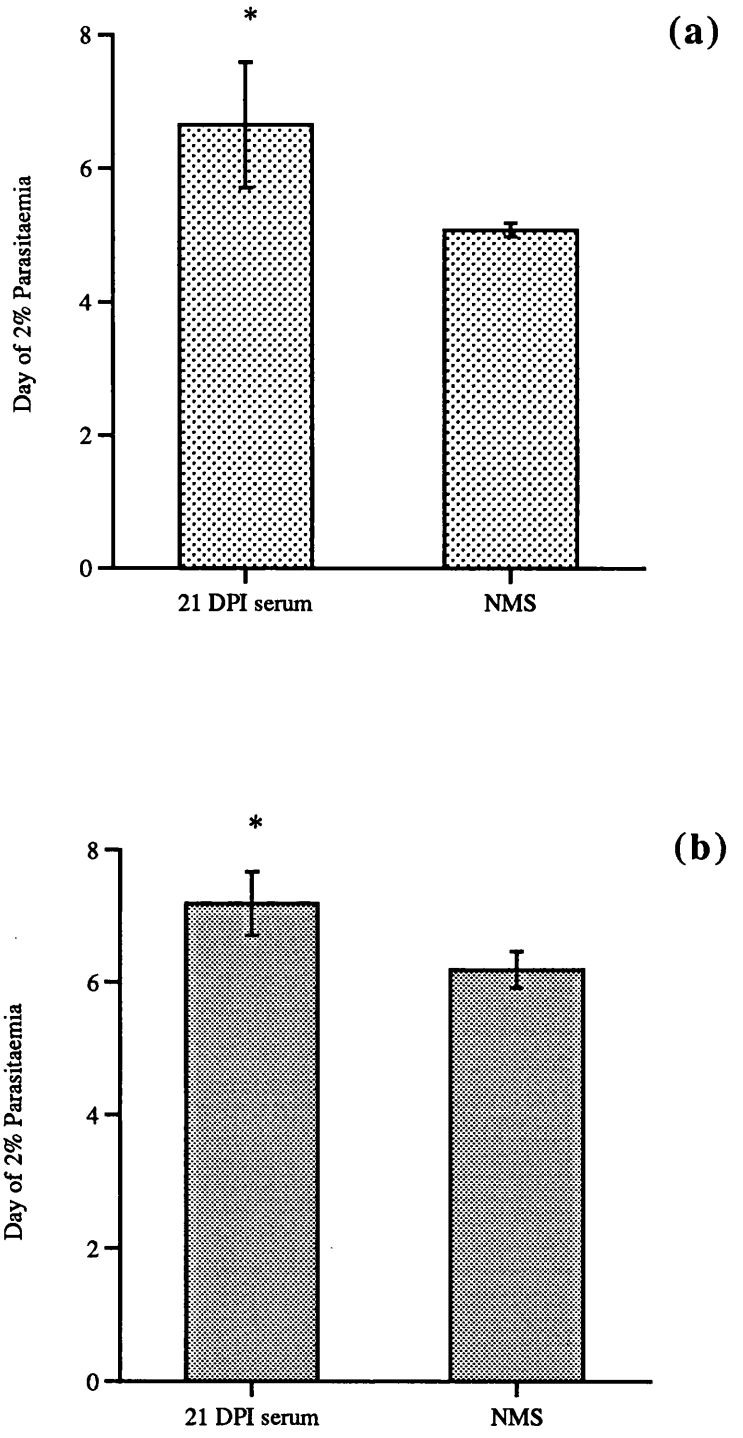


FIGURE 5.3.1
Delay in reaching 2% parasitaemia (days) in mice receiving either 21 DPI serum or NMS (no chloroquine treatment), challenged with (a) 5 x 104 pRBCs of the infecting population or (b) 5 x 104 recrudescent parasites of *P. chabaudi* CB.
* indicates significant difference ($p<0.05$)

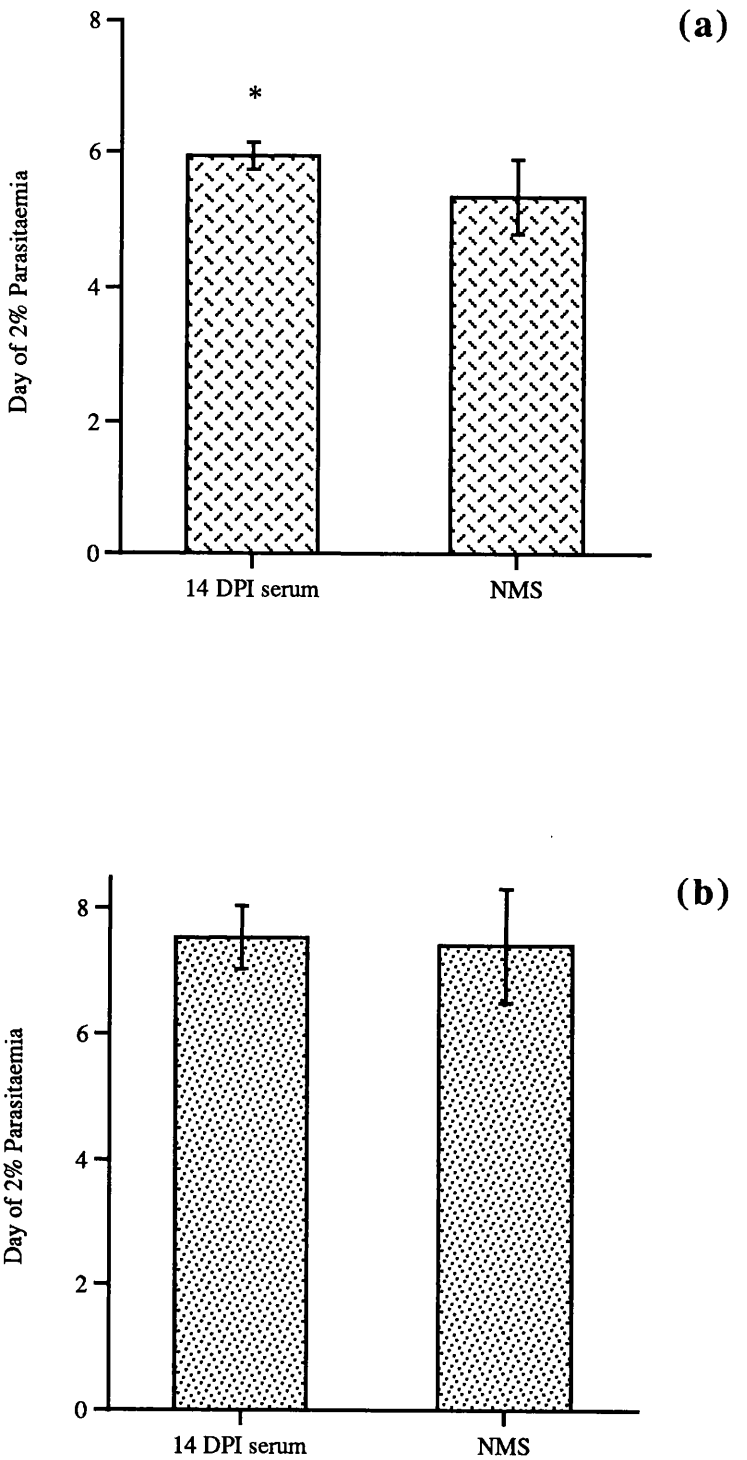


FIGURE 5.3.2
Delay in reaching 2% parasitaemia (days) in mice receiving either 14 DPI serum or NMS, challenged with (a) 5 x 10^4 pRBCs of the infecting population or (b) 5 x 104 recrudescent parasites, from chloroquine treated mice, of *P. chabaudi* CB.
* indicates significant difference ($p<0.05$)

These results show that subpatent sera from both naturally recovering and chloroquine treated mice are protective upon challenge with parent parasites. They also show that subpatent serum from naturally recovering mice can protect against challenge with recrudescent parasites, while subpatent serum is not protective against challenge with recrudescent parasites collected from chloroquine treated mice. This indicates that these recrudescent parasites are insensitive to the antiparasite activity of 14 DPI subpatent serum. This suggests that antigenic variation, as manifest by serum sensitivity, is demonstrable during *P. c. chabaudi* CB infection only following subcurative chloroquine treatment.

5.4 Analysis of recrudescent parasite populations

The recrudescent parasite populations collected from naturally recovered and chloroquine treated mice were analysed further by following their behaviour, with respect to the course of infection, *in vivo*, and by using a live IFAT to analyse the antigenic composition of the uncloned recrudescent parasites.

Three groups of six age-matched female mice were infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB of either the infecting parasite population, recrudescent parasites collected from naturally recovering mice or recrudescent parasites from chloroquine treated mice. The parasites used were the same stabilates as those used in the passive transfer experiment. The course of infection in each of the groups was followed and is illustrated in Figure 5.4.1. Both recrudescent populations behaved very similarly to the infecting parasite population. There was no obvious increase or decrease in virulence, the peak parasitaemia occurring 9 DPI at approximately 40% in each group, and the course of the primary parasitaemia was not significantly different ($p>0.05$) in any of the groups until after 20 DPI. Mice infected with recrudescent parasites from chloroquine treated animals, however, showed a delayed onset in the appearance of a recrudescence. The peak of this recrudescent patency was significantly depressed ($p<0.05$) compared to the other two groups (Figure 5.4.1c). This provides further evidence that subcurative chloroquine treatment of *P. c. chabaudi* CB results in the expression of antigenic variation that cannot be detected during the course of infection in naturally recovering mice.

Table 5.4.1 shows the results of a live IFAT using subpatent sera from naturally recovering mice and chloroquine treated mice against all of the recrudescent parasites collected from these groups of mice. Those parasites used in the passive transfer experiment are highlighted in bold. The reciprocal antibody titres for 21 DPI and 14 DPI subpatent sera against the infecting population were 320 and 160 respectively. The 21 DPI serum reacted with all the recrudescent parasites collected from naturally recovered mice, but the antibody titres varied. The titres for WEP 1267 and WEP 1283 recrudescent parasites (collected 32 DPI and 25 DPI respectively) were low and

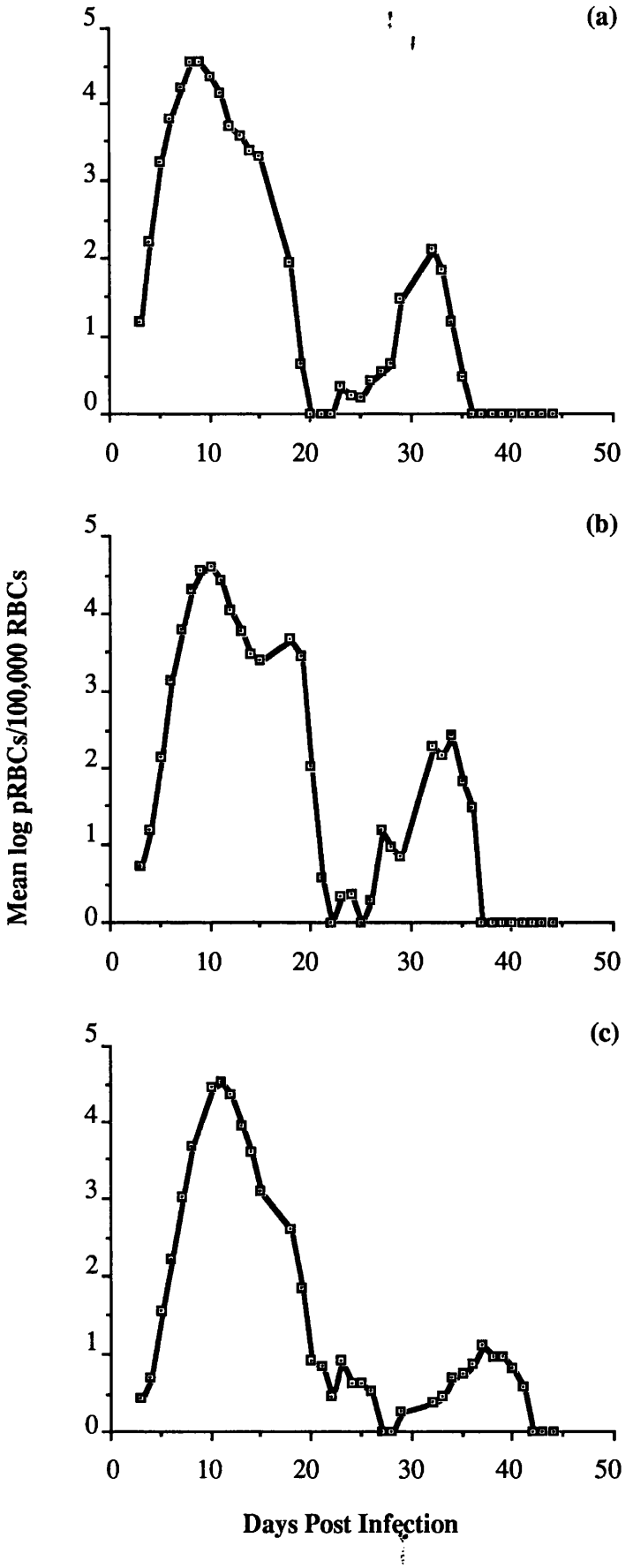


FIGURE 5.4.1 The courses of infection in NIH mice receiving 5 x 10^4 pRBCs of (a) infecting parasite population, (b) recrudescent parasites from naturally recovering mice or (c) recrudescent parasites from chloroquine treated mice.

PARASITE (WEP No.)	RECIPROCAL ANTIBODY TITRE	
	DAY 21 SERUM [a]	DAY 14 SERUM [b]
1224 (Infecting population)	320	160
1		
1265	160	N/D
1266	160	N/D
1267	10*	N/D
1283	80*	N/D
2		
1278	N/D	-
1284	N/D	-
1263 (pooled from 4 mice)	N/D	10*

[a] Subpatent serum collected from naturally recovering mice
[b] Subpatent serum collected from chloroquine treated mice

1 Recrudescent parasites collected from naturally recovering mice
2 Recrudescent parasites collected from chloroquine treated mice

* Indicates very few positive pRBCs (<5% positive pRBCs compared to positive controls (hyperimmune serum))

N/D = Not done

TABLE 5.4.1 - Positive titres in live IFAT of subpatent sera against recrudescent parasite populations of *P. c. chabaudi* CB collected from naturally recovered and chloroquine treated mice.

in each case <5% pRBCs showed positive fluorescence compared to the positive controls (hyperimmune serum - not shown). The level and proportion of reactivity of hyperimmune serum (40-50%) was the same for all populations tested. The lack of reactivity in some recrudescent populations, therefore, was specific for that pool of subpatent serum. WEP 1265 (used in the passive transfer) and WEP 1266, however, collected 24 and 28 DPI respectively, reacted with 21 DPI serum with a titre almost the same as the infecting population. 14 DPI serum from chloroquine treated mice

did not react with either WEP 1278 or WEP 1284, but showed slight positive fluorescence at a titre of 1:10 to WEP 1263.

All the recrudescent parasite populations collected from chloroquine treated mice, therefore, appeared to be antigenic variants of the infecting population. Only, two out of four recrudescences from naturally recovering mice, however, appeared by IFAT to be antigenically different from the infecting parasites. These IFAT results indicate that antigenic variation does occur in mice naturally recovering from *P. c. chabaudi* CB infection but was only demonstrated in two of the collected recrudescent populations.

5.5 Discussion

The results described in this chapter have demonstrated that subpatent serum from both naturally recovering and chloroquine treated mice is successful in transferring immunity to naive, immunocompetent recipients. Protective activity in the passively transferred serum was seen as a delay in the onset of the patent parasitaemia. The pattern of the patent parasitaemias in the mice receiving protective serum was, however, similar to that in recipients of normal serum (data not shown). A previous report by McLean *et al.* (1982a) demonstrated that the passive transfer of immunity to *P. c. chabaudi* AS with sera collected from mice at different times during the course of infection gave a measure of the changing level of protective activity in the serum. Their results demonstrated that the level of serum protective activity against the infecting population at the time that the primary parasitaemia had almost been reduced to subpatent levels was significantly higher than the level recorded at the time that the first recrudescent parasitaemia became patent.

Although the antimalarial Ab levels detected by slide IFAT were very similar in naturally recovering or chloroquine treated mice during the respective subpatent periods, IFAT, however, will measure a large proportion of antibodies that are not protective. No attempt was made to fractionate the serum used in passive serum transfers in this study. The major part of the protective activity of the serum probably lies in the immunoglobulin fraction of the serum. Diggs & Osler (1969) demonstrated that the protective activity of anti-*P. berghei* hyper-immune serum could be removed with anti-rat Ig antiserum. Likewise, Phillips & Jones (1972), Green & Kreier (1978) and Reese & Motyl (1979) showed that the protective activity of malaria immune serum was confined to the immunoglobulin fraction. It is unlikely that the protective activity observed in these passive transfer experiments was due to soluble non antibody factor(s) which may be produced in acutely infected mice and lead to intracellular killing of the parasites (Clark *et al.*, 1976; 1979; Allison & Eugui, 1982). There have been no direct reports that this factor can be transferred in serum, but nitric oxide produced during malaria infection (see chapter 6) can react with the

oxygen anion radical O_2-• to form a peroxynitrite anion which decays rapidly once protonated to form the reactive hydroxyl radical OH• and the stable free radical nitric oxide NO_2• (Liew & Cox, 1991). This may be active upon passive transfer (F.E.G. Cox, personal communication). The protective activity of 14 DPI serum from chloroquine treated mice, however, was only demonstrated against the infecting parasite population while recrudescent parasites were unaffected by this serum. Soluble non-Ab factors would be expected to affect all recrudescences, as well as heterologous parasite challenge. Moreover, dialysis of the serum pools would probably elute non-Ab factors, including the nitric oxide free radical described above.

The difference in serum sensitivity of infecting parasites and recrudescent parasites of 14 DPI or 21 DPI mice could be due to changing *in vivo* antimalarial activity during the course of infection in chloroquine treated or untreated mice. During the subpatent period, before the recrudescence of *P. c. chabaudi* AS emerges, there is a marked decline in the ability of mice to control a large challenge of homologous parasites (R.S. Phillips, personal communication; McLean, 1985). Indeed, a large *P. c. chabaudi* CB parasite challenge on day 20 in chloroquine treated mice or naturally recovering mice resulted in very high parasitaemias in the former, where the recrudescence was ascending, with a more controlled parasitaemia resulting in the latter (prior to subpatency) (R.S. Phillips, personal communication). An investigation of the proportion of Ig secreting spleen cells would be of interest to more easily quantify the levels of Abs present in each serum pool.

In mice receiving 14 DPI serum and infected with recrudescent parasites, no protection was observed. This indicated that antigenic variation was occurring in *P. c. chabaudi* CB infection following subcurative chloroquine treatment. In contrast, 21 DPI serum from naturally recovering mice was equally effective at delaying parasitaemia in mice challenged with both parent parasites and recrudescent parasites. This raises two questions: (i) are there specific Abs in 21 DPI subpatent serum to both infecting and recrudescing parasites? and/or (ii) are recrudescent parasites in naturally recovering mice not undergoing antigenic variation? The former point is more easily reconciled, and due to the unpredictable course of *P. c. chabaudi* CB infection it is likely that the serum collected had experienced recrudescent parasites. This probably accounts for the differences in the passive transfer experiment and the live IFAT. Ideally, to answer these questions more fully it would be necessary to repeat the passive transfers using each of the collected recrudescent parasites and correlate the results of these with the results of the live IFAT. However, Brannan *et al.* (1993) showed that it was difficult to correlate passive transfer experiments with the live IFAT, since the number of antigenic variants determined for recrudescences of *P. c. chabaudi* AS was greater than that determined by passive transfer (McLean *et al.*,

1986a). It must also be stressed that the populations of recrudescent parasites tested were uncloned populations, presumably containing a mix of antigenic types (McLean *et al.*, 1986a).

Antigenic variation, however, was evident in both groups of mice using a live IFAT. Nevertheless, all recrudescent parasites from chloroquine treated mice were of a different antigenic composition than the parent parasites, while it appeared that only under certain circumstances could antigenic variation be detected in the recrudescences from naturally recovering mice.

Antigenic variation has been shown to occur in both the ascending (Brannan *et al.*, 1994) and descending (McLean *et al.*, 1990) phases of the primary parasitaemia of *P. c. chabaudi* AS infection. That antigenic variation was not demonstrated (by serum sensitivity) in the recrudescences of some of the mice naturally recovering from CB strain infection may be due to variant antigenic types present in the ascending and declining primary parasitaemia being the major component of the recrudescent, uncloned, parasite population. It must then be assumed that, if these parasites were present in the recrudescence, the protective immune response was inactive against them, or that the immune response was declining during the onset of recrudescence. Differences in the composition of the recrudescent populations may explain why the uncloned recrudescent parasites varied in their reactivity in the live IFAT. In addition, as mentioned above, 21 DPI serum may have experienced some of the recrudescent parasites present in the collected parasite populations and was, therefore, reactive with some of the populations tested by live IFAT.

Speculatively, chloroquine may induce a more easily observed antigenic variation by inhibiting T_H1 cell responses (see Chapter 6) thereby allowing induction of a T_H2 response. There have been some indications that Ab may induce antigenic variation (Brown, 1973; Brown & Hills, 1974). Since T_H2-derived cytokines regulate B cell differentiation, and therefore, indirectly, antibody secretion, it may be that T_H2 cells are involved in the induction of antigenic variation. The sequential appearance of T_H1 and T_H2 cells observed during *P. c. chabaudi* AS infection may be a parasite immune evasion mechanism, such that a parasite induced T_H2 response is generated to evade T_H1 dependent, cell-mediated parasite destruction. In mice rendered B cell-deficient by anti-μ treatment, however, *P. c. chabaudi* AS infection is controlled but the parasitaemia is not totally eliminated (Von der Weid & Langhorne, 1993b; Taylor-Robinson & Phillips, 1994b). Such anti-μ treated mice maintain a T_H1 cell response, sufficient to control but not eliminate parasitaemia, suggesting that depletion of B cells reduces the generation of the T_H2 cell subset. Whether antigenic variation occurs in these B-cell depleted mice, lacking detectable antimalarial Ab, has yet to be determined. Detailed analyses of Ig isotypes and cytokine production during

the course of infection should provide more information on the dynamics of the immune response developing in naturally recovering or chloroquine treated mice.

CHAPTER SIX

THE ROLE OF NITRIC OXIDE DURING THE COURSE OF INFECTION

6.1 Introduction

Nitric oxide (NO), a simple and unstable free radical, has recently been identified as a potent mediator produced by and acting on many cells of the body. NO is derived from molecular oxygen and the guanidino nitrogen of L-arginine, in a reaction catalysed by NO synthase. Most mammalian cells constitutively produce a low level of NO and a number of cell types, when activated by immunological stimuli, produce large amounts of NO which is an important effector in the destruction of pathogens and tumours and in potentially damaging immune responses (Moncada *et al.*, 1991; Clark *et al.*, 1992).

Pathogens reported to be inhibited by NO and its derivatives include herpes simplex virus (Sethi, 1983), *Cryptococcus neoformans* (Granger *et al.*, 1988; 1990), *Leishmania major* (Green *et al.*, 1990a,b), *Schistosoma mansoni* (James & Glaven, 1989), *Trypanosoma cruzi* (Gazzinelli *et al.*, 1992; Petray *et al.*, 1994), *T. brucei gambiense*, *T. b. brucei* (Vincendeau *et al.*, 1992) and *Plasmodium* liver stages (Green *et al.*, 1990a,b; Mellouk *et al.*, 1991; Nüssler *et al.*, 1991b). A role for reactive nitrogen intermediate (RNI) killing *in vivo* has also been demonstrated for *Leishmania major* (Liew *et al.*, 1990) and *Toxoplasma gondii* (Adams *et al.*, 1990). In addition, killing of *Plasmodium falciparum in vitro* by NO derivatives has been demonstrated (Rockett *et al*, 1991).

A role for NO has been indicated in *P. c. chabaudi* AS infection (Taylor-Robinson *et al.*, 1993). During the course of infection in NIH mice a sharp peak of nitrate is detected in the sera of mice which consistently coincides with the peak parasitaemia. Inhibiting the production of NO by L-NMMA results in mice developing a significantly elevated and extended primary parasitaemia. The results of this indicate that NO is produced and can control a rapidly escalating primary parasitaemia either by direct parasite killing or indirectly by causing blood vessel vasodilation leading to less efficient parasite sequestration (Taylor-Robinson *et al.*, 1993). These workers have also shown that T_H1 malaria-specific cloned T cell lines protect mice upon adoptive transfer by a NO dependent mechanism, activating macrophages to produce NO or producing NO themselves (Taylor-Robinson *et al.*, 1994).

The experiments described in this chapter were designed to evaluate the ability of cells from mice infected with the more virulent strain, *P. c. chabaudi* CB to produce NO, and a detailed analysis of NO (measured as nitrate production) over the peak of the primary parasitaemia is described. In addition, differences in the level of production of NO were measured in mice given a number of subcurative chloroquine treatment regimes. The effect of chloroquine *in vitro* on the production of NO by a murine macrophage cell line is also described and the results discussed with reference to the use of chloroquine as an anti-malarial disease therapy.

6.2 Production of nitric oxide during the course of infection

Two groups of twenty age-matched female NIH mice were infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB and the course of infection monitored by examination of Giemsa's stained thin blood smears. To determine if collection of serum or chloroquine treatment affected the blood picture or level of nitrate a further two groups of three uninfected mice were included: one group was chloroquine treated and the other left untreated. On day 10 post infection (12.45 pm) one group of infected mice was treated with 50 mg/kg chloroquine. Sera were collected by tail bleeding from two mice from each group on days 4, 6 and 8 post infection. During the peak parasitaemia from 4.00 pm 9 DPI to 8.00 am 10 DPI serum was taken at four hourly intervals by exsanguinating two mice on each occasion. From the latter occasion until 2.00 pm 11 DPI serum was collected in the same way every six hours. Thereafter, serum collection continued every two days. Differential parasite counts were carried out on blood smears which were prepared at the same time as the serum collections around peak parasitaemia. All serum samples were analysed for nitrate using the chemiluminescence method.

Figure 6.2.1 shows the mean serum nitrate levels from individual mice during the course of infection. Nitrate levels reflect the NO levels in the serum of infected mice (Moncada *et al.*, 1991) and were compared by the Student's *t* test. The nitrate levels plotted 9 to 11 DPI for each group represent the mean values for all samples collected on that day. In both naturally recovering and mice treated with chloroquine 10 DPI a peak of nitrate was detected in the sera of infected mice (620 ± 155 and 541 ± 179 nmol/ml, respectively) which coincided with the peak of the primary parasitaemia. There was no significant difference between these two values ($p>0.05$) but both were significantly higher than those on any other day of infection ($p<0.01$). In both groups of mice the peak of nitrate declined rapidly and had returned to background levels by 8.00 am 11 DPI. The peak parasitaemias of naturally recovering and chloroquine treated mice were 32.0% and 31.8% respectively and there was no significant difference between these values ($p>0.05$). Chloroquine treatment at the peak of infection, however, resulted in rapid parasite remission (Figure 6.2.1b) with all mice subpatent by 14 DPI. A recrudescence occurred immediately after, and peaked at a parasitaemia of 3.0% 21 DPI. In naturally recovering mice (Figure 6.2.1a) the primary parasitaemia was not cleared to subpatent levels until 21 DPI and the recrudescence that followed peaked at 0.9% 31 DPI. In both cases, however, there was no indication of a further NO increase associated with the recrudescences.

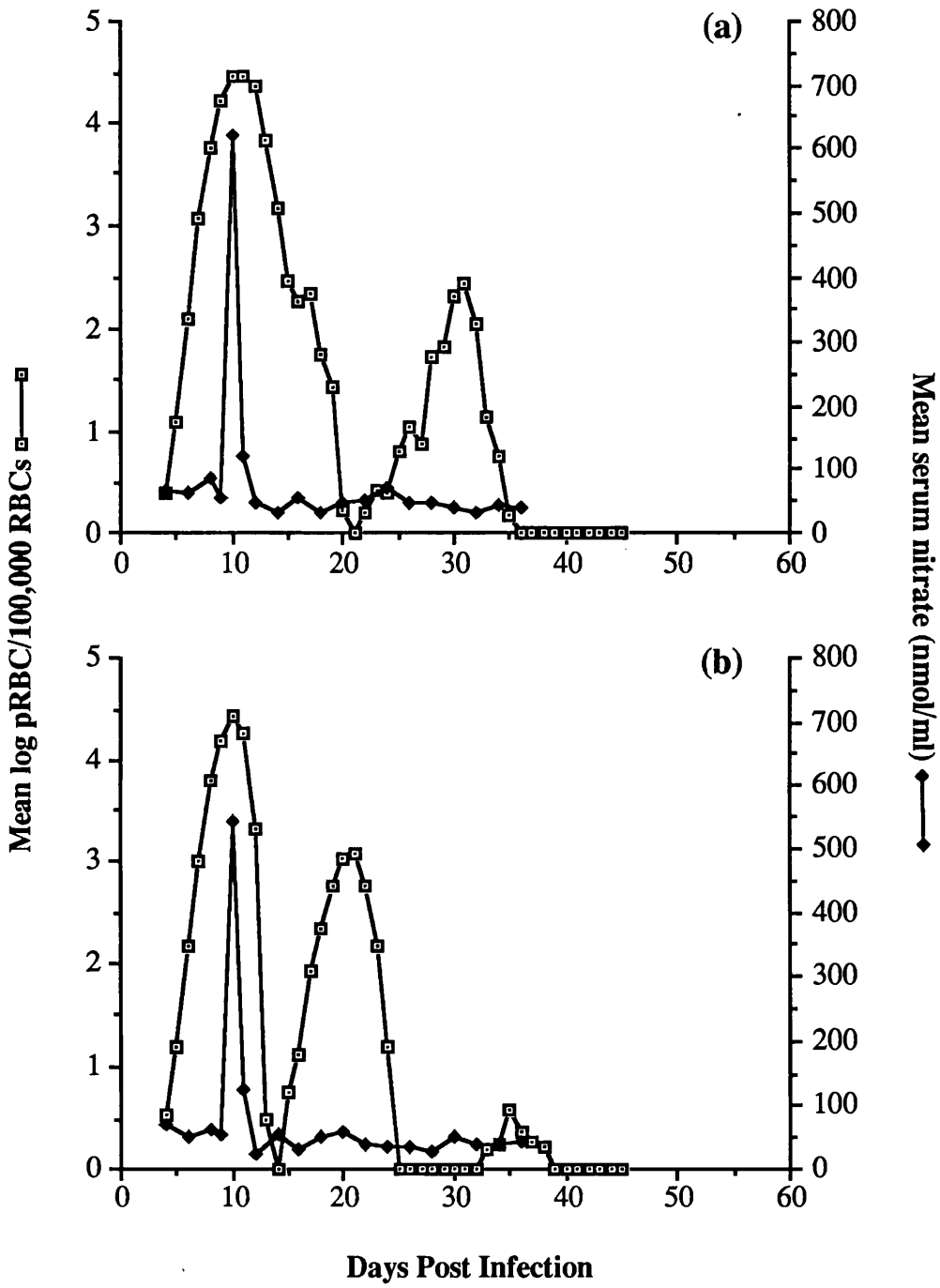


FIGURE 6.2.1 Serum nitrate levels during the course of infection in (a) naturally recovering mice and (b) chloroquine treated mice. Mice were infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB.

Figure 6.2.2 shows the nitrate levels of individual mice on day 10 post infection correlated with the percentage of ring stage parasites calculated from each time point. Although the data are presented as nitrate levels in naturally recovering (Figure 6.2.2a) or chloroquine treated (Figure 6.2.2b) mice the latter did not receive chloroquine treatment until three out of the five measurements were complete. Both graphs show that higher nitrate levels were detected when a large proportion of the parasites were at the ring-stage. The largest proportion of ring stage parasites were detected after midnight, the time at which schizogony occurred; as the parasites completed their 24 hour growth cycle and the % of rings decreased so too did the nitrate level in the sera of infected mice. Spearmann-Rank correlations were performed for each of the data. The correlation for the data presented in Figure 6.2.2a was 0.705, $p<0.02$; however, the correlation for chloroquine treated mice (Figure 6.2.2b) was somewhat less (0.462, $p>0.05$) and there was no significant difference detected for these values. Combining both sets of data, however, resulted in a correlation value of 0.643, $p<0.05$ but >0.02. A significant correlation, therefore, indicated that the highest nitrate levels at the peak parasitaemia were obtained at the earliest stage of parasite development, i.e., immediately after schizogony.

The data described in this section have shown that NO is produced in response to infection with *P. c. chabaudi* CB. Mice naturally recovering from infection or those treated with chloroquine on day 10 post infection produced significant levels of nitrate in their sera, the peak of which coincided with the peak of primary parasitaemia. Levels declined rapidly thereafter and remained at background levels throughout the course of infection. There were no differences in the levels of nitrate produced in naturally recovering mice or in mice following chloroquine treatment on day 10 post infection, despite differences in the course of infection. Chloroquine treated mice rapidly cleared the primary parasitaemia before a recrudescence of 3% occurred, while naturally recovering mice showed an extended primary parasitaemia and a recrudescence of less than 1%. No increase in nitrate levels was demonstrated during either recrudescence, indicating that NO is only involved in controlling the primary parasitaemia.

The measurement of nitrate levels in a narrow time range through the peak of infection showed that the initial rise in nitrate occurred just after midnight 9/10 DPI with levels significantly correlated with ring-stage parasites over the next 24 hour period. It appears, therefore, that preceding the peak of infection, nitrate is released during schizogony, which occurs around midnight in a normal cycle of infection, when merozoites are released into the blood stream and declines during the following period of parasite development.

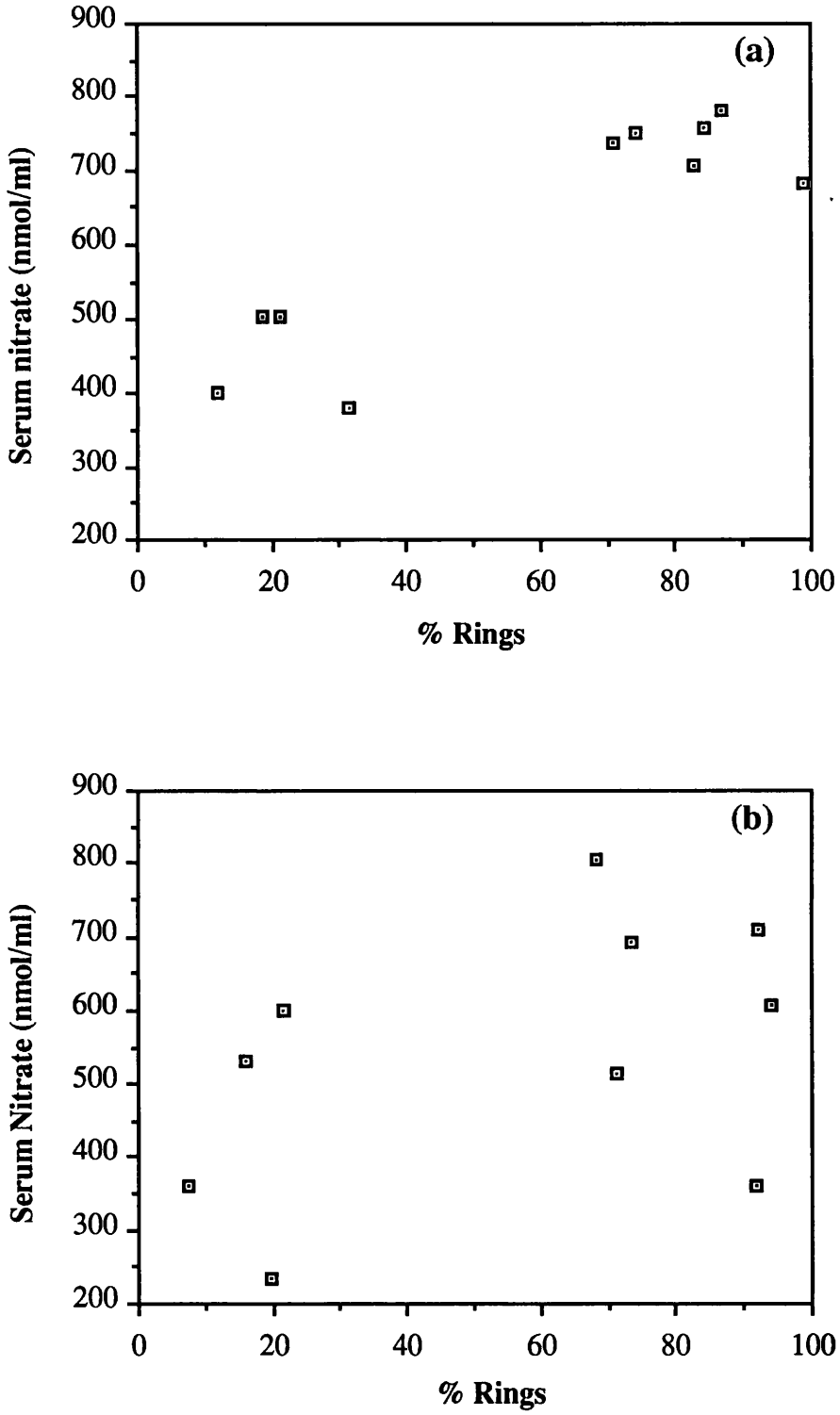


FIGURE 6.2.2
Correlation of individual serum nitrate levels from timed intervals 10 DPI (peak parasitaemia) with the percentage of ring-stage parasites for (a) naturally recovering mice and (b) chloroquine treated mice. Chloroquine treated (10 DPI, 12.45 pm) mice did not differ from naturally recovering mice for three out of the five measurements.

6.3 The effect of chloroquine treatment preceding peak parasitaemia on the production of nitric oxide.

Five groups of six age-matched female NIH mice were infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB and the course of infection followed. One group of mice was treated with chloroquine two days prior to the peak parasitaemia, another group was treated one day prior to the peak and a third group was treated on the day of the peak parasitaemia. The other two groups consisted of untreated mice or uninfected controls. Sera were collected by tail bleeding from two mice in each group every two days during the course of infection and every day around the peak parasitaemia and during the recrudescences. The sera were analysed for nitrate by chemiluminescence.

Figure 6.3.1 shows the levels of serum nitrate during the course of infection in the groups of mice. The peak parasitaemia occurred 8 DPI in untreated mice. Chloroquine, therefore, was administered 6, 7 or 8 DPI. In mice treated with chloroquine before the peak of parasitaemia the levels of nitrate in the sera remained at basal levels throughout the course of infection. In mice that were treated on the day of peak parasitaemia, or those that were untreated, a sharp peak of nitrate occurred on the same day as peak parasitaemia (688 ± 11 or 690 ± 21 nmol/ml nitrate, 8 DPI, respectively). No further elevation of nitrate levels was detected in any mice during the rest of the infection. Mice treated with chloroquine 6 DPI (parasitaemia=13.2%) or 7 DPI (parasitaemia=30.3%) quickly showed a reduction in parasitaemia but subpatency was not reached before a recrudescence occurred. In each case the parasitaemia of the recrudescence peaked at 3.4% or 3.1% respectively but with no associated rise in nitrate. It appears, therefore, that early chloroquine treatment resulting in a rapid decline in parasitaemia prevents any rise in serum nitrate levels during the course of infection, even when an appreciable recrudescence occurs.

To investigate this phenomenon further, the experiment was repeated and the levels of IFN-γ, a cytokine known to induce macrophages to produce NO, in Con A stimulated spleen cell supernatants were assessed. Mice were infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB as before, and the course of infection monitored. The peak parasitaemia occurred 10 DPI, so chloroquine treatment was administered 8 or 9 DPI. 7, 8, 9, 10 and 11 DPI spleen cells were prepared from individual infected mice or from naive controls and stimulated with Con A. 48 hours later, supernatants were collected and assayed for IFN-γ by ELISA. Spleen cells from chloroquine treated mice were prepared two hours following treatment.

Figure 6.3.2 shows the results. Throughout the measurements naive spleen cells consistently produced between 20 and 60 U/ml of IFN-γ when stimulated with Con A. In contrast, 7 DPI infected, untreated mice produced 147.3 ± 35.8 U/ml,

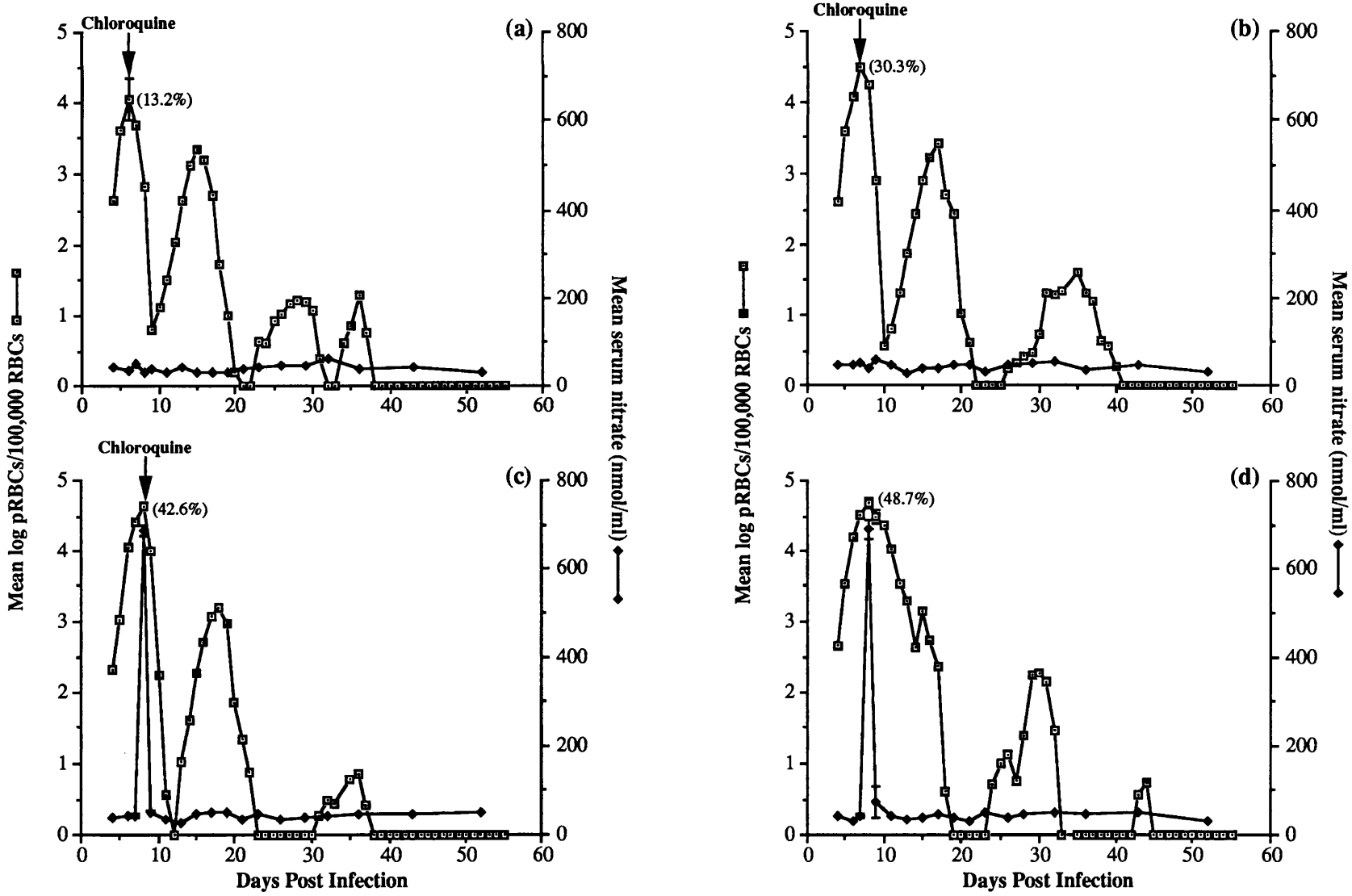


FIGURE 6.3.1 Serum nitrate levels during the course of *P. c. chabaudi* CB infection in mice treated with 50mg/kg chloroquine (a) 6 DPI, (b) 7 DPI, (c) 8 DPI or (d) untreated. The parasitaemia of each group on the day of treatment is shown in parenthesis.

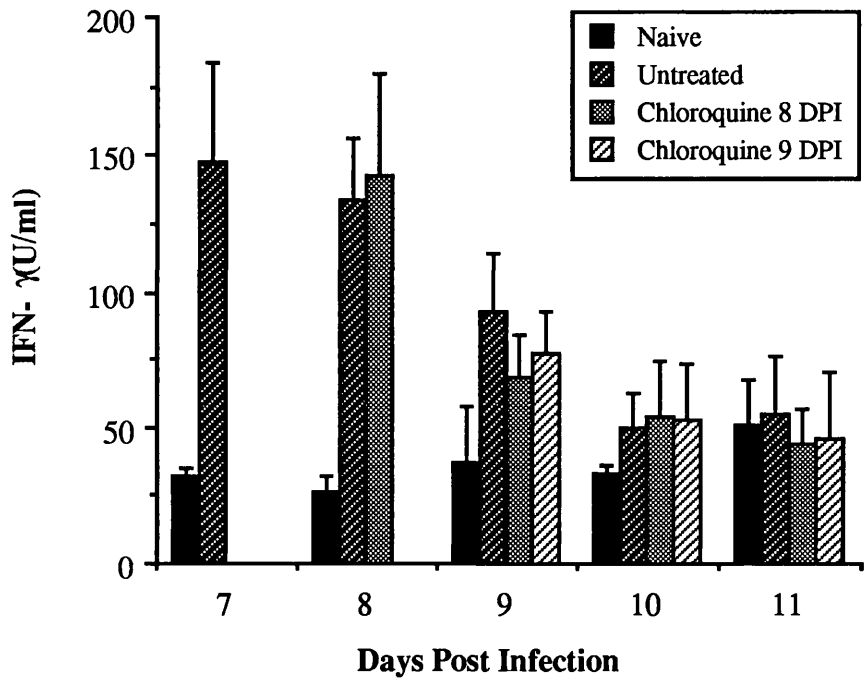


FIGURE 6.3.2 Interferon-gamma production by Con A stimulated spleen cells from chloroquine treated (8 or 9 DPI) or untreated mice infected with 5 x 10^4 pRBCs *P. c. chabaudi* CB. The peak parasitaemia occurred 10 DPI at 38.5% in untreated animals.

significantly higher ($p<0.01$) than naive controls. 8 DPI mice that were treated with chloroquine on that day showed no difference in the production of IFN-γ compared to untreated mice (142.3 ± 37.7 and 133.2 ± 23.3 U/ml, respectively). Both values were significantly higher than for naive controls ($p<0.01$). By 9 DPI levels of IFN-γ had dropped off in untreated mice and again there were no differences in mice treated with chloroquine 8 DPI or 9 DPI compared to untreated mice. This pattern remained through to 11 DPI with no significant differences detected between any groups, including naive mice.

These data show that although chloroquine treatment before the peak parasitaemia results in reduced NO production, no effect is observed on the production of IFN-γ. IFN-γ levels peaked over days 7 and 8 post infection, before chloroquine was administered, consistent with previous findings of Slade & Langhorne (1989) and Stevenson *et al.* (1990c). These investigators demonstrated that IFN-γ in serum and spleen cell supernatants respectively peaked approximately two days before the peak parasitaemia.

6.4 Chloroquine induced inhibition of nitric oxide *in vitro*

Following the results of early chloroquine treatment *in vivo* on the production of NO, the ability of chloroquine to inhibit NO production *in vitro* was investigated. J774 macrophage-like cells can be induced to produce NO upon stimulation with LPS and IFN-γ (Stuehr & Marletta, 1987). Macrophages were treated with various amounts of chloroquine either 24 hrs or 2 hrs before, or at the same time as stimulation with LPS and IFN-γ. The results are illustrated in Figure 6.4.1. These show that chloroquine, at doses above 1 μM, inhibits NO production by macrophages in a dose-dependent manner, and this was observed at all times when chloroquine was added to the cultures. The viability of the J774 cells remained at >90% during chloroquine treatment, so NO inhibition was not due to a cytotoxic effect on the cells, although following 48 hrs of stimulation some culture wells (those producing highest NO levels) looked grainy but maintained >65% viability. This is a usual consequence of NO production from these cells (C.A. O'Donnell, personal communication). Inhibition of NO synthesis was evident when chloroquine was added to macrophages at the same time as stimulation but was most effective when chloroquine was added to macrophages prior to stimulation. At each of the chloroquine pretreatment times, 1 μM chloroquine did not significantly inhibit NO production ($p>0.05$). Two hours preincubation with chloroquine (≥10 μM) before stimulation was enough to obtain a significant decrease in NO production. Preincubation of macrophages 24 hrs prior to stimulation caused a reduction of 25% in NO production with a dose of 10 μM chloroquine ($p<0.003$), 36% with 100 μM chloroquine ($p<0.0001$) and 58% with 1000 μM chloroquine ($p<0.0001$).

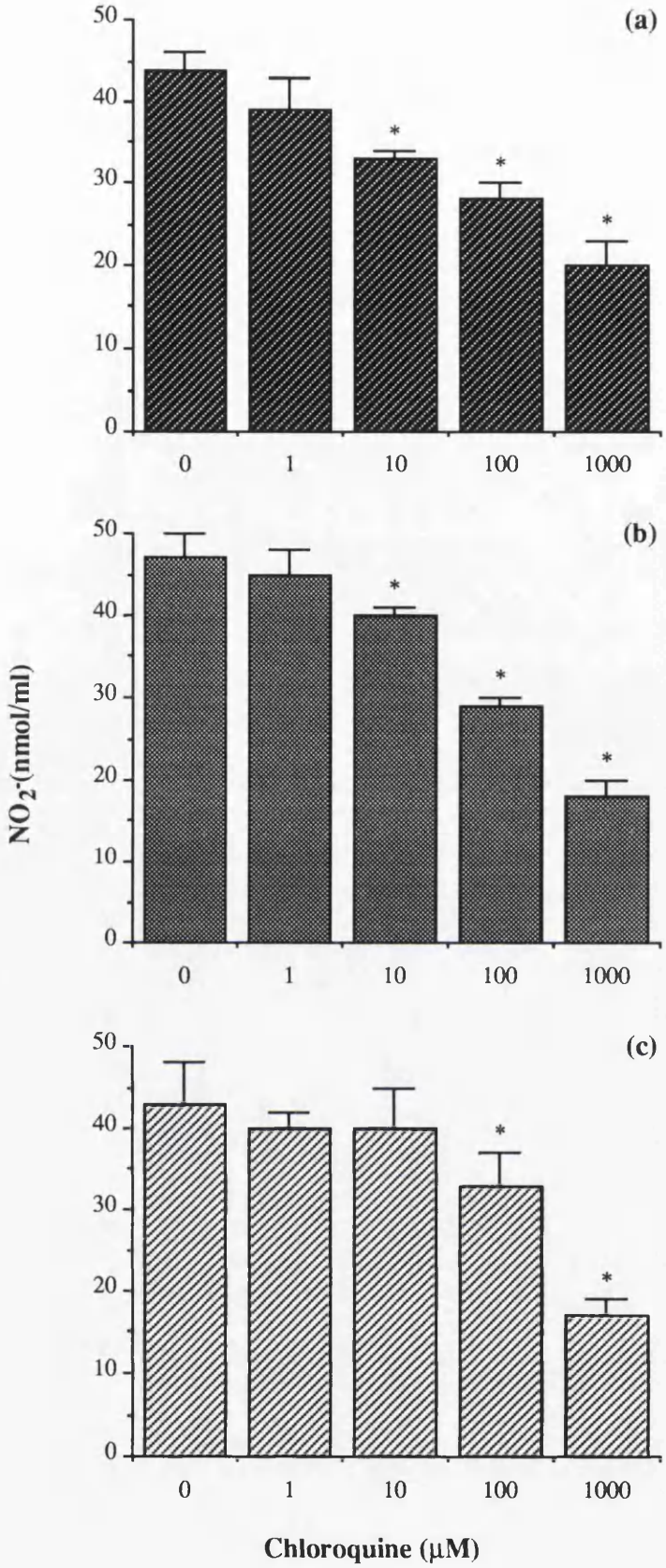


FIGURE 6.4.1 Chloroquine-induced inhibition of NO production from J774 macrophages stimulated with LPS and rIFN-γ: macrophages were pretreated with chloroquine for (a) 24 hours, (b) 2 hours before stimulation, or (c) treated at the same time as stimulation.
* indicates a significant difference compared to control well (0 μM chloroquine)

6.5 Discussion

The results reported in this chapter have demonstrated that NO is produced during infection with *P. c. chabaudi* CB with the same pattern as that observed during infection with the AS strain (Taylor-Robinson *et al.*, 1993). A significant peak of nitrate occurs in the serum of infected mice that coincides with the peak of parasitaemia. No further increase of nitrate is demonstrated throughout the rest of infection. This indicates that NO is produced to control the ascending primary peak parasitaemia but has no effect later in the course of infection (Taylor-Robinson *et al.*, 1993). This also supports the view of a sequential activation of T_H1 cells early in infection followed by T_H2 cells later in infection (Langhorne *et al.*, 1989; Taylor-Robinson & Phillips, 1992; 1993; 1994a). NO involvement may be mediated by T_H1 secretion of IFN-γ which may activate macrophages to produce large amounts of NO (Marletta *et al.*, 1988; Stuehr & Nathan, 1989) to kill parasites directly. In addition, malaria-specific cloned T_H1 cell lines have recently been shown to synthesise NO themselves (Taylor-Robinson *et al.*, 1994) and may add to direct parasite killing. Indirectly, NO may have an effect by causing blood vessel vasodilation (Knowles & Moncada, 1992) leading to less efficient parasite sequestration in deep tissue capillaries, allowing removal of the parasites by macrophages (Taylor-Robinson *et al.*, 1993).

NO and RNI may react in several ways to cause cell death. Once the oxides of nitrogen have diffused into erythrocytes, the formation of nitrosothiol groups on proteins could lead to the inactivation of enzymes or changes in protein function. These groups can react further to cross-link sulphydryl groups (Rockett *et al.*, 1991), one effect of which would be to oxidise glutathione and so reduce the parasite oxidant defense capacity. This process may create more nitrosothiol groups rather than form nitrite and nitrate (Mirna & Hoffmann, 1969) and thus initiate a chain reaction. *S*-nitrosothiol derivatives of cysteine and glutathione have been shown to be potent compounds in the *in vitro* killing of *P. falciparum* (Rockett *et al.*, 1991). In an erythrocyte, one target for RNI attack is oxyhaemoglobin. Nitrite reacts with this molecule to form either nitrosothiolhaemoglobin (Westenberger *et al.*, 1990) or methaemoglobin (Wallace & Caughey, 1975), and one product of the latter reaction is hydrogen peroxide, itself toxic to malaria parasites (Clark & Hunt, 1983; Dockrell & Playfair, 1983). This property of RNI to reduce metal ions is consistent with the concept that exposure of Fe-S groups to NO results in iron-nitrosyl complex formation (Hibbs *et al.*, 1988) causing the inactivation and degradation of Fe-S prosthetic groups of aconitase and complex I and II of the mitochondrial electron transport chain. In addition, NO and RNI can also form toxic alkylating agents by reacting with secondary amines (Iyengar *et al.*, 1987).

The results described herein, demonstrated that NO was released/detected in the

serum of infected mice during schizogony on the night preceding peak parasitaemia. Differential counts of blood smears showed that the highest nitrate levels occurred when parasites were at the early ring-stage. This probably indicates that NO is most active when merozoites are released from schizonts, free in the blood stream. The selectivity of target destruction may be explained by the fact that the NO molecule is very short-lived, and therefore its reactivity would necessarily be directed towards targets with which the activated macrophage is in close contact. Sequestration of the malaria parasite at sites such as the liver, would provide a microenvironment where endothelial cells, hepatocytes and kupffer cells are all in close proximity to the parasite; NO produced by these cells could destroy merozoites as they are released from schizonts (R.S. Phillips, personal communication).

Cytokines are involved in the induction of NO synthesis (Ding *et al*, 1988; Steuhr & Marletta, 1987). There is now general agreement that levels of TNF are increased in the sera of human malaria patients (Grau *et al.*, 1989c; Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990) and mouse models of malaria (Curfs *et al.*, 1989; Grau *et al.*, 1989b). Increase in RNI caused by TNF could contribute to the antimalarial effects of TNF *in vivo* (Clark *et al.*, 1987b) and the antiparasite effect may be a direct result of NO alone or RNI in synergy with oxygen radicals known to be toxic to malaria parasites (Clark & Hunt, 1983; Dockrell & Playfair, 1983). Alternatively, nitric oxide may go on to form chemical species that are even more toxic, such as peroxynitrite (Blough & Zafiriou, 1985; Gryglewski *et al.*, 1986) or hydroxyl radicals (Beckman *et al.*, 1990). Since a number of cell types produce NO, particularly endothelial cells, this mechanism could explain how P/J mice, whose macrophages have poor oxidative capacity, can kill *P. chabaudi* as effectively as can normal mice (Cavacini *et al.*, 1989).

T_H1 cell secretion of IFN-γ activates macrophages to produce large amounts of NO (Marletta *et al.*, 1988; Stuehr & Nathan, 1989). Taylor-Robinson *et al.* (1994) proposed that the protection upon adoptive transfer of a T_H1 cloned cell line was mediated by IFN-γ induced NO production. Inhibition of NO by L-NMMA elevated IFN-γ production, but increased parasitaemia. This is consistent with the idea that the effect of IFN-γ is mediated predominantly through NO production. Several earlier reports, however, have shown that IFN-γ production directly contributes to resistance to asexual blood stage malaria (Clark *et al.*, 1987a; Bienzle *et al.*, 1988; Shear *et al.*, 1989; Meding *et al.*, 1990; Curfs *et al.*, 1993). Furthermore, IFN-γ enhances the effect of antimalarial chemotherapy (Kremsner *et al.*, 1991) in *P. vinckei* infection. In a recent report by Curfs *et al.* (1993) treatment with IFN-γ of *P. berghei* K173-infected mice temporarily reduced body temperature, delayed patency, inhibited proliferation of the parasites, and also prevented development of cerebral lesions, depending on the regimen used. Anti-IFN-γ antibody caused increased proliferation

of the parasite. The mechanism of this protection may be due to the activation of ROI (Clark *et al.*, 1987a; Shear *et al.*, 1989) or NO (Taylor-Robinson *et al.*, 1993; 1994).

Clark *et al.* (1991; 1992) hypothesised a role for NO in the development of cerebral malaria. Overproduction of NO would cause brain vessel dilatation, responsible in turn for the increased intracranial pressure seen in cerebral malaria patients (Newton *et al.*, 1991). In addition, NO might be involved in another process expressed at the neuron level. NO could diffuse through the brain parenchyma and could activate the guanylate cyclase in neurons causing a disruption of the regulation of glutamate-induced neural NO. Senaldi *et al.* (1992), Asensio *et al.* (1993) and Kremsner *et al.* (1993), however, have reported recently that there is no influence of NO inhibitors on the development of cerebral malaria in the mouse model, *P. berghei* ANKA, even upon intracranial administration. Conversely, treatment with anti-IFN-γ antibody has been reported to prevent the development of the cerebral complication in *P. berghei* ANKA-infected CBA/Ca mice (Grau *et al.*, 1989a) when given before and during infection. A continuous delivery of IFN-γ, however, prevented the development of cerebral malaria in a recent study by Curfs *et al.* (1993), possibly downregulating its own production or that of TNF release. Distinct mechanisms of IFN-γ and NO protection may, therefore, exist.

Mice treated with chloroquine at the peak of infection did not show any differences in the production of nitrate following the resolution of the primary parasitaemia, despite the increase in parasitaemia of the recrudescence. Treatment of mice, however, before the peak parasitaemia resulted in a rapid parasite remission and although a high recrudescence followed quickly, no NO was detected in the serum of infected mice throughout the whole course of infection. Peak IFN-γ levels after Con A stimulation were detected in spleen cell supernatants of mice 7 and 8 DPI. In untreated mice levels had fallen off by day 9 post infection and chloroquine treatment 8 or 9 DPI did not affect the IFN-γ levels in any way. Slade & Langhorne (1989) and Stevenson *et al.* (1990c) have previously demonstrated similar kinetics in IFN-γ production in AS infected mice, that is peak levels occurring one or two days prior to the peak parasitaemia. In activated T_H1 cells the peak NO synthase activity occurs 12 hrs after activation (Taylor-Robinson *et al.*, 1994) and in infected mice peak NO occurs at the peak parasitaemia. This indicates that perhaps some factor other than IFN-γ is involved in activating macrophages and T_H1 cells to produce NO. This may be TNF, as mentioned above, which is detectable during a primary infection with *P. c. chabaudi* AS (Stevenson *et al.*, 1990b). Resolution of primary parasitaemia and recrudescence following subcurative chloroquine treatment occurred in all groups of mice regardless of the level of NO produced. NO may only be produced above a certain threshold of parasitaemia. Preventing the parasitaemia from reaching a critical

value by chloroquine treatment may be the reason for blocking the elevation of NO normally observed at the peak of infection.

Despite the occurrence of a peak of NO, in some cases the primary parasitaemia still took some time to be resolved. This indicates that other immune mechanisms that are NO (and IFN-γ) independent are operative to control the primary parasitaemia. A more detailed analysis of the kinetics of both T_H1 and T_H2 cytokine production would be of interest to evaluate further the role of both Ab-independent and Ab-dependent immune mechanisms during resolution of infection. Ab titres as measured by IFAT show that significant specific anti-malaria Ab are detected by day 7 post infection. By measuring the Ab isotypes that occur during infection and the effect of exogenous cytokines on these titres would provide useful evidence as to the mechanisms eliciting protection to *P. c. chabaudi* CB. Chloroquine treatment of mice at the peak parasitaemia (10 DPI) does not significantly alter the IFAT Ab levels in the serum of infected mice until after the recrudescence when the larger Ag stimulation in these mice causes an increase in the Ab titre.

In vitro, chloroquine was shown to inhibit the synthesis of LPS and IFN-γ induced NO from a murine macrophage cell line in a dose-dependent manner. Chloroquine easily penetrates into macrophages (Picot *et al.*, 1993) and has previously been shown to inhibit Il-1 secretion, presentation of antigens by accessory cells, to depress mitogen responsiveness and natural killer cell cytotoxicity and to display anti-inflammatory properties (Krogstad & Schlessinger, 1987). In addition, the action of prophylactic doses of chloroquine has been shown to inhibit the phagocytosis of IgG-coated sheep red blood cells (Osorio *et al.*, 1992). Recent observations by Picot *et al.* (1991; 1993) have shown that chloroquine also inhibits TNF and Il-6 production. This observation, combined with the inhibition of NO described herein, may be clinically relevant and chloroquine used at pharmacological concentrations could inhibit cytokine over-production *in vivo*. Although the pH was strictly controlled in all cultures containing chloroquine, elevated pH is a consequence of chloroquine accumulation in acidic intracellular compartments including lysosomes and endosomes. This in turn can cause alterations in intracellular trafficking, affecting receptor-mediated endocytosis, intralysosomal digestion, exocytosis and the biosynthesis of secretory proteins (Maxfield, 1982). However, reacidification of lysosomes occurs only three hours after chloroquine treatment (Tietz *et al.*, 1990), providing evidence that chloroquine-dependent TNF inhibition (Picot *et al.*, 1991; 1993) and NO inhibition is not due to the weak base properties of chloroquine. Picot *et al.* (1993) have hypothesised that chloroquine inhibits TNF secretion by disrupting iron haemostasis outside lysosomal compartments.

NO and TNF have both been postulated as mediators responsible for morbidity

and mortality associated with cerebral malaria (Clark *et al.*, 1991; 1992; Grau *et al.*, 1989b, c; Kwiatkowski *et al.*, 1990). Regardless of the effect on parasite killing by chloroquine, maintaining levels of these mediators below certain threshold levels at which cerebral manifestations occur, provides an approach for the continued use of chloroquine despite the rapid emergence of drug-resistance.

CHAPTER SEVEN

IN VITRO PROPAGATION OF MALARIA SPECIFIC T CELL LINES

7.1 Introduction

The previous chapters have reported, through depletion of T cells *in vivo* and to an extent by adoptive transfer experiments, that the immune response to the erythrocytic stages of the CB strain of *P. c. chabaudi* is absolutely dependent on the presence of CD4-bearing T cells. This confirms the work of Süss *et al.* (1988), Podoba & Stevenson (1991) and Taylor-Robinson (1991) working with *P. c. chabaudi* AS, and Vinetz *et al.* (1990) working with *P. yoelii*. These reports supported the pioneering work of Jayawardena *et al.* (1982), who implicated a role for $CD4^+$ but not $CD8^+$ T cells in acquired resistance to blood stage malaria parasites, a view which was later contradicted by Mogil *et al.* (1987). Working with the same strain of *P. yoelii* (17X) the discrepancy between the results of these investigators was thought to be due to attempts to dissect the contribution 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 advances in the methodology for propagating and maintaining Ag-specific T cell lines and clones *in vitro* (Gillis & Smith, 1977; Fathman & Fitch, 1982). 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). Thus, it has become possible to establish enriched populations of antigen-specific $CD4^+$ or $CD8^+$ T cells by repeated cycles of Ag stimulation followed by expansion either with Il-2 or with mAb against the CD3 T cell receptor (Van Wauve *et al.*, 1980). These 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 by a variety of techniques including cytokine release (Kappler *et al.*, 1980) and T cell proliferation (Seeger & Oppenheim, 1970). Using this technology, malaria specific T cell lines have been generated by a number of workers. Gross *et al.* (1984) have demonstrated that the transfer of a T cell line raised to *P. berghei* provides protection in rats to parasite challenge. Similarly, lines produced to *P. c. adami* infections in BALB/c mice (Brake *et al.*, 1986) also transfer protection. In this latter study, one of the $CD4^+$ cell lines was cloned with no loss of adoptive protection (Brake *et al.*, 1988). More recently, Taylor-Robinson (1991) and Taylor-Robinson & Phillips (1992, 1993, 1994a) have described the generation of T_H1 and T_H2 cell lines and clones specific for *P. c. chabaudi* AS which are protective upon adoptive transfer and have been characterised in much detail with respect to their function.

The adoptive transfer experiments described in Chapter 4 demonstrated the effectiveness with which T cells could transfer protection to mice challenged with *P. c. chabaudi* CB. It was therefore decided to attempt to raise T cell lines with spleen cells isolated from donor animals at time points similar to those used for the adoptive

transfers. Table 7.1.1 shows the time points chosen. It was hoped that these lines, if generated successfully, would have a similar immunological status as the enriched T cell populations used in the transfers, but would have the advantage of being cultivated *in vitro* as relatively homogeneous populations suitable for further investigation such as cytokine 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. Thus, the generation of these lines in mice primed to *P. c. chabaudi* CB to different degrees, and therefore, being of varying immunological competence, would allow the study of specific cellular responses during the course of infection.

The work described in this chapter outlines the preparation of T_H cell lines from different time points during a *P. c. chabaudi* CB infection in NIH mice and following subcurative chloroquine treatment. Although the generation of these cell lines proved problematic, the initiation and maintenance of one cell line is detailed. The ability of the cultured T_H cells to respond to specific antigenic stimulation is described as is the adoptive transfer of this line into immunocompromised syngeneic mice.

NATURALLY RECOVERING MICE	CHLOROQUINE TREATED MICE
16 DPI	14 DPI (subpatency)
21 DPI (subpatency)	30 DPI
31 DPI	60 DPI
60 DPI (WEP 1153)	After 1 reinfection
After 1 reinfection	After 2 reinfections
After 2 reinfections	

TABLE 7.1.1 Selected time points for generation of T cell lines from naturally recovered and chloroquine treated mice.

7.2 Optimisation of T cell line culture conditions

The preparation of responder T cells, with APCs, for long-term culture requires rigorous attention to conditions necessary for optimal lymphocyte proliferation. Therefore, the number of responder cells, the concentration of APCs as well as inhibition of APC proliferation, and the concentration of antigen for cell stimulation must be assessed before initiation of specific T cell lines. Within this study, optimisation of these conditions was carried out in consultation with Dr A.W. Taylor-Robinson. Features such as the number of responder cells and APCs remained as predetermined by Taylor-Robinson & Phillips (1992). The dose of gamma irradiation for inhibition of those cells in the APC population capable of proliferation was maintained at 30 Gy (3000 rads) - a dose which has no adverse effects on the capacity

of the APCs to present Ag in a suitable manner for recognition and activation of responder cells, but does completely abrogate cellular proliferation.

At the outset of this investigation two sources of antigen were used for stimulation of T cells *in vitro*. These were whole pRBCs and a crude solubilised lysate of pRBCs of *P. c. chabaudi* CB. To elucidate whether both these Ag preparations could provide a suitable source of antigenic stimulus/determinants against which a cellular response could be mounted, assays were performed to demonstrate a specific proliferative response of spleen cells to these Ags. These also determined the optimum number of pRBCs or the optimum protein concentration of the lysate necessary to elicit the maximal level of lymphocyte proliferation. In addition, the use of nRBCs and a nRBC lysate was included to determine if lymphocyte growth was in response to parasite-specific Ag(s).

The Ag concentration assays were performed on spleen cell suspensions from mice recently recovered (60 DPI) from infection with 5 x 10^4 pRBCs *P. c. chabaudi* CB. These cells were cultured at 2 x 10^6 cells/well in a 96 well microtitre plate and assayed for proliferation by the incorporation of methyl [^{3}H] thymidine. The proliferative response of the cells to both sources of antigen is shown in Figure 7.2.1 a & b. In each case the response is dose-dependent and the activation induced by either pRBC preparation was significantly greater than that induced by nRBCs ($p<0.001$), indicating that the stimulus for cell growth was parasite specific and not due to a common determinant on all RBCs (compare to response seen later with cultured T cells, 7.5). Maximal proliferation was attained with 1 x 10^6 pRBCs/ml or 250 μg/ml pRBC lysate; figures 7.2.1a and 7.2.1b respectively. Therefore, for routine stimulation of T cell lines *in vitro*, cultures were stimulated with the above concentrations of the respective Ag.

7.3 Initiation and maintenance of helper T cell lines

Having determined the optimum Ag concentration as described above, it was 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* CB immune response. The lines established *in vitro* were raised by the protocol described in Chapter 2. Table 7.1.1 shows the stages of infection from which it was attempted to prepare T cell lines. At the outset, cultures were set up in parallel, stimulated with either whole pRBCs or the pRBC lysate. As the study progressed it became apparent that cell growth was not supported by the lysate preparation. This has been reported previously (Khansari *et al.*, 1981; Gross & Frankenburg, 1989; Riley *et al.*, 1989b) and therefore, it was decided to use whole infected pRBCs as the sole source of Ag. Several splenic lymphocyte lines were raised, but only one was successively

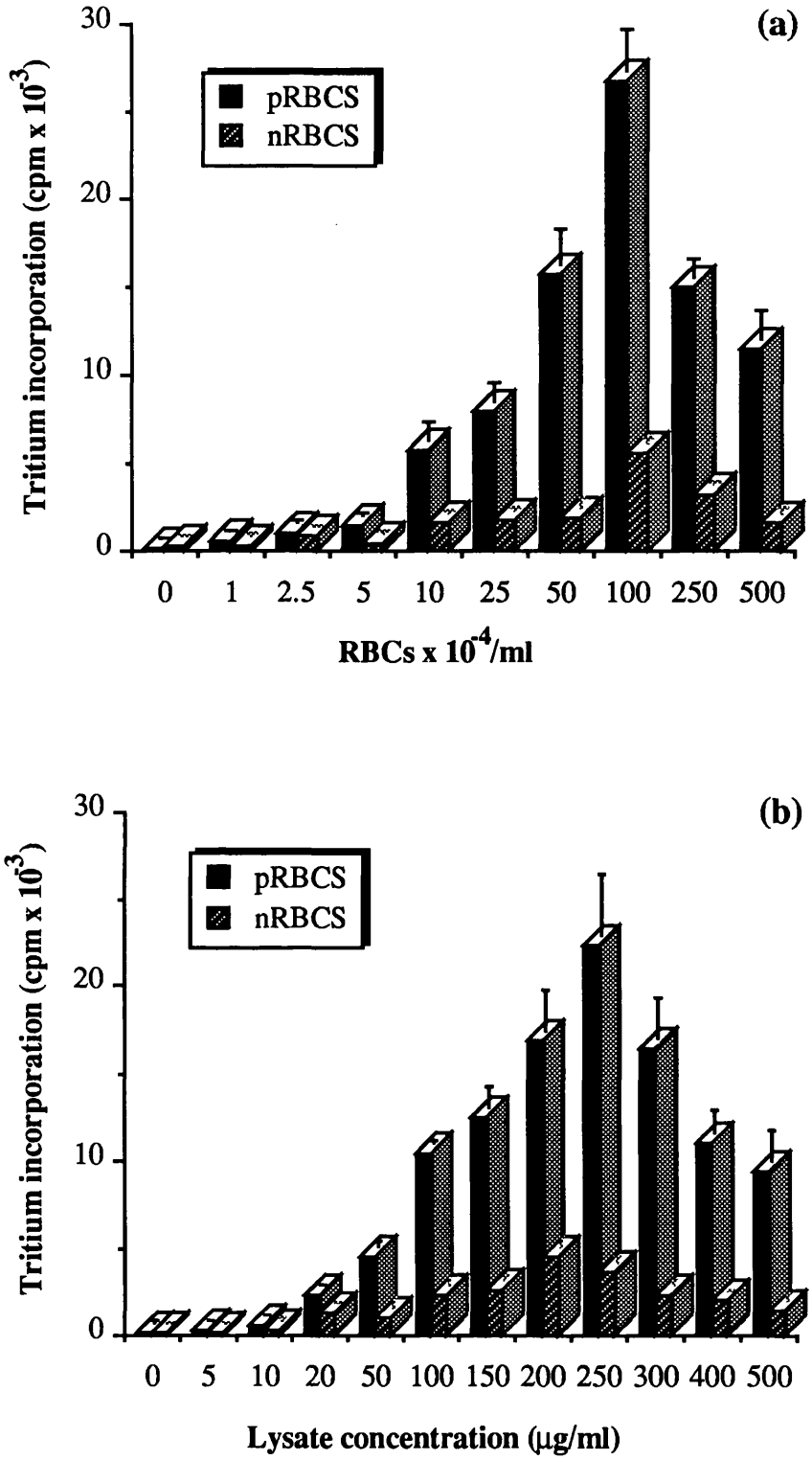


FIGURE 7.2.1
Proliferative response of post-infective splenic lymphocytes to (a) pRBCs, and (b) lysate preparations of *P. c. chabaudi* CB, to determine optimum Ag concentrations for T cell line stimulation.

maintained. This was prepared from naturally recovered mice at 60 DPI and was designated WEP 1153. The other lines succumbed to contamination or simply would not grow after the second or third stimulation *in vitro*. Following cryopreservation, none of the lines responded to stimulation upon recovery from liquid nitrogen.

7.4 Surface phenotyping of WEP 1153

The successfully generated T cell line was phenotyped by immunofluorescence (2.30). After 5 rounds of antigenic stimulus with pRBCs the cell line was tested against a panel of mAbs specific for CD4 and CD8 surface determinants, and a monoclonal to the lymphocyte marker Thy-1.2. By this method, WEP 1153 contained 96% $CD4^+$ and 4% $CD8^+$ T cells, all possessing the Thy-1.2 marker.

7.5 Antigen specific T cell proliferation

In vitro studies must include a T_H cell proliferation assay to measure the ability of cultured T_H cells to respond to specific antigenic stimulation, to demonstrate that the T cell line generated was primed to the asexual erythrocytic stages of *P. c. chabaudi* CB to which it was raised. This assay was used not only to confirm the antigenic reactivity of the newly generated T cell line, but also to examine the maintenance of specificity during long-term culture. Cellular proliferation was measured by the incorporation of [^{3}H-T] and the methodology is described in detail in section 2.31. Careful attention was paid to the inclusion of negative control wells, for statistical analysis and enumeration of background responses.

Figure 7.5.1 shows the proliferative response of WEP 1153. Proliferation was measured after 6 cycles of stimulation, 5 days after the last addition of Ag (Figure 7.5.1a) or after 7 cycles of stimulation, 7 days after the last stimulus (Figure 7.5.1b). In the former assay high background counts were observed with cells + Ag and cells + APCs (Figure 8.5.1a) indicating that the cells were still responding to residual Ag from previous stimulation in culture and/or that APCs were still able to present fresh Ag to the responding WEP 1153 cells. The assay was, therefore, repeated with cells that had been deprived of Ag for longer before the assay was carried out (Figure 7.5.1b). Here, the background responses were negligible, although a significant response was seen with cells + APCs ($p<0.05$) compared to cells alone or medium alone wells. However, this response was significantly lower ($p<0.01$) than all other test wells.

In both assays, a trend was observed, with a dose dependent response to pRBCs: the greatest response seen with 2.5 x 10^6 pRBCs/ml; maximal cellular growth, therefore, being achieved by a greater number of pRBCs than that used to give optimal proliferation of unfractionated 60 DPI spleen cells (Figure 7.2.1). This was only significant ($p<0.01$) for the second assay (Figure 7.5.1b). However, for both

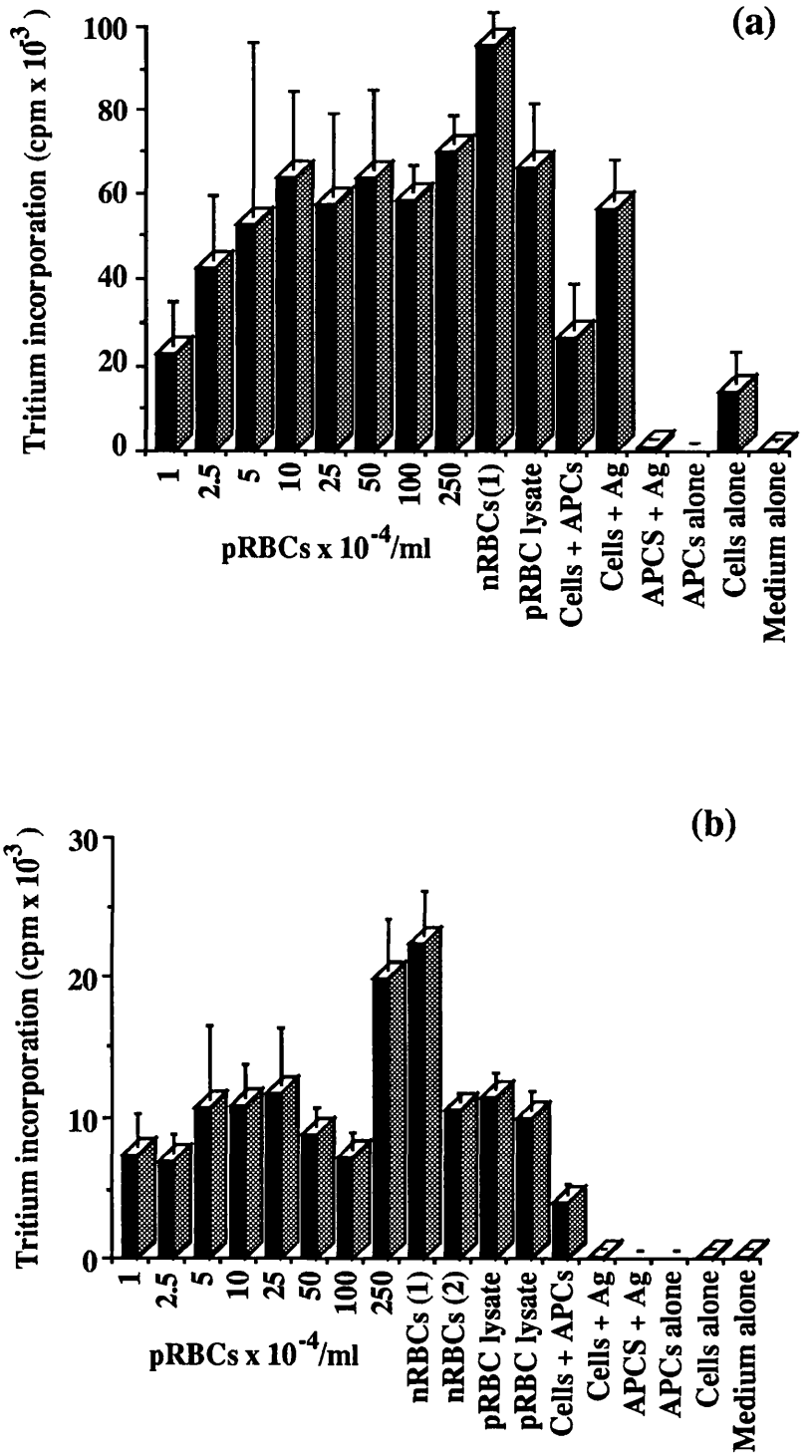


FIGURE 7.5.1
Proliferative response of WEP 1153 T lymphocytes to antigenic stimulation with pRBCs of *P. c. chabaudi* CB. Cells were deprived of antigen for either (a) 5 days or (b) 7 days before the assay.
nRBCs (1) = 2.5 x 10^6/ml
nRBCs (2) = 2.5 x 10^5/ml

assays, the greatest proliferative response was to nRBCs, (2.5 x 10^6/ml) showing a significant difference to control wells ($p<0.001$) but this response was not significantly different to that obtained with the highest concentration of pRBCs. At a lower concentration of red cells (nRBCs (2) - Figure 7.5.1b) the response was not as great, although it was comparable to those cells responding to the lower doses of pRBCs and to pRBC lysate (250 μg/ml). These responses strongly suggest that the proliferation of the established cell line WEP 1153 was not due entirely to stimulation by cocultured parasite-determined antigenic determinants, but to a nRBC/altered red cell Ag. Throughout the assay, as the number of pRBCs increased so too did the number of nRBCs within the preparation and this was perhaps the reason for the apparent dose response seen with pRBCs. The response to pRBCs, soluble pRBC lysate or nRBCs, however, was dependent on the availability of APCs, since no proliferation was observed when T cells were stimulated without APCs (Figure 7.5.1b).

7.6 Adoptive transfer of WEP 1153 T cell line to immunocompromised recipients

Following pRBC Ag stimulation *in vitro*, the effect of adoptive T cell transfer of host resistance to a primary infection with *P. c. chabaudi* CB in sublethally irradiated, syngeneic mice was assessed. The study documented herein was carried out to assess if *in vitro* lymphoproliferation correlated with *in vivo* protection against parasite challenge.

WEP 1153 was propagated *in vitro* in bulk cultures prior to adoptive transfer to gain sufficient viable lymphocytes for inoculation. Enough cells were harvested, while the cells were still dividing logarithmically, to inoculate 5 age-matched female NIH mice with 2 x 10^7 viable cells, i.v.. These mice had been exposed, on the same day of transfer, to 4 Gy (400 rads) γ irradiation. A control group of irradiated mice received the same size inoculum of enriched T cells prepared from non-immune naive donor mice. All animals were infected with 1 x 10^4 pRBCs *P. c. chabaudi* CB immediately after adoptive transfer, and the consequent course of infection monitored by examination of daily blood smears.

The resultant course of infection in both groups of mice is shown in Figure 7.6.1. Detectable protection against parasite challenge after adoptive transfer was observed with WEP 1153. This was manifested as a delay in onset of patency and a lower peak parasitaemia, the peak occurring 13 DPI in both groups, the mean parasitaemia being 29.9% and 39.1% for WEP 1153 and naive T cell recipients respectively. These features, however, were not significant ($p>0.05$). However, there was a much more rapid parasite remission in recipients of WEP 1153, $p<0.05$ on days

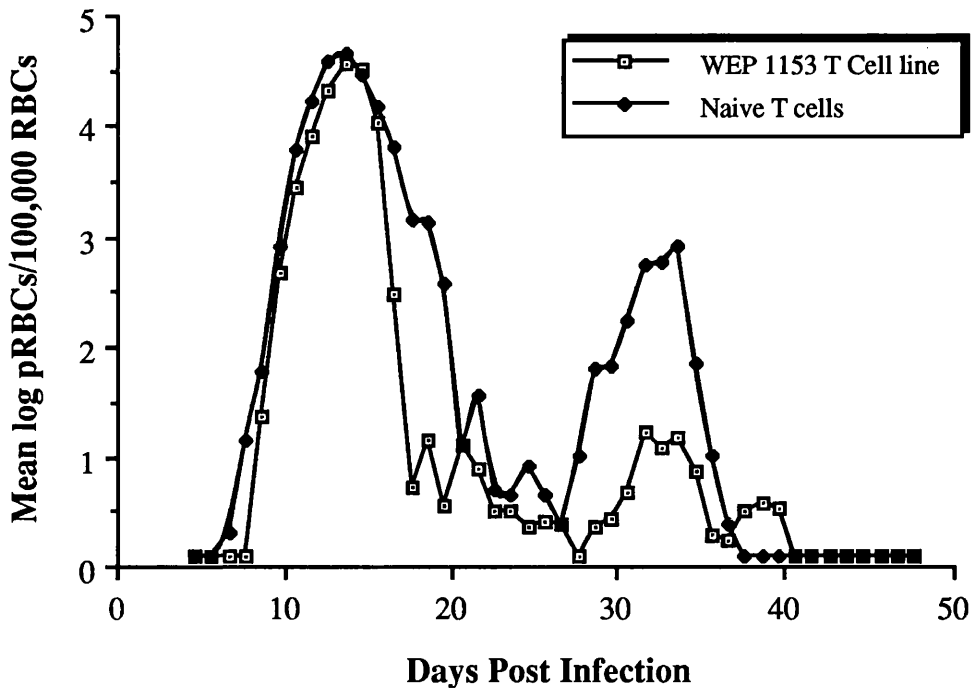


FIGURE 7.6.1 The course of infection in 4Gy irradiated recipients of 2 x 10^7 WEP 1153 T cells. Mice were challenged with 1 x 10^4 pRBCs *P. c. chabaudi* CB.

16, 17, 18 and 19 post infection. The recrudescence observed in WEP 1153 recipients was significantly lower ($p<0.05$) than that observed in naive T cell recipients.

7.7 Discussion

This chapter describes the successful initiation and propagation of one T cell line, WEP 1153, raised from splenic T lymphocytes taken from donor mice at 60 DPI. By immunofluorescent staining, this line was predominantly of the $CD4^+$ phenotype. An attempt was made to generate other lines at the selected time points shown in Table 7.1.1. However, these were unsuccessful due either to contamination or difficulties in maintaining a *P. c. chabaudi* CB specific proliferation *in vitro*.

WEP 1153 was raised by stimulating the cultures with pRBCs as the source of Ag, due to the unresponsiveness of the T cells to proliferate in bulk culture to lysate preparations. Possible reasons for this include factors associated with the lysate that may downregulate T-cell proliferation (Gross & Frankenburg, 1989; Khansari *et al.*, 1981; Riley *et al.*, 1989b), act directly as T-cell toxins or induce nonspecific cytotoxicity, eg. by altering the pH or osmolality of the medium (Wasserman *et al.*, 1993). Wasserman *et al.* (1993) have shown that the responsiveness of malaria specific T lymphocyte lines is markedly enhanced by the use of syngeneic APCs that have preprocessed an antigen lysate and subsequently washed prior to culture with T cells *in vitro*. Thus, this provides a possible new method for the successful initiation of malaria-specific T cell lines, with the immediate availability of preprocessed Ags for the stimulation of T cells enhancing T-cell responsiveness, as has been described for other systems (Unanue, 1989; Corradin, 1990; Cresswell, 1990). Brake *et al.* (1988) found that coculturing APCs, with the same soluble Ag as that used to stimulate proliferation, for 3hrs before irradiation gave the highest cell growth response. Furthermore, Chemtai *et al.* (1984a,b) showed an increased proliferative response to *P. c. chabaudi* pRBC lysate using lysate-fed macrophages. However, APC sensitisation has been shown to reduce the radiosensitivity of the APCs (Ashwell *et al.*, 1988).

Previous findings using T cell lines raised against *P. berghei* (Gross *et al.*, 1984; Gross & Frankenburg, 1988), *P. c. adami* (Brake *et al.*, 1986; 1988) and *P. c. chabaudi* AS (Taylor-Robinson, 1991; Taylor-Robinson & Phillips, 1992, 1993, 1994a) have shown that lymphocyte lines (and daughter clones) retain their Ag specificity *in vitro*, as evidenced by the fact they proliferate in response to parasite stimulation but not to nRBC preparations. These studies indicated that parent lymphocyte lines could be established from mice at a variety of times after infection; Taylor-Robinson (1991) and Taylor-Robinson & Phillips (1992; 1993; 1994a) demonstrating that anti-malaria lines could be raised as early as 16 DPI after challenge of the donor animals with *P. c. chabaudi* AS. Chemtai *et al.* (1984a) had

previously raised lymph node cell lines specific for the virulent IPPCI strain of *P. c. chabaudi* from mice primed for either 8 or 14 days. The proliferative response of these established lines was parasite specific. Langhorne & Simon (1989) using a limiting dilution assay of undefined splenic lymphocytes measured a specific response as early as 7 DPI. The above information was encouraging in attempting to generate *P. c. chabaudi* CB specific T cell lines from donor mice at different times throughout infection. Interestingly, Chemtai *et al.* (1984a) found no difference between the ability of whole or lysed pRBCs to induce lymphocyte activation, as was found in this study. These latter workers, however, failed to establish pRBC-specific lines *in vitro* using splenic lymphocytes.

Preliminary investigations to determine the optimal conditions for *in vitro* culture resulted in the use of whole, unlysed pRBCs at 1 x 10^6/ml. This was in agreement with other experimenters (Gross *et al.*, 1984; Brake *et al.*, 1986; 1988). The APC concentration used in this study was 1-2 x 10^6 APCs/ml, a value close to that of 10^6 peritoneal macrophages/ml quoted by Chemtai *et al.* (1984a) but differing from those of Gross *et al.* (1984) using 6 x 10^6 spleen cells/ml and Brake *et al.* (1986; 1988) using only 2 x 10^5 spleen cells/ml. This may reflect the difficulty in generating T cell lines in this study, but the variation in numbers used successfully above is more difficult to explain. Spleen cell concentration is probably not a direct measure of APC concentration, rather, the functional frequency of cells which fulfill the role of APC to different types of responder cells may vary according to such factors as the state of purification or differentiation of the T lymphocyte population. The variation in numbers, however, is probably not critical, the magnitude of Ag-induced T cell proliferation in one study not being affected directly by APC frequency (Goodacre *et al.* (1987).

The *in vitro* proliferative response of WEP 1153 unusually showed maximal response to a high number of nRBCs (2 x 10^6/ml) and a positive response was also demonstrated with a lower concentration of nRBCs (2.5 x 10^5/ml). This has not been documented previously and it appears that the WEP 1153 T cell line produced was raised to a pRBC Ag that cross-reacted with a nRBC/altered red cell Ag. Sherman & Winograd (1990) proposed that changes to the surface proteins of the red cells on infection with *P. falciparum* were parasite-induced modifications to host proteins. They suggested that changes to naturally occurring host red cell proteins, especially band 3, result in subtle changes to the antigenicity of infected red cells. There is evidence that alterations in band 3, the principal membrane protein of the red cell, do occur and contribute to changes in the antigenicity of parasitised cells. Intramembranous band 3 particles are specifically redistributed in the region of a *P. falciparum*-induced knob (Allred *et al.*, 1986) and immunoelectron microscopic studies have shown that band 3 is clustered in the region of knobs (Sherman *et al.*,

1989). Autoantibodies to band 3 occur naturally (Winograd *et al.*, 1989) and have been used to immunoprecipitate a surface iodinatable protein of >240 kDa (PfEMP1, a *P. falciparum* vaccine candidate, is exposed on the surface of the infected red cell and is >240 kDa), which by peptide mapping has been shown to be a truncated form of band 3 (Winôgrad & Sherman, 1989). Another truncated form of band 3, of 85 kDa, can be immunoprecipitated by a mAb that specifically reacts with the surface of live knobby cells, but not uninfected cells (Winograd & Sherman, 1989). Cytoadherence, may also be affected by band 3 modifications (Winograd & Sherman, 1989; Crandall & Sherman, 1994). A 65 kDa altered form of band 3 present in a knobless cytoadherent line of parasites is recognised by a mAb that also blocks cytoadherence (Winograd & Sherman, 1989).

It is debatable whether this information helps to explain the occurrence of maximal proliferation of WEP 1153 to nRBCs. It is possible, however, that the dominant response of the T cell line was to such an altered nRBC component and that within the proliferation assays, the concentration of red cells was high enough that proliferation was induced. That there was a dose response to pRBCs may be due to the presence of the altered Ag itself as well as an increase in the number of uninfected cells in the stimulating preparation. Such a large number of uninfected red cells (or FCS within the system) may also act as mitogens increasing proliferative responses. Con A, a T cell mitogen, has been shown to recognise sugar residues on band 3, and addition of Con A to knobby pRBCs *in vitro* enhances the agglutination of such cells (Sherman & Greenan, 1986). It is possible that FCS or nRBCs in the microtitre assay recognise band 3 and cause an alteration in antigenicity. Further studies on these responses were, however, impossible as WEP 1153 suffered contamination problems and would not grow upon recovery from liquid nitrogen.

That the cell line became unresponsive after recovery from liquid N_2 may have been due to the T cells becoming anergic. If the APC concentration was too low for the responding cells, the T cells may act as APCs, but following this they cannot respond to subsequent specific antigenic challenge (Prof. R. Lechler, personal communication). If the T cells become anergic they may act as Ag-specific suppressor cells, or consume either exogenous or endogenous Il-2 preventing proliferation of responsive T cells (Prof. R. Lechler, personal communication).

The adoptive transfer of WEP 1153 T cells resulted in protection against challenge with *P. c. chabaudi* CB in recipient immunocompromised mice. Gross *et al.* (1984), Brake *et al.* (1986) and Taylor-Robinson (1991), raised T cell lines against *P. berghei, P. c. adami* and *P. c. chabaudi* AS respectively, and showed that these lines were protective upon adoptive transfer. These workers used a variety of recipient mice, from naive to irradiated, CD4 depleted and congenitally T cell-deficient nude mice. The use of sublethally irradiated hosts in this study was to tally

with the previous adoptive transfer experiments, and served to amplify the protection conferred. After a slight delay in onset of patency which was not significant compared to recipients receiving naive T cells, the ascending parasitaemia of WEP 1153 cell recipients did not differ from the control animals. It would appear, therefore, that the transferred cells did not function immediately as effector cells, but rather that they took time for expansion to numbers necessary to affect the course of infection, or that they activated other mechanisms within the irradiated but recovering host, or possibly elsewhere in the donor population, to effect resolution of infection. Such activation is probably a result of cytokine activation, as first proposed by Brake *et al.* (1986). Unfortunately, this study did not permit the characterisation of cytokine secretion of WEP 1153 and it is impossible to tell whether the T cell line was predominantly T_H1 or T_H2.

Control animals in this adoptive transfer experiment received a population of enriched naive T cells. A more rigorous control would have been to include mice receiving CD4 enriched cells, since a population of naive T cells will include a significant proportion of $CD8^+$ cells. These may exert an effect by suppressing the development of immunity to *P. c. chabaudi* CB. It is unlikely, however, that such a contribution by these cells would exist, since the removal of $CD8^+$ cells by mAb treatment (Chapter 3) did not alter the course of a normal infection.

The protection demonstrated by the transfer of WEP 1153 was much less than that observed with the transfer of enriched splenic T cells from donor mice 75 DPI (Chapter 4). In the latter case protection was manifested as a significant reduction in the peak parasitaemia, rapid parasite remission and no recrudescences of infection. WEP 1153 induced protection was exhibited by a more rapid parasite remission compared to control animals, but a recrudescence did occur although it was significantly lower than in control mice. This probably reflects the difference in transferring a heterogeneous population of cells, albeit enriched populations, in the case of the adoptive transfer experiments described in Chapter 4, and the transfer of *in vitro* propagated Ag-specific T cell lines. The former populations of cells always contained an undefined proportion of contaminating cells which may either have been activated by the T cells within the population or contributed to immunity by themselves.

Although the cellular events occurring during the prepatent period are not known, it was assumed that the trigger for the activation of the transferred WEP 1153 T cells was *P. c. chabaudi* CB. However, this cannot be stated with certainty due to the proliferative response observed *in vitro*; while a pRBC response was demonstrated, which may have been maintained *in vivo*, it is difficult to explain the results to include the proliferation to nRBCs. Proliferation, however, demonstrated *in vitro* to pRBCs is likely to be maintained *in vivo*. Spasmodic regrowth of donor

tumour specific T cells has been reported by Chen *et al.* (1990) with the number of lymphocytes of donor origin maintained at the same or greater level for longer than a month. There is no reason why such a proliferative response *in vivo* should not occur with transferred T cells through a natural *P. c. chabaudi* CB infection (R.S. Phillips, personal communication). The homing patterns of the transferred cells are important although they have not been studied within this experiment. Lymphocyte migration patterns have been examined for the host lymphocyte reservoir during malaria infection (Kumararatne *et al.*, 1987), but 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. Transferred effector lymphocytes, if they still possess markers associated with activation, will probably home to the the tissue of their origin (Mackay, 1993). Many cloned T cells, however, when injected intravenously, display aberrant trafficking patterns, a phenomenon that 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 distribute to immunologically critical sites. As the spleen is critical in the resolution of malaria infections, it is important that the grafted lymphocytes reach the spleen, and perhaps even microenvironments within it, in order to fulfill their protective capacity.

CHAPTER EIGHT

GENERAL DISCUSSION

The work reported in this thesis has demonstrated the importance of T lymphocytes in the development of protective immunity to the asexual blood stages of *Plasmodium chabaudi chabaudi* CB. Depletion of specific subsets by mAb treatment showed that the $CD4^+$ T cell subset is absolutely critical in the resolution of infection (Chapter 3). $CD4^+$ T cells can be separated into two major subsets, T_H1 and T_H2, based upon the repertoire of cytokines secreted following stimulation, with the distinct cytokine profiles that they display indicating their function (Mosmann & Coffman, 1987). Both subsets of lymphocytes, for the *P. c. chabaudi* CB/NIH mouse system, will recognise plasmodial peptides which have been processed and presented on H-2 class II molecules of APCs (Chapter 7). T_H1 cells produce Il-2, IFN-γ and TNF-β and through these activate macrophages and mediate delayed-type hypersensitivity responses. T_H2 cells produce Il-4, Il-5, Il-6 and Il-10 and provide help for B-cell Ab production (Mosmann & Coffman, 1987; 1989). Hence, T_H1 and T_H2 cells mediate qualitatively distinct immune responses, containing largely nonoverlapping cell-mediated and humoral elements. Infection of mice with the AS strain of *P. c. chabaudi* generates a host protective immune response which involves $CD4^+$ T cells of both the T_H1 and T_H2 phenotypes (Langhorne, 1989). The relative contributions of these two subsets change during the course of infection (Langhorne *et al.*, 1989a, b; Taylor-Robinson & Phillips, 1992; 1993; 1994a) such that T_H1 cells predominate during the primary parasitaemia, while T_H2 cells predominate during the later stages of infection and reinfection.

The adoptive transfer experiments described in Chapter 4 indicated that immunity could be transferred with enriched splenic T cell populations prepared from donor mice at different stages of malarial infection. Although a detailed analysis of the function of the cells was not carried out it was apparent that both subsets of T_H cells were involved in eliciting immunity. Increased levels of malaria-specific Abs were detected in the sera of mice that received 'immune' T cells from all the stages of infection from which spleen cell populations were prepared. A prolonged secretion of IFN-γ from Con A stimulated spleen cells was observed in mice receiving 21 DPI T cells (Chapter 4). An attempt at generating *P. c. chabaudi* CB specific T cell lines (Chapter 7) was undertaken with the aim of obtaining homogeneous cell lines of either the T_H1 or T_H2 phenotype, to characterise further the T cell responses present during different stages of malarial infection.

When T_H1 cells are activated they secrete amongst other cytokines, IFN-γ. IFN-γ is a potent activator of macrophages, which leads to their expression of increased levels of MHC class II determinants (King & Jones, 1983; Fernandez-Botran *et al.*, 1988). They, thereby, become more efficient APCs (Zlotnik *et al.*, 1983). Macrophage activation also results in enhanced phagocytosis (Zlotnik *et al.*, 1983)

and in the release of cytokines such as TNF, Il-1 and Il-6 (Dockrell & Playfair, 1983, reviewed by Stevenson *et al.*, 1990b), reactive oxygen intermediates (Clark *et al.*, 1987a) and reactive nitrogen intermediates (Green *et al.*, 1990a). All these effects of macrophage activation have been noted as contributing towards clearance of malaria pRBCs. Activated macrophages phagocytose effectively *Plasmodium*-infected RBCs (Ockenhouse & Shear, 1983; Shear, 1984). TNF, Il-1 and Il-6 can induce fever which may be directly damaging to parasites (Dinarello, 1989; Kwiatkowski, 1989), influence the production of acute phase proteins (Dinarello, 1989), and enhance further cell-mediated and Ab responses by activating T cells, B cells and macrophages, sometimes by acting in a synergistic manner (Titus *et al.*, 1991). These cytokines also contribute to the pathology of malaria.

TNF-α can be induced in macrophages by direct stimulation with erythrocytic stages of the parasite (Bate *et al.*, 1988; 1989; Taverne *et al.*, 1990). This cytokine is also detectable during a primary infection with *P. c. chabaudi*, *P. vinckei*, *P. berghei* and *P. yoelii*, both in the plasma and in the supernatants of splenic cells taken from infected animals and cultures *in vitro* (Stevenson *et al.*, 1990c; Taverne *et al.*, 1986; Grau *et al.*, 1987). Treatment with exogenous TNF-α at the beginning of infection protects against an otherwise lethal infection of *P. c. chabaudi* in susceptible strains of mice (Stevenson & Ghadirian, 1989), and delays patency and reduces peak parasitaemias in *P. c. adami* infections (Clark *et al.*, 1987b).

Mice infected with *P. berghei* have elevated serum levels of Il-6 and the production of Il-6 can be modulated by injection of infected mice with an Ab against IFN-γ and TNF-α (Grau *et al.*, 1990). Although serum levels of Il-1 have not been found to be elevated in malaria infection, Il-1 has important immunoregulatory activities (Arai *et al.*, 1990; Beutler & Cerami, 1988; Snick, 1990). Some subsets of $CD4^+$ T cells have receptors for Il-1 (Kaye *et al.*, 1984; Greenbaum *et al.*, 1988; Lichtman *et al.*, 1988; Munoz *et al.*, 1990; Taylor-Robinson & Phillips, 1994c) and thus may be preferentially expanded in the presence of this cytokine. Indeed, Taylor-Robinson & Phillips (1994c) have shown that the expression of the Il-1 receptor discriminates T_H2 from T_H1 cloned T cells specific for *P. c. chabaudi* AS, providing a possible marker for phenotyping, depletion or selection of $CD4^+$ T cells of either the T_H1 or T_H2 subset.

Oxygen intermediates and NO released from activated macrophages are toxic for intraerythrocytic parasites *in vitro* (Dockrell & Playfair, 1983; Clark & Hunt, 1983; Rockett *et al.*, 1991) and may be important for killing parasites *in vivo* (Rockett *et al.*, 1992; Taylor-Robinson *et al.*, 1993). Free oxygen radicals may be important in killing parasites evidenced by mice treated with the free radical scavenger butylated hydroxyanisole during *P. c. adami* infection having higher parasitaemias than

untreated mice (Clark *et al.*, 1987a). In contrast, however, P/J mice which are defective in macrophage activation and production of oxygen intermediates are able to resolve *P. c. chabaudi* infections (Cavacini *et al.*, 1989). The importance of NO *in vivo* has been demonstrated by Taylor-Robinson *et al.* (1993), T_H1 T cell clones specific for *P. c. chabaudi*, upon adoptive transfer, protect *in vivo* by a NO dependent mechanism. *In vivo* production of NO has also been demonstrated during infection with *P. c. chabaudi* CB (Chapter 6).

T_H1 $CD4^+$ lymphocytes, through their synthesis and secretion of IFN-γ (and NO (Taylor-Robinson *et al.*, 1994)) are, therefore, effective initiators 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 prior to peak parasitaemia (Slade & Langhorne, 1989, Stevenson *et al.*, 1990a). Experiments described in Chapter 4 and Chapter 6 demonstrated that IFN-γ production in mice infected with *P. c. chabaudi* CB was also produced maximally one or two days prior to the peak parasitaemia. This concurs with reports that T_H1 cells predominate during the resolution of the primary parasitaemia and that this resolution is independent of specific Ab production, probably through the synthesis of NO (Taylor-Robinson *et al.*, 1993; 1994). It also demonstrates that the kinetics of the immune response to infection with *P. c. chabaudi* CB are similar to those of the AS strain. The Ab levels during the course of infection (Chapter 5) show that although detectable Abs appear early in infection, peak Ab titres occur following resolution of the primary parasitaemia. T_H1 lymphocytes are thought to be unable to stimulate resting B cells (Boom *et al.*, 1988) while T_H2 cells, the frequency of which is very low in the first two weeks of *P. c. chabaudi* AS infection (Langhorne *et al.*, 1989a), can induce the activation of resting B cells. The Ab that is detectable during the primary parasitaemia is of the IgG_{2a} isotype (Langhorne *et al.*, 1984; 1985), the synthesis of which is positively regulated by IFN-γ (Snapper & Paul, 1987). IgG_1 synthesis is detected later in malarial infection, and indeed the suppressed levels of IgG_1 Abs elaborated during the primary patency (Langhorne *et al.*, 1984; 1985; Langhorne & Asofsky, 1986; Falanga *et al.*, 1987) are probably due to T_H1-produced IFN-γ via its antagonistic effects on Il-4-dependent responses (Rabin *et al.*, 1986; Snapper & Paul, 1987; Snapper *et al.*, 1988).

Studies by Langhorne *et al.* (1989a), Taylor-Robinson (1991) and Taylor-Robinson & Phillips (1992, 1993, 1994a) have shown that $CD4^+$ populations specific for *P. c. chabaudi* AS obtained late in infection belonged to the T_H2 subset and provided considerable help for specific Ab synthesis. It has also been shown that cloned T_H2 malaria-specific cells protect immunocompromised mice upon adoptive transfer by the enhancement and accelerated production of specific IgG_1 Ab (Taylor-

Robinson *et al.*, 1993). T_H2-type CD4-bearing lymphocytes are activated by Il-2 and Il-1 secreted from activated T_H1 cells and activated macrophages respectively (Mosmann & Coffman, 1989). Following activation T_H2 cells then secrete their own growth factor, Il-4. After stimulation with malaria Ags, T_H2 cells provide help, in the form of Il-4 and Il-5, for B cells to produce specific Ab (Mosmann & Coffman, 1987). Ig specific for blood stage malaria has been shown to serve several functions. Circulating Abs against pRBC membrane-bound determinants may cause elimination of infected RBCs by agglutination (Brown *et al.*, 1970b) or complement-mediated lysis, or alternatively, function in a neutralising capacity by blocking reinvasion of merozoites into RBCs (Butcher *et al.*, 1978). In addition, circulating Ig acts as an opsonin for the Fc receptor-mediated phagocytosis of pRBCs (Ockenhouse & Shear, 1984). In turn, this signals further mononuclear phagocytic cell activation and the subsequent release of toxic mediators into the surrounding localities of the liver and spleen. Alternatively, B lymphocyte membrane-bound Ig specific for surface Ags of free merozoites or pRBCs may function as a reservoir for parasite material, thereby facilitating the uptake, processing and presentation of plasmodial Ags by available APCs to $CD4^+$ T cells (Langhorne, 1989). This would serve to recruit other effector cells to the parasite-directed immune response.

Several studies have indicated the important role played by Ab in the elimination of plasmodia *in vivo*. Successful passive transfer experiments (Chapter 5) have been undertaken using rodent models and immune serum (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 T_H2 cells (Langhorne *et al.*, 1989b). With this in mind, it is perhaps not surprising that the immunity transferred by 21 DPI serum from naturally recovering mice was greater than that transferred with 14 DPI subpatent serum from chloroquine treated mice (Chapter 5), given that there is more likelihood of a greater frequency of T_H2 cells being present by 21 DPI.

As the primary infection of *P. c. chabaudi* AS (and presumably *P. c. chabaudi* CB) progresses there is, therefore, a clear shift in the pattern of the $CD4^+$ T cell response. Host immunity appears to combine both Ab-independent and Ab-dependent mechanisms, T_H1 and T_H2 cells appearing sequentially during a primary erythrocytic infection. The T_H1 response may control acute phase parasitaemias through Ab-independent means via macrophage activation and production of parasiticidal mediators. T_H2 cells which appear later become the major effector mechanism of protective immunity by producing the appropriate cytokines for B cells to expand and to differentiate into malaria-specific Ab-producing cells (Langhorne, 1989). The signals which differentially regulate the activation of T_H1 and T_H2 cells

following challenge are not completely understood. The knowledge of the signals which differentially regulate activation of these cells is necessary for an understanding of how cellular and humoral responses are generated and regulated during an infection and for the design of a subunit vaccine which induces the appropriate effector pathway.

Several lines of evidence indicate that the major controlling factors for the selective activation of T_H subsets are secreted cytokines (Swain *et al.*, 1991; Abehsira-Amar *et al.*, 1992), the nature of the APC involved (Gajewski *et al.*, 1991; DeKruyff *et al.*, 1992; Schmitz *et al.*, 1993), the processing of the Ag for presentation to the T cell (Soloway *et al.*, 1991), and the antigenic load (DeKruyff *et al.*, 1992; Mamula & Janeway, 1993). The latter may provide an explanation for the relatively large recrudescent patency observed in *P. c. chabaudi* CB-infected, chloroquine treated mice. In mice naturally recovering from infection, the parasitaemia is not resolved to subpatent levels until around 21 DPI; it is possible that the drop in parasitaemia may commit the immune response to T_H2-regulated mechanisms during the subpatent period and later stages of infection. For chloroquine treated mice the circulating-Ag dose is reduced quickly, perhaps providing the signal for a switch from the T_H1 to T_H2 phenotype before other appropriate signals are operative, hence leading to an increase in parasitaemia.

Two independent *in vivo* studies (Von der Weid & Langhorne, 1993b; Taylor-Robinson & Phillips, 1994b) on mice rendered B cell-deficient by life-long treatment with anti-μ Abs strongly suggested that the signal(s) mediating the switch from T_H1 to T_H2 cells is provided by B cells. In both studies, anti-μ mice infected with *P. c. chabaudi* AS maintained a strong T_H1 response throughout the whole course of infection. That the progression from a T_H1-regulated to a T_H2-regulated immune response fails to occur in B cell-depleted mice, suggests that B cells are required for the downregulation of T_H1 mediated and/or the generation of T_H2-mediated protective immunity to *P. c. chabaudi*. This hypothesis is supported by the concept that T_H1 cells respond optimally to Ag presented on macrophages whereas T_H2 cells respond better to peptides presented on B cells (Gajewski *et al.*, 1989; Hsieh *et al.*, 1992; DeKruyff *et al.*, 1992; Schmitz *et al.*, 1993).

Chapter 6 demonstrated that subcurative chloroquine treatment could alter the production of NO, a T_H1 cell-mediated immune mediator. Few studies have considered the interactions between chemotherapy and immunity to malaria although it is increasingly recognised that antimalarial drugs can interact in both positive and negative ways. Chloroquine has previously been shown to inhibit Il-1 secretion, presentation of antigens by accessory cells, to depress mitogen responsiveness and natural killer cell cytotoxicity and to display anti-inflammatory properties (Krogstad

& Schlessinger, 1987). Prophylactic doses of chloroquine have also been shown to inhibit the phagocytosis of IgG-coated sheep RBCs (Osorio *et al.*, 1992). The results presented in this thesis and those of Picot *et al.* (1991; 1993) have shown that chloroquine, at doses achievable *in vivo,* is capable of inhibiting a number of cytokines and immune mediators. This is of great interest and warrants further investigation, not only because it provides an approach for the use of chloroquine as an anti-disease therapy for malaria and auto-immune disorders, but also because it may influence the balance of T_H1 and T_H2 cells described above. This may affect the ability of the malaria parasite to undergo antigenic variation (Chapter 5) or induce an early T_H2 response, which may increase the susceptibility of a host to malaria infection (Stevenson & Tam, 1993).

Reiterating points made in the previous chapters and within this chapter, future work on *P. c. chabaudi* CB infection of the NIH mouse will allow factors involved in the interplay between cells of the two $CD4^+$ T cell subsets to be investigated. Immunity to most infectious agents can be broadly categorised into a predominant protective response of either the T_H1 or T_H2 phenotype. Experimental infections with *Leishmania*, *Candida* and *Listeria* evoke a protective T_H1 response but a disease-exacerbating T_H2 response. In contrast, for nematode infections, there is a significant relationship between T_H2 responses and worm expulsion and between T_H1 responses and chronic infection (Sher & Coffman, 1992). The dichotomy of response during a primary infection of *P. c. chabaudi* infection, therefore, is an ideal system to study the mechanisms of immunity evoked by either/both T cell subset.

The relative contributions of each T cell subset in immunity to *P. c. chabaudi* CB could be analysed in a variety of ways. Adoptive transfer of T cell clones, if successfully derived from different stages of infection, as well as from different tissues (eg., liver, spleen and peripheral blood), will enable the mechanisms leading to the recipient's control of infection to be elucidated. Titration of the numbers of cells, as described in Chapter 4, would determine the threshold number of malaria-specific T cells required to confer protection. In addition, to examine any synergy between the two subsets in eliciting immunity, adoptive transfer of suboptimal doses of cells alone or in combination could be performed. Direct screening of *P. c. chabaudi* CB cDNA libraries with parasite-specific $CD4^+$ T cell clones would identify the antigenic determinants to which the protective cells respond. 'Recognised' fragments could then be sequenced and used in protection experiments. Using the AS strain of *P. c. chabaudi* and the protective T_H1 and T_H2 cell clones derived by Taylor-Robinson & Phillips (1992; 1993; 1994a), such studies are underway. A comparison between fragments recognised by these clones and T_H clones specific for *P. c. chabaudi* CB could then be made, and the strain differences determined. This would not only help

in the elucidation of the mechanisms of protective immunity of anti-plasmodial T cell clones, but would evaluate their worth with a view to subunit vaccine development.

Detailed analysis of the cytokine profiles during the course of infection would allow the flux in levels of T_H1 and T_H2 cells to be followed, perhaps highlighting the switch between these two phenotypes. Delivery of exogenous cytokines by *Salmonella* mutants, a project underway in the laboratory for AS strain infection, will also provide information on the roles of T_H1 and T_H2 specific cytokines and their regulation to be investigated. Experiments described in this thesis demonstrated that chloroquine had an interesting effect on the course of infection, antigenic variation and NO production. Chloroquine chemotherapy could be used as a tool to investigate in more detail the induction of different T cell immune responses. By varying treatment regimes, and hence the antigenic load at any given time, the resulting cytokine patterns would indicate the predominant T cell responses present.

To conclude, the elucidation of the T cell-mediated immune mechanisms, with and without chloroquine therapy, involved in the killing or growth inhibition of malarial parasites could provide new avenues for therapeutic intervention as well as prophylactic immunisation. Studies using the mouse malaria *P. c. chabaudi* go some way to achieving this aim and allow analogies to human malaria to be made.

APPENDIX

CHLOROQUINE
5 mg/ml chloroquine diphosphate (Sigma) made up in PBS (pH 7.2)
0.25 ml/mouse (each mouse weighing approx. 25-28 g)

GIEMSA'S BUFFER
3.0 g Na_2HPO_4
0.6 g KH_2PO_4
Made up to 1 l with distilled water and adjusted to pH 7.4

GREISS REAGENT
The Greiss reagent is obtained by mixing in equal volumes, the following two solutions:
(a) 0.1% alpha-naphthyl-amine (Sigma) in distilled water
(b) 1% sulphanilamide (Sigma) in 5% phosphoric acid.
Store both solutions in the dark. Prepare fresh Greiss reagent every time.

NITRATE CONVERSION BUFFER
Stock reagents

NADPH (Sigma)	5 mg/ml in PBS or distilled water, stored at -20°C
FAD (Sigma)	41.5 mg/ml in PBS or distilled water, stored at -20°C
Nitrate reductase (Sigma)	34 mg/ml - lyophilised powder diluted in distilled water. Aliquots of 50 µl stored at -70°C.
KH_2PO_4 (anhydrous)	0.5 M, pH7.5

Buffer
For 50 samples and standards in duplicate:
500 µl NADPH
500 µl FAD
500 µl KH_2PO_4
500 µl distilled water
50 µl nitrate reductase + 450 µl distilled water to dilute enzyme
enzyme added to buffer last, then buffer is immediately added to samples.

PHOSPHATE BUFFERED SALINE (PBS)
Stock Solution
60.0 g $Na_2HPO_4.12H_2O$
13.6 g $Na_2HPO_4.2H_2O$
8.5 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

RPMI
RPMI Malaria Stock Medium
10.39 g RPMI 1640 powdered medium (with L-glutamine) (Gibco)
5.94 g (25 mM) N2-hydroxyethylpiperazine-N'-2 ethane sulphonic acid (HEPES) (Sigma)
Made up to 960 ml with distilled water and filter-sterilised (Millipore/Gelman filter size 0.22 µm).
RPMI Malaria Incomplete
Stock, aliquoted in 100 ml volumes to which the following supplements are added:

4.2 ml 5% w/v $NaHCO_3$
0.25 ml Gentamicin (Sigma)

RPMI Cell Culture Stock Medium
10.39 g RPMI 1640 powdered medium (with L-glutamine) (Gibco)
5.96 g (25 mM) N2-hydroxyethylpiperazine-N'-2 ethane sulphonic acid (HEPES) (Sigma)
Made up to 1 l with distilled water and filter-sterilised (Millipore/Gelman filter size 0.22 μm), pH adjusted to 7.2
RPMI Cell Culture Incomplete
Stock supplemented with the following additives:
11 ml L-glutamine (Gibco)
5.5 ml $NaHCO_3$ (3.5%)
0.55 ml 2-Mercaptoethanol (.1 M)
22 ml Fungizone (Gibco)
2.2 ml Gentamycin (Sigma)
Aliquots of 85 ml dispensed into 100 ml bottles.

Both Complete media contained 10% FCS (Gibco)

SORBITOL-GLYCEROL
380 g glycerol (Sigma)
39 g sorbitol (BDH)
6.3 g NaCl

TRIS-AMMONIUM CHLORIDE
0.17 M Tris(hydroxymethyl)aminomethane (20.60 g/l)
0.16 M ammonium chloride (8.30 g/l)

10 ml Tris added to 90 ml ammonium chloride and the 0.83% stock solution adjusted to pH 7.4

REFERENCES

ABEHSIRA-AMAR, O., GIBERT, M., JOLIY, M., THEZE, J. & JANKOVIC, D.L. (1992) Il-4 plays a dominant role in the differential development of T_H0 into T_H1 and T_H2 cells. *Journal of Immunology*, **148**, 3820.

ABELE, D.C., TOBLE, J.E., HILL, A.J., CONTACOS, P.G. & EVANS, C.B. (1965) Alterations in serum proteins and antibody production during the course of induced malarial infections in man. *American Journal of Tropical Medicine and Hygiene*, **14**, 191.

ADAM, C., GENITEAU, M., GOUGEROT-POCIDALO, M., VERROUST, P., LEBRAS, J., GIBERT, C. & MOREL-MAROGER, L. (1981) Cryoglobulins, circulating immune complexes and complement activation in cerebral malaria. *Infection and Immunity*, **31**, 530.

ADAMS, L.B., HIBBS, J.B., TAINTOR, R.R. & KRAHENBUHL, J.L. (1990) Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*: role for synthesis of inorganic nitrogen oxides from L-arginine. *Journal of Immunology*, **144**, 2725.

ADU, D., WILLIAMS, D.G., QUAKYI, I.A., VOLLER, A., ARUM-ADDO, Y., BRUCE-TAGOE, A.A., JOHNSON, G.O. & HOLBOROW, E.J. (1982) Anti-ss DNA and anti-nuclear antibodies in human malaria. *Clinical and Experimental Immunology*, **49**, 310.

AIKAWA, M., MILLER, L.H., JOHNSON, J. & RABBEGE, J. (1978) Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. *Journal of Cell Biology*, **77**, 72.

AIKAWA, M., SUSUKI, M. & GUTIERREA, Y. (1980) Pathology of malaria. In: *Malaria* (ed. J.P. Krier), p.47. Academic Press, New York, San Francisco & London.

ALGER, N.E. (1963) Distribution of schizonts of *Plasmodium berghei* in tissues of rats, mice and hamsters. *Journal of Protozoology*, **10**, 6.

ALLISON, A.C. & EUGUI, E.M. (1982) A radical interpretation of immunity to malaria parasites. *Lancet*, **ii**, 1431.

ALLISON, A.C. & EUGUI, E.M. (1983) The role of cell-mediated immune response in resistance to malaria with special reference to oxidant stress. *Annual Review of Immunology*, **1**, 361.

ALLISON, A.C. (1954) Protection afforded by sickle-cell trait against subtertian malarial infection. *British Medical Journal*, **i**, 190.

ALLRED, D., GRUENBERG, J.E. & SHERMAN, I.W. (1986) Dynamic rearrangements of erythrocyte membrane internal architecture induced by infection with *Plasmodium falciparum*. *Journal of Cell Science*, **81**, 1.

ANDERS, R.F. & SMYTHE, J.A. (1989) Polymorphic antigens in *Plasmodium falciparum*. *Blood*, **74**, 1865.

ANDERS, R.F. (1986) Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective immunity against malaria. *Parasite Immunology*, **8**, 529.

AQEL, N.M., CLARK, M., COBBOLD, S.P. & WALDMANN, H. (1984) Immunohistological screening in the selection of monoclonal antibodies: the use of isotype-specific antiglobulins. *Journal of Immunological Methods*, **69**, 207.

ARAI, K., LEE, F., MIYJAMA, A., MIYATAKE, S., ARAI, N. & YOKOTA, T. (1990) Cytokines: coordinators of immune and inflammatory responses. *Annual Review of Biochemistry*, **59**, 738.

AREEKUL, S., KASEMSUTH, R. & KANAKAHORN, K. (1984) Studies on the transcapillary escape rate of fibrinogen and capillary permeability in patients with *Plasmodium falciparum* malaria. *Tropical Geographical Medicine*, **36**, 151.

ARNOT, D.E., BARNWELL, J.W., TAM, J.P., NUSSENZWEIG, R.S. & ENEA, V. (1985) Circumsporozoite protein of *Plasmodium vivax*: gene cloning and characterisation of the immunodominant epitope. *Science*, **230**, 815.

ASARNOW, D.M., GOODMAN, T., LeFRANCOIS, L. & ALLISON, J.P. (1989) Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. *Nature*, **341**, 60.

ASENSIO, V.C., OSHIMA, H. & FALANGA, P.B. (1993) *Plasmodium berghei*: Is nitric oxide involved in the pathogenesis of mouse cerebral malaria? *Experimental Parasitology*, **77**, 111.

ASHWELL, J.D., JENKINS, M.K. & SCHWARTZ, R.H. (1988) Effect of gamma radiation on resting B lymphocytes. II. Functional characterisation of the antigen-presentation defect. *Journal of Immunology*, **141**, 2536.

BALLET, J.J., JAUREGUIBERRY, G., DELORON, P. & AGRAPART, M. (1987) Stimulation of T lymphocyte-dependent differentiation of activated human B lymphocytes by *Plasmodium falciparum* supernatants. *Journal of Infectious Diseases*, **155**, 1037.

BALLOU, W.R., SHERWOOD, J.A., NEVA, F.A., GORDON, D.M., WIRTZ, R.A., WASSERMAN, G.G., DIGGS, C.L., HOFFMAN, S.L., HOLLINGDALE, M.R., HOCKMEYER, W.T., SCHNEIDER, I., YOUNG, J.F., REEVE, P. & CHULAY, J.D. (1987) Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. *Lancet*, **ii**, 1277.

BANNISTER, L.H., BUTCHER, G.A. & MITCHELL, G.H. (1977) Recent advances in understanding the invasion of erythrocytes by merozoites of *Plasmodium knowlesi*. *Bulletin of the World Health Organisation*, **55**, 163.

BANNISTER, L.H., BUTCHER, G.A., DENNIS, E.D. & MITCHELL, G.H. (1975) Structure and invasive behaviour of *Plasmodium knowlesi* merozoites *in vitro*. *Parasitology*, **71**, 483.

BANNISTER, L.H., MITCHELL, G.H., BUTCHER, G.A. & DENNIS, E.D. (1986) Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium falciparum*: a clue to the mechanisms of invasion. *Parasitology*, **92**, 291.

BANYAL, H.S. & INSELBURG, J. (1985) Isolation and characterisation of parasite-inhibitry *Plasmodium falciparum* monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene*, **34**, 1055.

BARKER, L.R. & POWERS, K.G. (1971a) Impairment of antibody response and recovery in malarial rodents by antilymphocyte serum. *Nature*, **229**, 429.

BARKER, L.R. & POWERS, K.G. (1971b) Immunosuppression in rodent malaria. Effect upon recovery and antibody response. *American Journal of Tropical Medicine and Hygiene*, **20**, 389.

BATE, C.A.W., TAVERNE, J. & PLAYFAIR, J.H.L. (1988) Malarial parasites induce TNF production by macrophages. *Immunology*, **64**, 227.

BATE, C.A.W., TAVERNE, J. & PLAYFAIR, J.H.L. (1989) Soluble malarial antigens are toxic and induce the production of tumour necrosis factor *in vivo*. *Immunology*, **66**, 600.

BEALE, G.H., CARTER, R. & WALLIKER, D. (1978) Genetics. In: *Rodent Malaria* (eds. R. Killick-Kendrick & W. Peters), pp.213. Academic Press, New York, San Francisco & London.

BEAUDOIN, R.L., STROMA, C.P.A., PALMER, T.T. & BOWDEN, N. (1975) Immunogenicity of sporozoites of the ANKA strain of *Plasmodium berghei* following different treatments. *American Society for Parasitology*, **231**, 98.

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedins of the National Academy of Science* USA, **87**, 1620.

BEHR, C. & DUBOIS, P. (1992) Preferential expansion of V gamma 9 V delta 2 T-cells following stimulation of peripheral blood lymphocytes with extracts of *Plasmodium falciparum*. *International Immunology*, **4**, 361.

BEKESSY, A., MOLINEAUX, L. & STOREY, J. (1976) Estimation of incidence and recovery rates of *Plasmodium falciparum* parasitaemia from longitudinal data. *Bulletin of the World Health*

Organisation, **54**, 685.

BENJAMIN, R. & WALDMANN, H. (1986) Induction of tolerance by monoclonal antibody therapy. *Nature*, **320**, 449.

BERENDT, A.R., SIMMONS, D., NEWBOLD, C.I., TANSEY, J. & MARSH, K. (1989) Intercellular adhesion molecule 1 (ICAM-1) is an endothelial cytoadherence receptor for *Plasmodium falciparum*. *Nature*, **341**, 57.

BEUTLER, B. & CERAMI, A. (1988) Tumor necrosis, cachexia, shock, and inflammation: a common mediator. *Annual Review of Biochemistry*, **57**, 505.

BEUTLER, E., WEST, C. & BLUME, K.G. (1976) The removal of leucocytes and platelets from whole blood. *Journal of Laboratory and Clinical Medicine*, **88**, 328.

BIENZLE, U., FRITSCH, K.G., HOTH, G., ROZDZINSKI, E., KOHLER, K., KALINOWSKI, M., KREMSNER, P., ROSENKAIMER, F. & FELDMEIER, H. (1988) Inhibition of *Plasmodium vinckei* malaria in mice by recombinant murine interferon-γ. *Acta Tropica*, **45**, 289.

BIGGS, B.A., GOOZE, L., WYCHERLEY, K., WOLLISH, W., SOUTHWELL, B., LEECH, J.H. & BROWN, G.V. (1991) Antigenic variation in *Plasmodium falciparum*. *Proceedings of the National Academy of Science* USA, **88**, 9171.

BLOUGH, N.V. & ZAFIRIOU, O.C. (1985) Reaction of superoxide with nitric oxide to form peroxonitrite in alkaline aqueous solution. *Inorganic Chemistry*, **24**, 3502.

BOOM, W.H., LIANO, D. & ABBAS, A.K. (1988) Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin-4 and interleukin-2-producing T cell clones on resting B lymphocytes. *Journal of Experimental Immunology*, **167**, 1352.

BOONPUCKNAVIG, S. & EKAPANYAKUL, G. (1984) Autoantibodies in sera of Thai patients with *Plasmodium falciparum* infection. *Clinical and Experimental Immunology*, **58**, 77.

BOUHAROUN-TAYOUN, H., ATTANATH, P., SABCHAREON, A., CHONGSUPHAJAISIDDHI, T. & DRUILHE, P. (1990) Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *Journal of Experimental Medicine*, **172**, 1633.

BOYLE, W. (1968) An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. *Transplantation*, **6**, 761.

BRADFORD, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248.

BRAKE, D.A., LONG, C.A. & WEIDANZ, W.P. (1988) Adoptive protection against *Plasmodium chabaudi adami* malaria in athymic nude mice by a cloned T cell line. *Journal of Immunology*, **140**, 1989.

BRAKE, D.A., WEIDANZ, W.P. & LONG, C.A. (1986) Antigen-specific, interleukin 2-propagated T lymphocytes confer resistance to a murine malaria parasite, *Plasmodium chabaudi adami*. *Journal of Immunology*, **137**, 347.

BRANNAN, L.R., McLEAN, S.A. & PHILLIPS, R.S. (1993) Antigenic variants of *Plasmodium chabaudi chabaudi* AS and the effects of mosquito transmission. *Parasite Immunology*, **15**, 135.

BRANNAN, L.R., TURNER, C.M.R. & PHILLIPS, R.S. (1994) Malaria parasites undergo antigenic variation at high rates *in vivo*. *Proceedings of the Royal Society of London* B, **256**, 71.

BRAY, R.S. & GARNHAM, P.C.C. (1982) The life cycle of primate malaria parasites. *British Medical Bulletin*, **38**, 117.

BRINKMANN, V., KAUFMANN, S.H.E. & SIMON, M.M. (1985) T-cell-mediated immune response in murine malaria: differential effects of antigen-specific Lyt T-cell subsets in recovery from

Plasmodium yoelii infection in normal and T-cell-deficient mice. *Infection and Immunity*, **47**, 737.

BRISSETTE, W.H., COLEMAN, R.M. & RENCRICA, N.J. (1978) Depressed splenic T lymphocyte numbers and thymocyte migratory patterns in murine malaria. *Proceedings of the Society for Experimental Biology and Medicine*, **159**, 317.

BRODEUR, B.R., TSANG, P. & LAROSE, Y. (1984) Parameters affecting ascites tumour formation in mice and monoclonal antibody production. *Journal of Immunological Methods*, **71**, 265.

BROWN, I.N. & PHILLIPS, R.S. (1974) Immunity to *Plasmodium berghei* in rats: passive serum transfer and the role of the spleen. *Infection and Immunity*, **10**, 1213.

BROWN, I.N., ALLISON, A.C. & TAYLOR, R.B. (1968a) *Plasmodium berghei* infections in thymectomised rats. *Nature*, **219**, 292.

BROWN, K.N. & BROWN, I.N. (1965) Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature*, **208**, 1286.

BROWN, K.N. & KREIER, J.P. (1982) *Plasmodium berghei* malaria: blockage by immune complexes of macrophage receptors for opsonised plasmodia. *Infection and Immunity*, **37**, 1227.

BROWN, K.N. & PHILLIPS, R.S. (1971) The role of thymus derived lymphocytes in protective immunity to malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **65**, 7.

BROWN, K.N. (1973) Antibody induced variation in malaria. *Nature*, **242**, 49.

BROWN, K.N. (1974) Antigenic variation and immunity to malaria. In: *Parasites in the Immunised Host. Ciba Foundation Symposium*, **25**, 35. Associated Scientific Publishers, Amsterdam.

BROWN, K.N., BROWN, I.N. & HILLS, L.A. (1970a) Immunity to malaria. I. Protection against *Plasmodium* shown by monkeys sensitised with drug-suppressed infections or by dead parasites in Freund's adjuvant. *Experimental Parasitology*, **28**, 304.

BROWN, K.N., BROWN, I.N., TRIGG, P.I., PHILLIPS, R.S. & HILLS, L.A. (1970a) Immunity to malaria. II. Serological response of monkeys sensitised by drug-suppressed infection or by dead parasitised cells in Freund's complete adjuvant. *Experimental Parasitology*, **28**, 304.

BROWN, K.N. & HILLS, L.A. (1974) Antigenic variation and immunity to *Plasmodium knowlesi*: antibodies which induce antigenic variation and antibodies which destroy parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **68**, 139.

BROWN, K.N., HILLS, L.A. & JARRA, W. (1976b) Preliminary studies of artificial immunisation of rats against *Plasmodium berghei* and adoptive transfer of this immunity by splenic T and T&B cells. *Bulletin of the World Health Organisation*, **54**, 149.

BROWN, K.N., JARRA, W. & HILLS, L.A. (1976a) T-cells and protective immunity to *Plasmodium berghei* in rats. *Infection and Immunity*, **14**, 858.

BRUCE, J., SYMINGTON, F.W., McKEARN, T.J. & SPRENT, J. (1981) A monoclonal antibody discriminating between subsets of T and B cells. *Journal of Immunology*, **127**, 2496.

BRUCE-CHWATT, L.J. (1979) Man against malaria: conquest or defeat. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **73**, 605.

BURKITT, D.P. (1969) Etiology of Burkitt's lymphoma - an alternative hypothesis to a vectored virus. *Journal of the National Cancer Institute*, **42**, 19.

BUTCHER, G.A. (1992) HIV and malaria: A lesson in immunology? *Parasitology Today*, **8**, 307.

BUTCHER, G.A., MITCHELL, G.H. & COHEN, S. (1978) Antibody mediated mechanisms of immunity to malaria induced by vaccination with *Plasmodium knowlesi* merozoites. *Immunology*, **34**, 77.

BYGBERG, I.C. & FLACHS, H. (1986) Effect of chloroquine on human lymphocyte proliferation. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 231.

CANTRELL, W. & ELKO, E.E. (1966) Effect of splenectomy on phagocytic activation by *Plasmodium berghei*. *Journal of Infectios Diseases*, **116**, 429.

CANTRELL, W., ELKO, E.E. & HOPFF, B.M. (1970) *Plasmodium berghei*: phagocytic hyperactivity of infected rats. *Experimental Parasitology*, **28**, 291.

CARTER, R. & CHEN, D.H. (1976) Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature*, **263**, 57.

CARTER, R. & DIGGS, C.L. (1977) Plasmodia of rodents. In: *Parasitic Protozoa* (ed J.P. Kreier) **111**, 359. Academic Press, New York, San Francisco & London.

CARTER, R. & NIJHOUT, M.M. (1977) Control of gamete formation (exflagellation) in malaria parasites. *Science*, **195**, 407.

CARTER, R. & WALLIKER, D. (1975) New observations on the malaria parasites of rodents of the Central African Republic - *Plasmodium vinckei petteri* sub. sp. nov. and *Plasmodium chabaudi* Landau 1965. *Annals of Tropical Medicine and Parasitology*, **69**, 187.

CARTER, R., GWADZ, R.W. & GREEN, I. (1979b) *Plasmodium gallinaceum*: transmission blocking immunity in chickens. II. The effect of antigamete antibodies *in vitro* and *in vivo* and their elaboration during infection. *Experimental Parasitology*, **47**, 194.

CARTER, R., GWADZ, R.W. & McAULIFFE, F.M. (1979a) *Plasmodium gallinaceum*: transmission-blocking immunity in chickens. I. Comparative immunogenicity of gametocyte- and gamete-containing preparations. *Experimental Parasitology*, **47**, 185.

CARTER, R., KUMAR, N., QUAKYI, I., GOOD, M., MENDIS, K., GRAVES, P. & MILLER, L.H. (1988) Immunity to asexual stages of malaria parasites. *Progress in Allergy*, **41**, 193.

CAVACINI, L.A., GUIDOTTI, M., PARKE, L.A., MELANCON-KAPLAN, J. & WEIDANZ, W.P. (1989) Reassessment of the role of splenic leucocyte oxidative activity and macrophage activation in expression of immunity to malaria. *Infection and Immunity*, **57**, 3677.

CAVACINI, L.A., LONG, C.A. & WEIDANZ, W.P. (1986) T-cell immunity in murine malaria: adoptive transfer of resistance to *Plasmodium chabaudi adami* in nude mice with splenic cells. *Infection and Immunity*, **52**, 637.

CAVACINI, L.A., PARKE, L.A. & WEIDANZ, W.P. (1990) Resolution of acute malarial infections by T cell-dependent non-antibody-mediated mechanisms of immunity. *Infection and Immunity*, **58**, 2946.

CHAPMAN, W.L. & HANSON, W.L. (1971) *Plasmodium berghei* infection in neonatally thymectomised hamsters. *Journal of Parasitology*, **57**, 24.

CHAYEN, A. & PARKHOUSE, R.M.E. (1982) Preparation and properties of a cytotoxic monoclonal rat anti-mouse Thy-1 antibody. *Journal of Immunological Methods*, **49**, 17.

CHEMTAI, A.K., HAMERS-CASTLEMAN, C., HAMERS, R. & DE BAETSELIER, P. (1984a) T cell-mediated immunity in murine malaria. II. Induction of protective immunity to *Plasmodium chabaudi* by antigen fed macrophages and antigen educated lymphocytes. *Parasite Immunology*, **6**, 469.

CHEMTAI, A.K., VAECK, M., HAMERS-CASTLEMAN, C., HAMERS, R. & DE BAETSELIER, P. (1984b) T-cell mediated immunity in murine malaria. I. Induction of T-cell dependent proliferative responses to *Plasmodium chabaudi*. *Parasite Immunology*, **6**, 51.

CHEN, D.H., TIGELAAR, R.E. & WEINBAUM, F.I. (1977) Immunity to sporozoite induced malaria infection in mice. *Journal of Immunology*, **118**, 1322.

CHEN, W., REESE, V.A. & CHEEVER, M.A. (1990) Adoptively transferred antigen-specific T cells can be grown and maintained in large numbers *in vivo* for extended periods of time by intermittent restimulation with specific antigen plus Il-2. *Journal of Immunology*, **144**, 3659.

CHEUNG, A., LEBAN, J., SHAW, A.R., MERKIL, B., STOCKER, J., CHIZZOLINI, C., SANDER, C. & PERRIN, L.H. (1986) Immunisation with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies. *Proceedings of the National Academy of Science USA.*, **83**, 8328.

CLARK, I.A. & ALLISON, A.C. (1974) *Babesia microti* and *Plasmodium berghei yoelii* infections in nude mice. *Nature*, **252**, 328.

CLARK, I.A. & HUNT, N.H. (1983) Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infection and Immunity*, **39**, 1.

CLARK, I.A. (1979) Resistance to *Babesia* spp. and *Plasmodium* spp. in mice pretreated with an extract of *Coxiella burnetti*. *Infection and Immunity*, **24**, 319.

CLARK, I.A. (1987) Cell-mediated immunity in protection and pathology of malaria. *Parasitology Today*, **3**, 300.

CLARK, I.A., ALLISON, A.C. & COX, F.E.G. (1976) Protection of mice against *Babesia* and *Plasmodium* with BCG. *Nature*, **259**, 309.

CLARK, I.A., COWDEN, W.B. & BUTCHER, G.A. (1983) Free oxygen radical generators as antimalarial drugs. *Lancet*, **i**, 234.

CLARK, I.A., COWDEN, W.B., BUTCHER, G.A. & HUNT, N.H. (1987b) Possible roles of tumor necrosis factor in the pathology of malaria. *American Journal of Pathology*, **129**, 192.

CLARK, I.A., COX, F.E.G. & ALLISON, A.C. (1977) Protection of mice against *Babesia* spp. and *Plasmodium* spp. with killed *Corynebacterium parvum*. *Parasitology*, **74**, 9.

CLARK, I.A., HUNT, N.H., BUTCHER, G.A. & COWDEN, W.B. (1987a) Inhibition of murine malaria (*Plasmodium chabaudi*) *in vivo* by recombinant interferon-γ or tumour necrosis factor, and its enhancement by butylated hydroxyanisole. *Journal of Immunology*, **139**, 3493.

CLARK, I.A., ROCKETT, K.A. & COWDEN, W.B. (1991) Proposed link between cytokines, nitric oxide and human cerebral malaria. *Parasitology Today*, **7**, 205.

CLARK, I.A., ROCKETT, K.A. & COWDEN, W.B. (1992) Possible central role of nitric oxide in conditions clinically similar to cerebral malaria. *Lancet*, **340**, 894.

CLARK, I.A., VIRELIZIER, J.L., CARSWELL, E.A. & WOOD, P.R. (1981) Possible importance of macrophage derived mediators in acute malaria. *Infection and Immunity*, **32**, 1058.

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. & HORNICK, R.B. (1973) Specificity of protection of man immunised against sporozoite-induced falciparum malaria. *American Journal of Medical Science*, **266**, 398.

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. & WOODWARD, W.E. (1975) Immunisation of man against falciparum and vivax malaria by use of attenuated sporozoites. *American Journal of Tropical Medicine and Hygiene*, **24**, 397.

COBBOLD, S.P. (1983) *Monoclonal antibodies against cell surface antigens of the mouse haemopoietic system.* PhD thesis. University of Cambridge. pp.53.

COBBOLD, S.P., JAYASURIYA, A., NASH, A., PROSPERO, T.D. & WALDMANN, H. (1984) Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*. *Nature*, **312**, 548.

COBBOLD, S.P., MARTIN, G. & WALDMANN, H. (1986) Monoclonal antibodies for the prevention of graft-versus-host disease and marrow graft rejection. *Transplantation*, **42**, 239.

COBBOLD, S.P., MARTIN, G., LOVAT, P.E. & WALDMANN, H. (1985) Immunosuppression with monoclonal antibodies: rules for effective serotherapy. *Advances in Experimental and Medical Biology*, **186**, 789.

COCHRANE, A.H., SANTORO, F., NUSSENZWEIG, V., GWADZ, R.W. & NUSSENZWEIG, R.S. (1982) Monoclonal antibodies identify the protective antigens of sporozoites of *Plasmodium knowlesi*. *Proceedings of the National Academy of Science USA.*, **79**, 5651.

COGGLESHALL, L.T. & KUMM, H.W. (1937) Demonstration of passive immunity in experimental monkey malaria. *Journal of Experimental Medicine*, **16**, 177.

COHEN, S. & BUTCHER, G.A. (1969) Comments on immunisation. *Military Medicine*, **134** (Suppl), 1191.

COHEN, S. & McGREGOR, I.A. (1963) Gamma globulin and acquired immunity to malaria. In: *Immunity to Protozoa* (eds. P.C.C. Garnham, A.E. Pierce & I. Roitt), p. 123. Blackwell Scientific Publications, Oxford.

COHEN, S. (1979) Immunity to malaria. *Proceedings of the Royal Society of London B*, **203**, 323.

COHEN, S., BUTCHER, G.A., MITCHELL, G.H., DEANS, J. & LANGHORNE, J. (1977) Acquired immunity and vaccination in malaria. *American Journal of Tropical Medicine and Hygiene*, **26**, 223.

COHEN, S., McGREGOR, I.A. & CARRINGTON, S. (1961) Gamma globulin and acquired immunity to human malaria. *Nature*, **192**, 733.

COLLINS, W.E., ANDERS, R.F., PAPPAIOANOU, M., CAMPBELL, G.H., BROWN, G.V., KEMP, D.J., COPPEL, R.L., SKINNER, J.C., ANDRYSIAK, P.M., FAVALORO, J.M., CORCORAN, L.M., BRODERSON, J.R., MITCHELL, G.F. & CAMPBELL, C.C. (1986) Immunisation of *Aotus* monkeys with recombinant proteins of an erythrocyte surface antigen of *Plasmodium falciparum*. *Nature*, **323**, 259.

CONRAD, M.E. & DENNIS, L.H. (1968) Splenic function in experimental malaria. *American Journal of Tropical Medicine and Hygiene*, **17**, 170.

CONTRERAS, C.E., JUNE, C.H., PERRIN, L.H. & LAMBERT, P-H. (1980) Immunopathological aspects of *Plasmodium berghei* infection in five strains of mice. I. Immune complexes and other serological features during the infection. *Clinical and Experimental Immunology*, **42**, 403.

CORRADIN, G. (1990) Antigen processing and presentation. *Immunology Letters*, **25**, 11.

COTTRELL, B.J., PLAYFAIR, J.H.L. & DeSOUZA, B.J. (1977) *Plasmodium yoelii* and *Plasmodium vinckei*: the effects of non-specific immunostimulation on murine malaria. *Experimental Parasitology*, **43**, 45.

COTTRELL, B.J., PLAYFAIR, J.H.L. & DeSOUZA, B.J. (1978) Cell mediated immunity in mice vaccinated against malaria. *Clinical and Experimental Immunology*, **34**, 147.

COWMAN, A.F., COPPEL, R.F., SAINT, R.B., FAVALORO, J., CREWTHER, P.E., STAHL, H.D., BIANCO, A.E., BROWN, G.V., ANDERS, R.F. & KEMP, D.J. (1984) The Ring-infected Erythrocyte Surface Antigen (RESA) polypeptide of *Plasmodium falciparum* contains two separate blocks of tandem repeats encoding antigenic epitopes that are naturally immunogenic in man. *Molecular and Biochemical Medicine*, **3**, 207.

COX, H.W. (1962) The behaviour of *Plasmodium berghei* strains isolated from relapsed infections of white mice. *Journal of Protozoology*, **9**, 114.

COX, H.W. (1964) Immune response of rats and mice to trypanosome infections. *Journal of Parasitology*, **50**, 23.

COX, J. & HOMMEL, M. (1984) *Plasmodium chabaudi*: a possible model for the study of

sequestration in malaria. *Parasitology*, **89** (Suppl.), xxvii.

COX, J., SEMOFF, S. & HOMMEL, M. (1987) *Plasmodium chabaudi*: a rodent malaria model for *in vivo* and *in vitro* cytoadherence of malaria parasites in the absence of knobs. *Parasite Immunology*, **9**, 543.

CRANDALL, I. & SHERMAN, I.W. (1994) Cytoadherence-related neoantigens on *Plasmodium falciparum* (human malaria)-infected human erythrocytes result from the exposure of normally cryptic regions of the band 3 protein. *Parasitology*, **108**, 257.

CRESSWELL, P. (1990) Questions of presentation. *Nature*, **343**, 593.

CREWTHER, P.A., CULVENOR, J.G., SILVA, A., COOPER, J.A. & ANDERS, R.F. (1990) *Plasmodium falciparum*: two antigens of similar size are located in different compartments of the rhoptry. *Experimental Parasitology*, **70**, 193.

CRISWELL, B.S., TURNER, W.T., ROSSEN, R.D. & KNIGHT, V. (1971) Murine malaria: the role of humoral factors and macrophages in the destruction of parasitised erythrocytes. *Journal of Immunology*, **107**, 212.

CURFS, J.H.A.H., SCHETTERS, T.P.M., HERMSEN, C.C., JERUSALEM, C.R., VAN ZON, A.A.J.C. & ELING, W.M.C. (1989) Immunological aspects of cerebral lesions in murine malaria. *Clinical and Experimental Immunology*, **75**, 136.

CURFS, J.H.A.J., VAN DER MEIDE, P.H., BILLIAU, A., MEUWISSEN, J.H.E.Th. & ELING, W.M.C. (1993) *Plasmodium berghei*: recombinant interferon-γ and the development of parasitaemia and cerebral lesions in malaria-infected mice. *Experimental Parasitology*, **77**, 212.

DALLDORF, K.G., LINSELL, C.E. & BARNHART, F.E. (1964) An epidemiological approach to the lymphomas of African children and Burkitt's sarcoma of the jaws. *Perspectives in Biology and Medicine*, **7**, 435.

DAME, J.B., WILLIAMS, J.L., McCUTCHAN, T.F., WEBER, J.L., WIRTZ, R.A., HOCKMEYER, W.T., MALOY, W.L., HAYNES, J.D., SCHNEIDER, I., ROBERTS, D., SANDERS, G.S., REDDY, E.P., DIGGS, C.L. & MILLER, L.H. (1984) Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science*, **225**, 593.

DAVID, P.H., HOMMEL, M., MILLER, L.H., UDEINYA, I.J. & OLIGINO, L.D. (1983) Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proceedings of the National Academy of Science* USA, **80**, 5075.

DEANS, J.A. & COHEN, S. (1983) Immunology of malaria. *Annual Review of Microbiology*, **37**, 25.

DEANS, J.A., ALDERSON, T., THOMAS, A.W., MITCHELL, G.H., LENNOX, E.S. & COHEN, S. (1982) Rat monoclonal antibodies which inhibit the *in vitro* multiplication of *Plasmodium knowlesi*. *Clinical and Experimental Immunology*, **49**, 297.

DeKRUYFF, R.H., FANG, Y. & UMETSU, D.T. (1992) Il-4 synthesis by *in vivo* primed keyhole limpet haemocyanin-specific $CD4^+$ T cells. I. Influence of antigen concentration and antigen-presenting cell type. *Journal of Immunology*, **149**, 3468.

DEPIERREUX, M., HOCHMAN, A., HERRERA, S. & LAMBERT, P-H. (1987) Increased blood-brain permeability during cerebral malaria in rats. Correlation with immunological and histological features. *Immunology*, **61**, 142.

DESOWITZ, R.S & MILLER, L.H. (1980) A perspective on malaria vaccines. *Bulletin of the World Health Organisation*, **58**, 897.

DESOWITZ, R.S., MILLER, L.H., BUCHANAN, R.D. & PERENPANIK, B. (1969) The sites of deep vascular schizogony in *Plasmodium coatneyi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **38**, 289.

DIALYNAS, D.P., QUAN, Z.S., WALL, K.A., PIERRES, A., QINTANS, J., LOKEN, M.R., PIERRES, M. & FITCH, F.W. (1983) Characterisation of the murine T cell surface antigen designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *Journal of Immunology*, **131**, 2445.

DIGGS, C.L. & OSLER, A.G. (1969) Humoral immunity in rodent malaria. II. Inhibition of parasitaemia by serum antibody. *Journal of Immunology*, **102**, 298.

DIGGS, C.L., SHIN., H., BRIGGS, B.T., LAUDENSLAYER, K. & WEBER, R. (1972b) Antibody mediated immunity to *Plasmodium berghei* independent of the component of complement. *Proceedings of the Helminthological Society of Washington*, **39**, 456.

DIGGS, C.L., WELLDE, B.T., ANDERSON, S.J., WEHER, R.M. & RODRIGUEZ, E. (1972a) The protective effect of African human immunoglobulin G in *Aotus trivirgatus* infected with Asian *Plasmodium falciparum*. *Proceedings of the Helminthological Society of Washington*, **39**, 449.

DINARELLO, C.A. (1989) The endogenous pyrogens in host-defence interactions. *Hosp. Pract. Off. Ed.*, **24**, 111.

DING, A.H., NATHAN, C.F. & STUEHR, D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *Journal of Immunology*, **141**, 2407.

DOCKRELL, H. & PLAYFAIR, J.H.L. (1983) Killing of blood stage murine malaria parasites by hydrogen peroxide. *Infection and Immunity*, **39**, 456.

DOCKRELL, H.M., DeSOUZA, J.B. & PLAYFAIR, J.H.L. (1980) The role of the liver in immunity to blood-stage murine malaria. *Immunology*, **41**, 421.

DODGE, J.T., MITCHELL, C. & HANAHAN, D.J. (1963) The preparation and chemical characteristics of haemoglobin-free ghosts of human erythrocytes. *Archives of Biochemistry and Biophysics*, **100**, 119.

DOWNES,M.J., EDWARDS, M.W., ELSEY, T.S. & WALTERS, C.L. (1976) Determination of a non-volatile nitrosamine by using denitrosation and a chemiluminescence analyser. *Analyst*, **101**, 742.

DRUILHE, P. & KHUSMITH, S. (1987) Epidemiological correlation between levels of antibodies promoting merozoite phagocytosis of *Plasmodium falciparum* and malaria immune status. *Infection and Immunity*, **55**, 888.

DULANEY, A.D., PRIEST, J.B., ALMEDA, M.L. & PARKER, B. (1948) The complement content of human sera with special reference to malaria. *Journal of Clinical Investigation*, **27**, 320.

DUSTIN, M.L., ROTHLEIN, R., BHAN, A.K., DINARELLO, C.A. & SPRINGER, T.A. (1986) Induction by Il-1 and interferon, tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). *Journal of Immunology*, **137**, 245.

DVORAK, J.A., MILLER, L.H., WHITEHOUSE, W.C. & SHIROISHI, T. (1975) Invasion of erythrocytes by malaria merozoites. *Science*, **187**, 748.

EICHINGER, D.J., ARNOT, D.E., TAN, J.P., NUSSENZWEIG, V & ENEA, V. (1986) Circumsporozoite protein of *Plasmodium berghei*: gene cloning and identification of the immunodominant epitopes. *Molecular and Cellular Biology*, **6**, 3965.

EL NAHEL, H.M.S. & BRAY, R.S. (1963) The use of Evans Blue in the fluorescent staining of malaria parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **60**, 564.

ELING, W.M.C. (1979) *Plasmodium berghei*: effect of anti-thymocyte serum on induction of immunity in the mouse. *Experimental Parasitology*, **47**, 403.

ELLIS, J., OZAKI, L.S., GWADZ, R.W., COCHRNE, A.H., NUSSENZWEIG, V., NUSSENZWEIG,

R.S. & GODSON, G.N. (1983) Cloning and expression in *E. coli* of the malarial sporozoite surface antigen gene from *Plasmodium knowlesi*. *Nature*, **302**, 536.

ENEA, V., ELLIS, J., ZAVALA, F., ARNOT, D.E., QUAKYI, I. & NUSSENZWEIG, R.S. (1984) DNA cloning of *Plasmodium falciparum* circumsporozoit gene: amino acid sequence of repetitive epitope. *Science*, **225**, 628.

EPSTEIN, N., MILLER, L.H., KAUSHAL, D.C., UDEINYA, I.J., RENER, J., HOWARD, R.J., ASOFSKY, R., AIKAWA, M. & HESS, R.L. (1981) Monoclonal antibodies against a specific surface determinant on malarial (*Plasmodium knowlesi*) merozoites block erythrocyte invasion. *Journal of Immunology*, **127**, 212.

EUGUI, E.M. & ALLISON, A.C. (1980) Differences in susceptibility of various mouse strains to haemosporozoan infections: possible correlation with natural killer activity. *Parasite Immunology*, **2**, 277.

FAHEY, J.R. & SPITALNY, C.L. (1986) Immunity to *Plasmodium yoelii*: kinetics of the generation of T and B lymphocytes that passively transfer protective immunity against virulent challenge. *Cellular Immunology*, **98**, 486.

FAIRLEY, N.H. (1947) Sidelights on malaria obtained by subinoculation experiments. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **40**, 621.

FALANGA, P.B., D'IMPERIO LIMA, M.R., COUTINHO, A. & PEREIRA Da SILVA, L. (1987) Isotypic pattern of the polyclonal B cell response during primary infection by *Plasmodium chabaudi* and in immune-protected mice. *European Journal of Immunology*, **17**, 599.

FATHMAN, C.G. & KIMOTO, M. (1981) Studies utilising murine T cell clones: Ir genes, Ia antigens and MLR stimulating determinants. *Immunology Reviews*, **54**, 57.

FATHMAN, G. & FITCH, F.W. (1982) *Isolation, Characterisation and Utilisation of T lymphocyte Clones*. Academic Press, New York.

FAVALORO, J.M., COPPEL, R.L., CORCORAN, C.M., FOOTE, S.J., BROWN, G.V., ANDERS, R.F. & KEMP, D.J. (1986) Structure of the RESA gene of *Plasmodium falciparum*. *Nucleic Acid Research*, **14**, 8265.

FAVILA-CASTILLO, L., JARRA, W. & BROWN, K.N. (1990) The adoptive transfer of T-cell dependent immunity to *Plasmodium chabaudi chabaudi* in CBA/Ca mice is achieved only after superinfection of immune spleen cell donors. *Parasite Immunology*, **12**, 297.

FERNANDEZ-BOTRAN, R., SANDERS, V.M., MOSMANN, T.R. & VITETTA, E.S. (1988) Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *Journal of Experimental Medicine*, **168**, 543.

FERRARONI, J.J. & SPEER, C.A. (1982) Adoptive transfer of resistance to *Plasmodium berghei* with spleen cells and serum from fansidar-cured mice. *Infection and Immunity*, **36**, 1109.

FERREIRA, A., SCHOFIELD, L., ENEA, V., SCHELLEKENS, H., VAN DER MEIDE, P., COLLINS, W., NUSSENZWEIG, R. & NUSSENZWEIG, V. (1986) Inhbition of development of exoerythrocytic forms of malaria by gamma-interferon. *Science*, **161**, 802.

FINLEY, R.W., MACKAY, L.A. & LAMBERT, P-H. (1982) Virulent *Plasmodium berghei* malaria: prolonged survival and decreased cerebral pathology in T-cell deficient nude mice. *Journal of Immunology*, **129**, 2213.

FLEMING, A.F. (1990) Opportunistic infections in AIDS in developed and developing countries. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84** (Suppl. 1), 1.

FOLEY, M., TILLEY, L., SAWYER, W.H. & ANDERS, R.F. (1993) The ring-infected erythrocyte surface antigen of *Plasmodium falciparum* associates with spectrin in the erytrocyte membrane. *Molecular and Biochemical Parasitology*,

FRANZ, D.R., LEE, M., SENG, L.T., YOUNG, G.D., BAZE, W.B. & LEWIS, G.E. Jr (1987) Peripheral vascular pathophysiology of *Plasmodium berghei* infection: comparative study in the cheek pouch and brain of the golden hamster. *American Journal of Tropical Medicine and Hygiene*, **36**, 474.

FREEMAN, R.R., TREJDOSIEWICZ, A.J. & CROSS, G.A.M. (1980) Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite. *Nature*, **284**, 366.

FREMOUNT, H.N. & MILLER, L.H. (1975) Deep vascular schizogony in *Plasmodium fragile*: organ distribution and ultrastructure of erythrocytes adherent to vascular endothelium. *American Journal of Tropical Medicine and Hygiene*, **24**, 1.

GAJEWSKI, T.F., PINNAS, M., WONG, T. & FITCH, F.W. (1991) Murine T_H1 and T_H2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *Journal of Immunology*, **146**, 1750.

GAJEWSKI, T.F., SCHELL, S.R., NAU, G., FITCH, F.W. (1989) Regulation of T-cell activation: differences among T-cell subsets. *Immunology Reviews*, **111**, 79.

GALFRE, G., MILSTEIN, C. & WRIGHT, B. (1979) Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse Ig. *Nature*, **277**, 131.

GALLATIN, M., ST. JOHN, T.P., SIEGLEMAN, M., REICHERT, R., BUTCHER, E.C. & WEISSMAN, I.L. (1986) Lymphocyte homing receptors. *Cell*, **44**, 673.

GARNHAM, P.C.C. & BRAY, R.S. (1956) The influence of immunity upon the stages (including exoerythrocytic schizonts) of mammalian malaria parasites. *Revista Brasiliera de Malariologia e doencas tropicais*, **8**, 151.

GARNHAM, P.C.C. (1966) *Malaria Parasites and other Haemosporidia*. Blackwell Scientific Publications, Oxford.

GEARY, T.G. & JENSON, J.B. (1983) Lack of cross-resistance to 4-aminoquinolines in chloroquine-resistant *Plasmodium falciparum in vitro*. *Journal of Parasitology*, **69**, 97.

GEIGER, B. & GALLILY, R. (1974) Effect of X-irradiation on various functions of murine macrophages. *Clinical and Experimental Immunology*, **16**, 643.

GERY, I. & EIDINGER, D. (1977) Selective and opposing effects of cytochalasin B and other drugs on lymphocyte responses to different doses of mitogen. *Cellular Immunology*, **30**, 147.

GILKS, C.F. (1988) *The surface of Plasmodium chabaudi infected erythrocytes*. D Phil Thesis, University of Oxford, pp.76.

GILKS, C.F., JARRA, W., HARVEY-WOOD, K., McLEAN, S.A. & SCHETTERS, T. (1989) Host diet in experimental rodent malaria: a variable which can compromise experimental design and interpretation. *Parasitology*, **98**, 175.

GILKS, C.F., WALLIKER, D. & NEWBOLD, C.I. (1990) Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi* - a rodent malaria model. *Parasite Immunology*, **12**, 45.

GILLIS, S. & SMITH, K.A. (1977) Long term culture of tumour-specific cytotoxic T cells. *Nature*, **268**, 154.

GINSBURG, H., HANDELI, S., FRIEDMAN, S., GORODETSKY, R. & KRUGLIAK, M. (1986) Effects of red blood cell potassium and hypertonicity on th growth of *Plasmodium falciparum* in culture. *Z. Parasitenkd*, **72**, 185.

GOERLICH, R., HACKER, G., PFEFFER, K., HEEG, K. & WAGNER, H. (1991) *Plasmodium falciparum* merozoites promarily stimulate the V gamma 9 subset of human gamma-delta T-cells. *Immunology*, **21**, 2613.

GOLENZER, J., SPIRA, D.T. & ZUCKERMAN, A. (1975) Neutralising antibody in rodent malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **69**, 251.

GOOD, M.F., QUAKYI, I.A., SAUL, A., BERZOFSKY, J.A., CARTER, R. & MILLER, L.H. (1987) Human T clones reactive to the sexual stages of *Plasmodium falciparum* malaria: high frequency of gamete-reactive T cells in peripheral blood from non exposed donors. *Journal of Immunology*, **138**, 306.

GOODACRE, J.A., SIMPSON, J.M., JOHNSTONE, R. & ROBINSON, J.H. (1987) A limiting diltion assay to determine the frequency of antigen presenting cells. *Journal of Immunological Methods*, **103**, 103.

GOODIER, M., FEY, P., EICHMANN, K. & LANGHORNE, J. (1992) Human peripheral blood gamma-dalta T-cells respond to antigens of *Plasmodium falciparum*. *International Immunology*, **4**, 33.

GORONZY, J., WEYAND, C.M. & FATHMAN, C.G. (1986) Long-term humoral unresponsiveness *in vivo* induced by treatment with monoclonal antibody against L3T4. *Journal of Experimental Medicine*, **164**, 911.

GRANGER, D.L., HIBBS, J.B., PERFECT, J.R. & DURACK, D.T. (1988) Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. *Journal of Clinical Investigation*, **81**, 1129.

GRANGER, D.L., HIBBS, J.B., PERFECT, J.R. & DURACK, D.T. (1990) Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *Journal of Clinical Investigation*, **85**, 264.

GRAU, G.E., FAJARDO, L.F., PIGUET, P.F., ALLET, B., LAMBERT, P-H. & VASSALLI, P. (1987) Tumor necrosis factor (cachectin) as an essential mediator in murine malaria. *Science*, **237**, 1210.

GRAU, G.E., FREI, K., PIGUET, P.F., FONTANA, A., HEREMANS, H., BILLIAU, A., VASSALLI, P. & LAMBERT, P-H. (1990) Interleukin-6 production in experimental cerebral malaria: modulation by anticytokine antibodies and possible role in hypergammaglobulinaemia. *Journal of Experimental Medicine*, **172**, 1505.

GRAU, G.E., HEREMANS, H., PIQUET, P-F., POINTAIRE, P., LAMBERT, P-H., BILLIAU, A. & VASSALLI, P. (1989a) Monoclonal antibody against interferon-γ can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. *Proceedings of the National Academt of Science* USA, **86**, 5572.

GRAU, G.E., PIGUET, P-F, VASSALLI, P. & LAMBERT, P-H. (1989b) Involvement of tumour necrosis factor and other cytokines in immune-mediated vascular pathology. *International Archives of Allergy and Applied Immunology*, **88**, 34.

GRAU, G.E., PIGUET, P.F., ENGERS, H.D., LOUIS, J.A., VASSALLI, P. & LAMBERT, P-H. (1986) L3T4$^+$ lymphocytes play a major role in the pathogenesis of murine cerebral malaria. *Journal of Immunology*, **137**, 2348.

GRAU, G.E., TAYLOR, T.E., MOLYNEUX, M.E., WIRIMA, J.J., VASSALLI, P., HOMMEL, M. & LAMBERT, P-H. (1989c) Tumor necrosis factor and disease severity in children with falciparum malaria. *New England Journal of Medicine*, **320**, 1586.

GRAVELY, S.M. & KREIER, J.P. (1976) Adoptive transfer of immunity to *Plasmodium berghei* with immune T and B lymphocytes. *Infection and Immunity*, **14**, 184.

GRAVES, P.M., CARTER, R., BURKOT, T.R., QUAKYI, I.A. & KUMAR, N. (1988) Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera. *Parasite Immunology*, **10**, 209.

GRAY, C.D. & PHILLIPS, R.S. (1981) Use of sorbitol in the cryopreservation of *Babesia*. *Research*

in Veterinary Science, **30**, 388.

GREEN, S.J., MELLOUK, S., HOFFMAN, S.L., MELTZER, M.S. & NACY, C.A. (1990a) Cellular mechanisms of nonspecific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes. *Immunology Letters*, **25**, 15.

GREEN, S.J., MELTZER, M.S., HIBBS, J.B. & NACY, C.A. (1990b) Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *Journal of Immunology*, **144**, 278.

GREEN, T.J. & KREIER, J.P. (1978) Demonstration of the role of cytophilic antibody in resistance to malaia parasites (*Plasmodium berghei*) in rats. *Infection and Immunity*, **19**, 138.

GREENBAUM, L.A., HOROWITZ, J.B., WOODS, A., PASQUALINI, P., REICH, E.P. & BOTTOMLEY, K. (1988) Autocrine growth of $CD4^+$ T cells. Differential effects of Il-1 on helper and inflammatory T cells. *Journal of Immunology*, **140**, 1555.

GREENBURG, A.E., NGUYEN-DINH, P., MANN, J.M., KABOTE, N., COLEBUNDERS, R.L., FRANCIS, H., QUINN, T.C., BAUDOUX, P., LYAMBA, B., DAVACHI, F., ROBERTS, J.M., KABEYA, N., CURRAN, J.W. & CAMPBELL, C.C. (1988) The association between malaria blood transfusion, and HIV seropositivity in paediatric population in Khinshasa, Zaire. *Journal of the American Medical Association*, **259**, 545.

GREENWOOD, B.M. & BRUETON, M.J. (1974) Complement activation in children with acute malaria. *Clinical and Experimental Immunology*, **18**, 267.

GREENWOOD, B.M. (1974) Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis. *Lancet*, **i**, 435.

GREENWOOD, B.M. (1979) The tropical splenomegaly syndrome. In: *Role of the Spleen in the Immunology of Parasitic Diseases*. Tropical Diseases Research Series 1, p.229. Schwabe & Co., basel.

GREENWOOD, B.M., BRADLEY-MORSE, A.M., PALIT, A. & BRYCESON, A.D.M. (1972) Immunosuppression in children with malaria. *Lancet*, **i**, 169.

GREENWOOD, B.M., HERRICK, E.M. & HOLBOROW, E.J. (1970) Speckled anti-neclear factor in African sera. *Clinical and Experimental Immunology*, **7**, 75.

GREENWOOD, B.M., MULLER, A.S. & VALKENBURG, H.A. (1971) Rheumatoid factor in Nigerian sera. *Clinical and Experimental Immunology*, **8**, 155.

GREENWOOD, B.M.. (1984) Immunosuppression in malaria and trypanosomiasis. In: *Parasites in the Immunised Host: Mechanisms of Survival. Ciba Foundation Symposium*, **25**, 137.

GREINER, D.L., RAJAN, T.V. & SCHULTZ, L.D. (1992) Animal models for immunodeficiency diseases. *Immunology Today*, **13**, 116.

GROSS, A. & FRANKENBURG, S. (1989) *Plasmodium berghei*: immunosuppression of the cell-mediated immune response induced by non variable antigenic preparations. *Experimental Parasitology*, **68**, 83.

GROSS, A., FRANKENBURG, S. & LONDNER, M.V. (1984) Cell-mediated immunity in rats with an antimalaria T cell line. *Cellular Immunology*, **84**, 14.

GROTENDORST, C.A., KUMAR, N., CARTER, R. & KAUSAL, D.C. (1984) A surface protein expressed during the transformation of zygotes of *Plasmodium gallinaceum* is a target of transmission-blocking antibodies. *Infection and Immunity*, **45**, 775.

GRUN, J.L. & WEIDANZ, W.P. (1981) Immunity to *Plasmodium chabaudi adami* in the B-cell deficient mouse. *Nature*, **290**, 143.

GRUN, J.L. & WEIDANZ, W.P. (1983) Antibody-independent immunity to reinfection malaria in B-cell deficient mice. *Infection and Immunity*, **41**, 1197.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986) Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. *Nature*, **320**, 454.

GUTSTEIN, N.L., SEAMAN, W.E., SCOTT, J.H. & WOFSY, D. (1986) Induction of tolerance by administration of monoclonal antibody to L3T4. *Journal of Immunology*, **138**, 117.

GWADZ, R.W. & GREEN, I. (1978) Malaria immunization in rhesus monkeys: a vaccine effective against both the sexual and asexual stages of *Plasmodium knowlesi*. *Journal of Experimental Medicine*, **148**, 1311.

HALDANE, J.B.S. (1949) Disease and evolution. *La Ricercia Scientific*, **19** (Suppl.), 68.

HAMBURGER, J. & KREIER, J.P. (1976) Antibody mediated elimination of malarial parasites (*Plasmodium berghei*) *in vitro*. *Infection and Immunity*, **12**, 339.

HANDUNETTI, S.M., MENDIS, K.N. & DAVID, P.D. (1987) Antigenic variation of cloned *Plasmodium fragile* in its host *Macaca sinica*: sequential appearance of successive variant antigenic types. *Journal of Experimental Medicine*, **165**, 1269.

HANDUNNETTI, S.M., DAVID, P.H., PERERA, K.L.R.L. & MENDIS, K.N. (1989) Uninfected erythrocytes form rosettes around *Plasmodium falciparum* infected erythrocytes. *American Journal of Tropical Medicine and Hygiene*, **40**, 115.

HANDWERGER, B.S. & SCHWARTZ, R.H. (1974) Separation of murine lymphoid cells using nylon wool columns. Recovery of the B cell-enriched population. *Transplantation*, **18**, 544.

HARRISON, G. (1978) *Mosquitoes, Malaria and Man: A History of the Hostilities since 1880*. John Murray, London.

HARTE, P.G., ROGERS, N.C. & TARGETT, G.A.T. (1985a) Role of T cells in preventing transmission of rodent malaria. *Immunology*, **56**, 1.

HARTE, P.G., ROGERS, N.C. & TARGETT, G.A.T. (1985b) Monoclonal anti-gamete antibodies prevent transmission of murine malaria. *Parasite Immunology*, **7**, 607.

HARTE, P.G., ROGERS, N.C. & TARGETT, G.A.T. (1985c) Vaccination with purified microgamete antigens prevents transmission of rodent malaria. *Nature*, **316**, 258.

HAWKING, F. (1954) Milk, PABA and malaria in rats and monkeys. *British Medical Journal*, **i**, 425.

HAWKING, F., WORMS, M.J. & GAMMAGE, K. (1968) 24- and 48-hour cycles of malaria parasites in the blood: their purpose and production. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **62**, 731.

HAYNES, J.D., DIGGS, C.L., HINES, F.A. & DESJARDINS, R.E. (1976) Culture of human malaria parasites *Plasmodium falciparum*. *Nature*, **263**, 767.

HELMBY, H., CAVELIER, L., PETTERSON, U. & WAHLGREN, M. (1993) Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. *Infection and Immunity*, **61**, 284.

HERD, N. & JORDAN, T. (1981) An investigation of malaria during pregnancy in Zimbabwe. *Central African Journal of Medicine*, **27**, 62.

HERRINGTON, D.A., CLYDE, D.F., LOSONSKY, G., CORTESIA, M., MURPHY, J.R., DAVIS, J., BAQAR, S., FELIX, A.M., HEIMER, E.P., GILLESCUE, D., NARDIN, E., NUSSENZWEIG, R.S., NUSSENZWEIG, V., HOLLINGDALE, M.R. & LEVINE, M.M. (1987) Safety and immunogenicity in man of a synthetic peptide and malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature*, **328**, 257.

HIBBS, J.B., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochemical and Biophysical Research Communications*, **157**, 87.

HILL, A.V.S, ALLSOPP, C., KWIATKOWSKI, D., ANSTEY, N., TWUMASI, P., ROWE, P., BENNETT, S., BREWSTER, D., McMICHAEL, A. & GREENWOOD, B. (1991) Common West African HLA antigens are associated with protection from severe malaria. *Nature*, **352**, 595.

HILL, A.V.S., ELVIN, J., WILLIS, A., AIDOO, M., ALLSOPP, C., GOTCH, F., GAO, X., TAKIGUCHI, M., GREENWOOD, B.M., TOWNSEND, A., McMICHAEL, A. & WHITTLE, H. (1992) Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature*, **360**, 434.

HO, M. & WEBSTER, H.K. (1990) Immunology of human malaria. A cellular perspective. *Parasite Immunology*, **11**, 105.

HO, M., WEBSTER, H.K., LOOAREESUWAN, S., SUPANARANAND, W., PHILLIPS, R.E., CHANTHAVANICH, P. & WARRELL, D.A. (1986) Antigen-specific immunosuppression in human malaria due to *Plasmodium falciparum*. *Journal of Infectious Diseases*, **153**, 763.

HO, M., WEBSTER, H.K., TONGTAWE, P., PATTANAPANYASAT, K. & WEIDANZ, W.P. (1990) Increased γδ T cells in acute *Plasmodium falciparum* malaria. *Immunology Letters*, **25**, 139.

HOLDER, A.A. & FREEMAN, R.R. (1984) The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. *Journal of Experimental Medicine*, **160**, 624.

HOLDER, A.A. (1988) The precursor to major merozoite surface antigens: structure and role in immunity. *Progress in Allergy*, **41**, 72.

HOLLINGDALE, M.R., ZAVALA, F., NUSSENZWEIG, R.S. & NUSSENZWEIG, V. (1982) Antibodies to the protective antigen of *Plasmodium berghei* sporozoites prevent entry into cultured cells. *Journal of Immunology*, **128**, 1929.

HOMMEL, M. & SEMOFF, S. (1988) Expression and function of erythrocyte-associated surface antigens in malaria. *Biology of the Cell*, **64**, 183.

HOMMEL, M. (1985) The role of variant antigens in acquired immunity to *Plasmodium falciparum*. *Ann. Soc. Belge Méd. Trop.*, **65** (Suppl.2), 57.

HOMMEL, M., DAVID, P.H. & OLIGINO, L.D. (1983) Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. *Journal of Experimental Medicine*, **157**, 1137.

HOMMEL, M., HUGHES, M., BOND, P. & CRAMPTON, J.M. (1991) Antibodies and DNA probes used to analyse variant populations of the Indochina-1 strain of *Plasmodium falciparum*. *Infection and Immunity*, **59**, 3975.

HOOGENRAAD, N., HELMAN, T. & HOOGENRAAD, J. (1983) The effect of pre-injection of mice with pristane on ascites tumour formation and monoclonal antibody production. *Journal of Immunological Methods*, **61**, 317.

HOWARD, C.J., SOPP, P., PARSONS, K.R. & FINCH, J. (1989) *In vivo* depletion of BoT4 (CD4) and of non-T4/T8 lymphocyte subsets in cattle with monoclonal antibodies. *European Journal of Immunology*, **19**, 757.

HOWARD, R.J. & GILLADOGA, A.D. (1989) Molecular studies related to the pathogenesis of cerebral malaria. *Blood*, **74**, 2603.

HOWARD, R.J. (1984) Antigenic variation of blood stage malaria parasites. *Philosophical Transactions of the Royal Society of London* B, **307**, 141.

HOWARD, R.J. (1988) Malarial proteins at the membrane of *Plasmodium falciparum*-infected

erythrocytes and their involvement in cytoadherence to endothelial cells. *Progress in Allergy*, **41**, 98.

HOWARD, R.J., BARNWELL, J.W. & KAO, V. (1983) Antigenic variation in *Plasmodium knowlesi* malaria: identification of the variant antigen of infected erythrocytes. *Proceedings of the National Academy of Science* USA, **80**, 4129.

HOWARD, R.J., UNI, S., AIKAWA, M., ALEY, S.B., LEECH, J.H., LEW, A.M., WELLEMS, T.E., RENER, J. & TAYLOR, D.W. (1986) Secretion of a malarial histidine-rich protein (PfHRPII) from *Plasmodium falciparum*-infected erythrocytes. *Journal of Cellular Biology*, **103**, 1269.

HSIEH, C-S., HEIMBERGER, A.B., GOLD, J.S., O'GARRA, A. & MURPHY, K.M. (1992) Differential regulation of T helper phenotype development by interleukins 4 and 10 in an α/β T-cell-receptor transgenic system. *Proceedings of the National Academy of Science* USA, **89**, 6065.

HUNTER, K.W., FINKELMAN, F.D., STRICKLAND, G.T., SAYLES, P.C. & SCHER, I. (1979) Defective resistance to *Plasmodium yoelii* in CBA/N mice. *Journal of Immunology*, **123**, 133.

HURVITZ, D. & HIRSCHHORN, K. (1965) Suppression of *in vitro* lymphocyte responses by chloroquine. *New England Journal of Medicine*, **273**, 23.

IYENGAR, R., STUEHR, D.J. & MARLETTA, M.A. (1987) Macrophage synthesis of nitrite, nitrate and *N*-nitrosamines: precursors and role of the respiratory burst. *Proceedings of the National Academy of Science* USA, **84**, 6369.

JAHIEL, R.I., NUSSENZWEIG, R.S., VANDERBURG, J. & VILCEK, J. (1968a) Anti-malarial effect of interferon inducers at different stages of development of *Plasmodium berghei* in the mouse. *Nature*, **220**, 710.

JAHIEL, R.I., VILCEK, J. & NUSSENZWEIG, R.S. (1970) Exogenous interferon protects mice against *Plasmodium berghei* malaria. *Nature*, **227**, 1350.

JAHIEL, R.I., VILCEK, J., NUSSENZWEIG, R.S. & VANDERBURG, J. (1968b) Interferon inducers protect mice against *Plasmodium berghei* malaria. *Science*, **161**, 802.

JAMES, S.L. & GLAVEN, J. (1989) Macrophage cytotoxicity against *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *Journal of Immunology*, **143**, 4208.

JAMES, S.L. & HIBBS, J.B. Jr (1990) The role of nitrogen oxides as effector molecules of parasite killing. *Parasitology Today*, **6**, 303.

JARRA, W. & BROWN, K.N. (1985) Protective immunity to malaria: Studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. I. The effectiveness and inter- and intra-species specificity of immunity induced by infection. *Parasite Immunology*, **7**, 595.

JARRA, W. & BROWN, K.N. (1989) Invasion of mature and immature erythrocytes of CBA/Ca mice by a cloned line of *Plasmodium chabaudi chabaudi*. *Parasitology*, **99**, 157.

JARRA, W., HILLS, L.A., MARCH, J.C. & BROWN, K.N. (1986) Protective Immunity to malaria: Studies with cloned lines of *Plasmodium chabaudi chabaudi* and *P. berghei* in CBA/Ca mice. II. The effectiveness and inter- or intra-species specificity of the passive transfer of immunity with serum. *Parasite Immunolgy*, **8**, 239.

JAYAWARDENA, A.N. (1981) Immune responses in malaria. In: *Parasitic Diseases* Vol. 1 (ed. J.M. Mansfield), p.85. Marcel Dekker, New York & Basel.

JAYAWARDENA, A.N., JANEWAY, C.A. & KEMP, J.D. (1979) Experimental malaria in the CBA/N mouse. *Journal of Immunology*, **123**, 2532.

JAYAWARDENA, A.N., MOGIL, R, MURPHY, D.B., BURGER, D. & GERSHON, R.K. (1983) Enhanced expression of H-2K and H-2D antigens on reticulocytes infected with *Plasmodium yoelii*. *Nature*, **302**, 623.

JAYAWARDENA, A.N., MURPHY, D.B., JANEWAY, C.A. & GERSHON, R.K. (1982) T cell mediated immunity in malaria. The Ly phenotype of T cells mediating resistance to *Plasmodium yoelii*. *Journal of Immunology*, **129**, 377.

JAYAWARDENA, A.N., TARGETT, G.A.T., CARTER, R.,LEUCHARS, E. & DAVIES, A.J.S. (1977) The immunological response of CBA mice to *Plasmodium yoelii*. I. General characteristics, the effect of T-cell deprivation and reconstitution with thymus grafts. *Immunology*, **32**, 849.

JAYAWARDENA, A.N., TARGETT, G.A.T., LEUCHARS, E. & DAVIES, A.J.S. (1978) The immunological response of CBA mice to *Plasmodium yoelii*. II. The passive transfer of immunity with serum and cells. *Immunology*, **34**, 157.

JAYAWARDENA, A.N., TARGETT, G.A.T., LEUCHARS, E., CARTER, R.L., DOENHOFF, M.J. & DAVIES, A.J.S. (1975) T-cell activation in murine malaria. *Nature*, **258**, 149.

JEFFREY, G.M. (1966) Epidemiological significance of repeated infections with homologous and heterologous strains and species of *Plasmodium*. *Bulletin of the World Health Organisation*, **35**, 873.

JENSEN, J.B. & TRAGER, W (1977) *Plasmodium falciparum* in culture: use of outdated erythrocytes and description of the candle jar method. *Journal of Parasitology*, **63**, 883.

JENSEN, J.B., BOLAND, M.T. & AKOOD, M. (1982) Induction of crisis forms in cultured *Plasmodium falciparum* in human serum from Sudan. *Science*, **216**, 1230.

JENSEN, J.B., BOLAND, M.T., ALLAN, J.S., CARLIN, J.M., WAA, J.A.V., DIVO, A.A. & AKOOD, M.A.S. (1983) Association between human semi-induced crisis forms in cultured *Plasmodium falciparum* and clinical immunity to malaria in Sudan. *Infection and Immunity*, **41**, 1302.

JERNE, N.K., HENRY, C., NORDIN, A.A., FUJI, H., KOROS, A.M.C. & LEI-KAVITS, J. (1974) Plaque forming cells: methodology and theory. *Transplantation Reviews*, **18**, 130.

JULIUS, M.H., SIMPSON, E. & HERZENBERG, L.A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. *European Journal of Immunology*, **3**, 645.

KABILAN, L., TROYE-BLOMBERG, M., PATARROYO, M.E., BJORKMAN, A. & PERLMANN, P. (1987) Regulation of the immune response in *Plasmodium falciparum* malaria. IV. T cell dependent production of immunoglobulin and anti-*P. falciparum* antibodies *in vitro*. *Clinical and Experimental Immunology*, **68**, 288.

KAPPLER, J.W., SKIDMORE, B., WHITE, J. & MARRACK, P. (1980) Antigen-inducible H-2-restricted, interleukin-2-producing T cell hybridomas: lack of independent antigen and H-2 recognition. *Journal of Experimental Medicine*, **153**, 1198.

KASLOW, D.C., BATHURST, I.C. & BARR, P.J. (1992) Malaria transmission-blocking vaccines. *Trends in Biotechnology*, **10**, 388.

KASLOW, D.C., ISAACS, S.N., QUAKYI, I.A., GWADZ, R.W., MOSS, B. & KEISTER, D.B. (1991) Introduction of *Plasmodium falciparum* transmission-blocking antibodies by recombinant vaccinia. *Science*, **252**, 1310.

KASLOW, D.C., QUAKYI, I.A., CHIANG, S., RAUM, M.G., KEISTER, D.B., COLIGAN, J.E., McCUTCHAN, T.F. & MILLER, L.H. (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. *Nature*, **333**, 74.

KASPER, L.H. & ALGER, N.E. (1973) Adoptive transfer of immunity to *Plasmodium berghei* by spleen and lymph node cells from young and old mice. *Journal of Protozoology*, **20**, 445.

KAYE, J., GILLIS, S., MIZEL, S.B., SHEVACH, E.M., MALEK, T.R., DINARELLO, C.A., LACHMAN, L.B. & JANEWAY, C.A. (1984) Growth of a cloned helper T cell line induced by

a monoclonal antibody specific for the antigen receptor: interleukin 1 is required for the expression of receptors of interleukin 2. *Journal of Immunology*, **133**, 1339.

KERN, P., HEMMER, C.J., VAN DAMME, J., GRUSS, H-J. & DIETRICH M. (1989) Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. *American Journal of Tropical Medicine and Hygiene*, **87**, 139.

KHANSARI, N., SEGRE, M. & SEGRE, D. (1981) Immunosuppression in murine malaria: a soluble immunosuppressive factor derived from *Plasmodium berghei* infected blood. *Journal of Immunology*, **127**, 1889.

KHARAZMI, A., JEPSEN, S. & VALERIUS, N.H. (1984) Polymorphonuclear leucocytes defective in oxidative metabolism inhibit *in vitro* growth of *Plasmodium falciparum*. Evidence against an oxygen dependent mechanism. *Scandanavian Journal of Immunology*, **20**, 93.

KHUSMITH, S. & DRUILHE, P. (1983) Antibody dependent ingestion of *Plasmodium falciparum* merozoites by human blood monocytes. *Parasite Immunology*, **5**, 357.

KHUSMITH, S., DRUILHE, P. & GENTILINI, M. (1982) Enhanced *Plasmodium falciparum* merozoite phagocytosis by monocytes from immune individuals. *Infection and Immunity*, **35**, 874.

KIMOTO, M. & FATHMAN, C.G. (1980) Antigen-reactive T cell clones I. Transcomplementing hybrid I-A-region gene products function effectively in antigen presentation. *Journal of Experimental Medicine*, **152**, 759.

KING, D.P. & JONES, P.P. (1983) Induction of Ia and H-2 antigens on a macrophage cell line by immune interferon. *Journal of Immunology*, **131**, 315.

KITCHEN, S.F. & DILUZIO, N.R. (1971) Influence of *Plasmodium berghei* infections on phagocytic and humoral recognition factor activity. *Journal of the Reticuloendothelial Society*, **9**, 237.

KITCHEN, S.F. (1949) Symptomatology; *falciparum* malaria; and *vivax* malaria. In: *Malariology* Vol II (ed. M.F. Boyd), p.66. W.B. Saunders, London, Philadelphia & Toronto.

KNELL, A.J. (1991) *Malaria*, Oxford University Press, Oxford.

KNOWLES, R.G. & MONCADA, S. (1992) Nitric oxide as a signal in blood vessels. *Trends in Biochemical Sciences*, **17**, 399.

KOLLER, B.H., MARRACK, P., KAPPLER, J.W. & SMITHIES, O. (1990) Normal development of mice deficient in β_2M, MHC class I proteins and $CD8^+$ T cells. *Science*, **248**, 1227.

KOPF, M., LE GROS, G., BACHMANN, M., LAMERS, M.C., BLUETHMANN, H. & KOHLER, G. (1993) Disruption of the murine Il-4 gene blocks T_H2 cytokine responses. *Nature*, **362**, 245.

KREMSNER, P.G., NEIFER, S., CHAVES, M.F., RUDOLPH, R. & BIENZLE, U. (1992) IFN-γ induced lethality in the late phase of *Plasmodium vinckei* malaria despite effective parasite clearance by chloroquine. *European Journal of Immunology*, **22**, 2873.

KREMSNER, P.G., NEIFER, S., SCHERMUCK, S., FERREIRA CHAVES, M., SLIWA, K. & BIENZLE, U. (1991) Interferon-γ enhances the effect of antimalarial chemotherapy in murine *Plasmodium vinckei* malaria. *Journal of Infectious Diseases*, **163**, 1161.

KREMSNER, P.G., NUSSLER, A., NEIFER, S., CHAVES, M.F., BIENZLE, U., SENALDI, G. & GRAU, G.E. (1993) Malaria antigen and cytokine-induced production of reactive nitrogen intermediates by murine macrophages: no relevance to the development of experimental cerebral malaria. *Immunology*, **78**, 286.

KROGSTAD, D.J. & SCHLESSINGER, P.H. (1987) The basis of anti-malarial action: non weak base effects of chloroquine on acid vesicle pH. *American Journal of Tropical Medicine and Hygiene*, **36**, 213.

KROTOSKI, W.A., COLLINS, W.E., BRAY, R.S., GARNHAM, P.C.C., COGSWELL, F.B., GWADZ, R.W., KILLICK-KENDRICK, R., WOLF, R., SINDEN, R.S., KOONTZ, L.C. & STANFILL, P.S. (1982a) Demonstration of hypnozoites in sporozoite transmitted *Plasmodium vivax* infection. *American Journal of Tropical Medicine and Hygiene*, **31**, 191.

KROTOSKI, W.A., COLLINS, W.E., BRAY, R.S., GARNHAM, P.C.C., COGSWELL, F.B., GWADZ, R.W., KILLICK-KENDRICK, R., WOLF, R., SINDEN, R.S., KOONTZ, L.C. & STANFILL, P.S. (1982b) Observations on early and late post sporozoite tissue stages in primate malaria. II. The hypnozoite of *P. cynomolgi bastienelli* from 3 to 105 days after infection and detection of 36 to 40 hour pre erythrocytic forms. *American Journal of Tropical Medicine and Hygiene*, **31**, 211.

KUMAR, S., GOOD, M.F., DONTFRAID, F., VINETZ, J.M. & MILLER, L.H. (1989) Interdependence of $CD4^+$ T cells and malarial spleen in immunity to *Plasmodium vinckei vinckei.* Relevance to vaccine development. *Journal of Immunology*, **143**, 2017.

KUMARARATNE, D.S., PHILLIPS, R.S., SINCLAIR, D., PARROTT, M.V.D. & FORRESTER, J.B. (1987) Lymphocyte migration in murine malaria during the primary patent parasitaemia of *Plasmodium chabaudi* infections. *Clinical and Experimental Immunology*, **68**, 65.

KUMARATILAKE, L.M. & FERRANTE, A. (1992) Il-4 inhibits macrophage-mediated killing of *Plasmodium falciparum* in vitro: A possible parasite-immune evasion mechanism. *Journal of Immunology*, **149**, 194.

KUMARATILAKE, L.M., FERRANTE, A. & RZEPCZYK, C.M. (1991) The role of T lymphocytes in immunity to *Plasmodium falciparum*: Enhancement of neutrophil-mediated parasite killing by lymphotoxin and IFN-γ: comparisons with tumour necrosis factor effects. *Journal of Immunology*, **146**, 762.

KUMARATILAKE, L.M., FERRANTE, A., JAEGER, T. & RZEPCZYK, C.M. (1992) Effects of cytokines, complement and antibody on the neutrophil respiratory burst and phagocytic response to *Plasmodium falciparum* merozoites. *Infection and Immunity*, **60**, 3731.

KURNICK, J.T., GRONVIK, K.O., KIMURA, A.K., LINDBLOM, J.B., SKOOG, V.T., SJOBERG, O. & WIGZELL, H. (1979a) Long term growth *in vitro* of human T cell blasts with maintenance of specificity and function. *Journal of Immunology*, **122**, 1255.

KURNICK, J.T., OSTBERG, L., STEGAGNO, M., KIMURA, A.K., ORN, A. & SJOBERG, O. (1979b) A rapid method for the preparation of functional lymphoid cell populations of human and animal origin on PVP-silica (Percoll) density gradients. *Scandinavian Journal of Immunology*, *10*, *563*.

KWIATKOWSKI, D. (1989) Febrile temperatures can synchronise the growth of *Plasmodium falciparum in vitro. Journal of Experimental Medicine*, **169**, 357.

KWIATKOWSKI, D. (1991) Cytokines and anti-disease immunity to malaria. *Research in Immunology*, **142**, 707.

KWIATKOWSKI, D. (1992) Malaria: becoming more specific about non-specific immunity. *Current Opinion in Immunology*, **4**, 425.

KWIATKOWSKI, D., HILL, A.V.S., SAMBOU, I., TWUMASI, P., CASTRACANE, J., MANOGUE, K.R., CERAMI, A., BREWSTER, D.R. & GREENWOOD, B.M. (1990) TNF concentrations in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet*, **336**, 1201.

LAL, A.A., DE LA CRUZ, V.F., WELSH, J.A., CHAROENVIT, Y., MALOY, W.L. & McCUTCHAN, T.F. (1987) Structure of the gene encoding the circumsporozoite protein of *Plasmodium yoelii. Journal of Biological Chemistry*, **262**, 2937.

LANDAU, I. (1965) Description de *Plasmodium chabaudi* n.sp., parasite de rongeurs africains. *C. r. Habd. Séanc. Acad. Sci. Paris*, **260**, 3758.

LANGHORNE, J. & ASOFSKY, R. (1986) Influence of *Plasmodium chabaudi adami* on the isotype distribution of the antibody response to sheep erythrocytes. *Immunobiology*, **174**, 432.

LANGHORNE, J. & SIMON, B. (1989) Limiting dilution analysis of the T cell response to *Plasmodium chabaudi chabaudi* in mice. *Parasite Immunology*, **11**, 545.

LANGHORNE, J. (1989) The role of $CD4^+$ T-cells in the immune response to *Plasmodium chabaudi*. *Parasitology Today*, **5**, 362.

LANGHORNE, J., BUTCHER, G.A., MITCHELL, G.H. & COHEN, S. (1979) Preliminary investigations on the role of the spleen in immunity to *Plasmodium knowlesi*. In: *Role of the Spleen in the Immunology of Parasitic Diseases*. Tropical Diseases Research Series I, p. 205. Schwabe & Co., Basel.

LANGHORNE, J., EVANS, C.B., ASOFSKY, R. & TAYLOR, D.W. (1984) Immunoglobulin isotype distribution of malaria-specific antibodies produced during infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii*. *Cellular Immunology*, **87**, 452.

LANGHORNE, J., GILLARD, S., SIMON, B., SLADE, S. & EICHMANN, K. (1989a) Frequencies of $CD4^+$ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with T_H1 and T_H2 characteristics during infection. *International Immunology*, **1**, 416.

LANGHORNE, J., GOODIER, M., BEHR, C. & DUBOIS, P. (1992) Is there a role for $\gamma\delta$ T cells in malaria? *Immunology Today*, **13**, 298.

LANGHORNE, J., KIM, K-J. & ASOFSKY, R. (1985) Distribution of immunoglobulin isotypes in the non-specific B-cell response induced by infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii*. *Cellular Immunology*, **90**, 251.

LANGHORNE, J., MEDING, S.J., EICHMANN, K. & GILLARD, S.S. (1989b) The response of $CD4^+$ T cells to *Plasmodium chabaudi chabaudi*. *Immunology Reviews*, **112**, 71.

LANGHORNE, J., SIMON-HAARHAUS, B. & MEDING, S.J. (1990) The role of $CD4^+$ T cells in the protective immune response to *Plasmodium chabaudi in vivo*. *Immunology Letters*, **25**, 101.

LEDBETTER, J.A. & HERZENBERG, L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunology Reviews*, **47**, 63.

LEECH, J.H., BARNWELL, J.W., MILLER, L.H. & HOWARD, R.J. (1984) Identification of a strain-specific malarial Ag exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. *Journal of Experimental Medicine*, **159**, 1567.

LEGORRETA-HERRERA, M., FIALLOS-LEON, C., CEDILLO-BARRON, L., MARTINEZ-GOMEZ, F., FOSTER-CUEVAS, M. & FAVILA-CASTILLO, L. (1993) Anti-Thy-1 treated and irradiated spleen cells from (BALB/c x C57Bl/6) F_1 mice infected with *Plasmodium chabaudi chabaudi* can transfer protection into irradiated hosts. *Parasite Immunology*, **15**, 143.

LEIST, T.P., COBBOLD, S.P., WALDMANN, H., AGUET, M. & ZINKERNAGEL, R.M. (1987) Functional analysis of T lymphocyte subsets in antiviral host defense. *Journal of Immunology*, **138**, 2278.

LELCHUK, R., ROSE, G. & PLAYFAIR, J.H.L. (1984) Changes in the capacity of macrophages and T cells to produce interleukins during murine malaria infection. *Cellular Immunology*, **84**, 253.

LELCHUK, R., TAVERNE, J., AGOMO, P.U. & PLAYFAIR, J.H.L. (1979) Development and suppression of a population of late-adhering macrophages in mouse malaria. *Parasite Immunology*, **1**, 61.

LICHTMAN, A.H., CHIN, J., SCHMIDT, J.A. & ABBAS, A.K. (1988) Role of interleukin 1 in the activation of T lymphocytes. *Proceedings of the National Academy of Science* USA, **85**, 9699.

LIEW, F.Y. & COX, F.E.G. (1991) Nonspecific defence mechanism: the role of nitric oxide.

Immunoparasitology Today, **7**, A17.

LIEW, F.Y., LI, Y., SEVERN, A., MILLOT, S., SCHMIDT, J., SALTER, M. & MONCADA, S. (1991) A possible novel pathway of regulation by murine T helper type-2 (T_H2) cells of a T_H1 cell activity via the modulation of the induction of NO synthase on macrophages. *European Journal of Immunology*, **21**, 2489.

LIEW, F.Y., MILLOT, S., PARKINSON, C., PALMER, R.M.J. & MONCADA, S. (1990) Macrophage killing of *Leishmania* parasites *in vivo* is mediated by nitric oxide from L-arginine. *Journal of Immunology*, **144**, 4794.

LONG, C.A. (1988) Immunity to murine malarial parasites. In: *The Biology of Parasitism* (eds. P.T. Englund & A. Sher), p.233. Alan R. Liss Inc., New York.

LOOSE, L.D. & DILUZIO, N.R. (1976) A temporal relationship between reticuloendothelial system phagocytic alterations and antibody responses in ice infected with *Plasmodium berghei* (NYU-2 strain). *American Journal of Tropical Medicine and Hygiene*, **25**, 221.

LOURIE, S.H. & DUNN, M.A. (1972) The effect of protective sera on the course of *Plasmodium berghei* in immunosuppressed rats. *Proceedings of the Helminthological Society of Washington*, **39**, 470.

LUCAS, S.B. (1990) Missing infections in AIDS. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84** (Suppl.1), 34.

LUCIA, H.L. & NUSSENZWEIG, R.S. (1969) *Plasmodium chabaudi* and *Plasmodium vinckei*: phagocytic activity of mouse reticulo-endothelial system. *Experimental Parasitology*, **25**, 319.

LUMSDEN, W.H.R. & HARDY, G.T.C. (1965) Nomenclature of living material. *Nature*, **250**, 1032.

LUPASCU, G.H., CONSTANTINESCU, P., NEGULICI, E., GARNHAM, P.C.C., BRAY, R.S., KILLICK-KENDRICK, R., SHUTE, P.G. & MARYON, M. (1967) The late primary exoerythrocytic stages of *Plasmodium malariae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **61**, 482.

LUSE, S.A. & MILLER, L.H. (1971) *Plasmodium falciparum* malaria: ultrastructures of parasitised erythrocytes in cardiac vessels. *American Journal of Tropical Medicine and Hygiene*, **20**, 655.

LUSTIG, H.J., NUSSENZWEIG, V. & NUSSENZWEIG, R.S. (1977) Erythrocyte membrane associated immunoglobulins during malaria infection of mice. *Journal of Immunology*, **119**, 210.

LUZZATO, L. (1979) Genetics of red cells and susceptibility to malaria. *Blood*, **54**, 961.

LUZZATTO, L., NWACHUKU, E.S. & REDDY, S. (1970) Increased sickling of parasitised erythrocytes as a mechanism of resistance against malaria in the sickle-cell trait. *Lancet*, **i**, 319.

MACKAY, C.R. (1993) Homing of naive, memory and effector lymphocytes. *Current Opinion in Immunology*, **5**, 423.

MACKEY, L.J., HOCHMAN, A., JUNE, C.H., CONTRERAS, C.E. & LAMBERT, P-H. (1980) Immunopathological aspects of *Plasmodium berghei* infection in five strains of mice. II. Immunopathology of cerebral and other tissue lesions during the infection. *Clinical and Experimental Immunology*, **42**, 412.

MacPHERSON, G.G., WARRELL, M.J., WHITE, N.J., LOOAREESUWAN, S. & WARRELL, D.A. (1985) Human cerebral malaria. A quantitative ultrastructural analysis of parasitised erythrocyte sequestration. *American Journal of Pathology*, **119**, 385.

MAHESHWARI, R.L., CZARNIECKI, C.W., DUTTA, G.P., PURI, S.K., DHAWAN, B.N. & FRIEDMAN, R.M. (1986) Recombinant human gamma interferon inhibits Simian malaria. *Infection and Immunity*, **53**, 628.

MAMULA, M.J. & JANEWAY, C.A. (1993) Do B cells drive the diversification of immune

response? *Immunology Today*, **14**, 151.

MANAWADU, B.R. & VOLLER, A. (1978) Standardisation of the indirect fluorescent antibody test for malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **72**, 456.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706.

MARSH, K. & HOWARD, R.J. (1986) Antigens induced on erythrocytes by *Plasmodium falciparum*: expression of diverse and conserved determinants. *Science*, **231**, 150.

MARSH, K., OTTO, L., HAYES, R.J., CARSON, D.C. & GREENWOOD, B.M. (1989) Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **83**, 293.

MARTIN, L.K., EINHEBER, A., SADUN, E.H. & WREN, R.E. (1967) Effect of bacterial endotoxin on the course of *Plasmodium berghei* infection. *Experimental Parasitology*, **20**, 186.

MATTEI, D. & SCHERF, A. (1992) The Pf332 gene of *Plasmodium falciparum* codes for a giant protein that is translocated from the parasite to the membrane of infected erythrocytes. *Gene*, **110**, 71.

MATTEI, D., HINTERBERG, K. & SCHERF, A. (1992) Pf111 and Pf332: Two giant proteins synthesised in erythrocytes infected with *Plasmodium falciparum*. *Parasitology Today*, **8**, 426.

MAXFIELD, F.R. (1982) Weak bases and ionophores rapidly and reversibly raise thepH of endocytic vesicles in cultured mouse fibroblasts. *Journal of Cell Biology*, **95**, 676.

McBRIDE, J.S., WALLIKER, D. & MORGAN, S. (1982) Antigenic diversity in the human malaria parasite *Plasmodium falciparum*. *Science*, **217**, 254.

McDONALD, V. & PHILLIPS, R.S. (1978) *Plasmodium chabaudi* in mice. Adoptive transfer of immunity with enriched populations of spleen T and B lymphocytes. *Immunology*, **34**, 821.

McDONALD, V. & SHERMAN, I.W. (1980) *Plasmodium chabaudi*: humoral and cell-mediated responses of immunised mice. *Experimental Parasitology*, **49**, 442.

McDONALD, V. (1977) *Studies on the protective immune response to Plasmodium chabaudi in mice*. PhD thesis, University of Glasgow.

McGHEE, R.B. (1960) An autoimmune reaction produced in ducklings in response to injections of duck embryo blood infected with *Plasmodium lophurae*. *Journal of Infectious Diseases*, **107**, 410.

McGREGOR, I.A. & BARR, M. (1962) Antibody response to tetanus toxoid inoculation in malarious and non-malarious Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **56**, 364.

McGREGOR, I.A. & WILLIAMS, K. (1978) Value of the gel-precipitation test in monitoring the endemicity in a rural African village. *Israel Journal of Medical Science*, **14**, 697.

McGREGOR, I.A. (1960) Demographic effects of malaria with special reference to the stable malaria of Africa. *West Africa Medical Journal*, **9**, 260.

McGREGOR, I.A., GILLES, H.M., WALTER, J.H. & DAVIES, A.H. (1956) Effects of heavy and repeated malarial infections of Gambian infants and children. Effects of erythrocyte parasitism. *British Medical Journal*, **iii**, 686.

McLEAN, S.A. (1985) *Survival of Plasmodium chabaudi in the semi-immune host.* PhD Thesis, University of Glasgow.

McLEAN, S.A., MacDOUGALL, L.M. & PHILLIPS, R.S. (1990) Early appearance of variant

parasites in *Plasmodium chabaudi* infections. *Parasite Immunology*, **12**, 97.

McLEAN, S.A., McDOUGALL, L. & PHILLIPS, R.S. (1986b) An indirect fluorescent antibody test which recognises antigenic variants of *Plasmodium chabaudi*. *IRCS Medical Science*, **14**, 896.

McLEAN, S.A., PEARSON, C.D. & PHILLIPS, R.S. (1982a) *Plasmodium chabaudi*: relationship between the occurrence of recrudescent parasitaemias in mice and the effective levels of acquired immunity. *Experimental Parasitology*, **54**, 213.

McLEAN, S.A., PEARSON, C.D. & PHILLIPS, R.S. (1982b) *Plasmodium chabaudi*: evidence of antigenic variation during recrudescent parasitaemias in mice. *Experimental Parasitology*, **54**, 296.

McLEAN, S.A., PEARSON, C.D. & PHILLIPS, R.S. (1986a) Antigenic variation in *Plasmodium chabaudi*: analysis of parent and variant populations by cloning. *Parasite Immunology*, **8**, 415.

McLEAN, S.A., PHILLIPS, R.S., PEARSON, C.D. & WALLIKER, D. (1987) The effect of mosquito transmission on antigenic variants of *Plasmodium chabaudi*. *Parasitology*, **94**, 443.

MEDING, S.J. & LANGHORNE, J. (1991) $CD4^+$ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi*. *European Journal of Immunology*, **21**, 1433.

MEDING, S.J., CHENG, S.C., SIMON-HAARHAUS, B. & LANGHORNE, J. (1990) Role of interferon-γ during infection with *Plasmodium chabaudi chabaudi*. *Infection and Immunity*, **58**, 3671.

MELLOUK, S., GREEN, S.J., NACY, C.A. & HOFFMAN, S.L. (1991) IFN-γ inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. *Journal of Immunology*, **146**, 3971.

MENDIS, K.N. & TARGETT, G.A.T. (1979) Immunization against gametes and sexual erythrocytic stages of rodent malaria parasites. *Nature*, **277**, 389.

MENDIS, K.N. & TARGETT, G.A.T. (1982) Vaccination to prevent transmission of *Plasmodium yoelii* malaria. *Parasite Immunology*, **4**, 117.

MENDIS, K.N., MUNESINGHE, Y.D., DeSILVA, Y.N.Y., KERAGALLA, I. & CARTER, R. (1987) Malaria transmission-blocking immunity induced by natural infections of *Plasmodium vivax* in humans. *Infection and Immunity*, **55**, 369.

MICHEL, J.C., HURTREL, B. & LANGRANGE, P.H. (1982) Inflammation and resistance of mice against *Plasmodium berghei*. *Annals of Institute Pasteur/Immunology*, **133C**, 97.

MILLER, L.H. (1969) Distribution of mature trophozoites and schizonts of *Plasmodium falciparum* in the organs of *Aotus trivirgatus* the night monkey. *American Journal of Tropical Medicine and Hygiene*, **18**, 860.

MILLER, L.H., AIKAWA, M. & DVORAK, J.A. (1975a) Malaria (*Plasmodium knowlesi*) merozoites: immunity and the surface coat. *Journal of Immunology*, **114**, 1237.

MILLER, L.H., DAVID, P.H., HUDSON, D.E., HADLEY, T.J., RICHARDS, R.L. & AIKAWA, M. (1984) Monoclonal antibodies to a 140,000-m.w. protein on *Plasmodium knowlesi* merozoites inhibit their invasion of rhesus erythrocytes. *Journal of Immunology*, **132**, 438.

MILLER, L.H., FREMOUNT, H.N. & LUSE, S.A. (1971a) Deep vascular schizogony of *Plasmodium knowlesi* in *Macaca mulatta*. *American Journal of Tropical Medicine and Hygiene*, **20**, 816.

MILLER, L.H., HOWARD, R.J., CARTER, R., GOOD, M.F., NUSSENZWEIG, V. & NUSSENZWEIG, R.S. (1986) Research towards malaria vaccines. *Science*, **234**, 1349.

MILLER, L.H., MASON, S.J., CLYDE, D.F. & McGINNIS, M.H. (1976) The resistance factor to *Plasmodium vivax* in Blacks: the Duffy blood-group genotype, F_yF_y. *New England Journal of*

Medicine, **295**, 302.

MILLER, L.H., MASON, S.J., DVORAK, J.A., McGINNIS, .H. & RATHMAN, I.K. (1975b) Erythrocyte receptors from (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science*, **189**, 561.

MILLER, L.H., POWERS, K.G. & SHIROISHI, T. (1977) *Plasmodium knowlesi*: functional immunity and antimerozoite antibodies in rhesus monkeys after repeated infections. *Experiemtnal Parasitology*, **41**, 105.

MILLER, L.H., USAMI, S. & CHIEN, S. (1971b) Alteration in the rheologic properties of *Plasmodium knowlesi*-infected red cells. A possible mechanism for capillary obstruction. *Journal of Clinical Investigation*, **50**, 1451.

MINOPRIO, P.S., ITOHARA, S., HEUSSER, C., TONEGAWA, S. & COUTINHOU, A. (1989) Immunobiology of murine *Trypanosoma cruzi* infection: the predominance of parasite non specific responses and the activation of TCR1 T cells. *Immunology Reviews*, **112**, 182.

MIRNA, A. & HOFMANN, K. (1969) The behaviour of nitrite in meat products. *Fleischwirtschaft*, **10**, 1361.

MITCHELL, G.H. & BANNISTER, L.H. (1988) Malaria parasite invasion: interactions with the red cell membrane. *Critical Reviews in Oncology/Haematology*, **8**, 255.

MITCHELL, G.H., BUTCHER, G.A., VOLLER, A. & COHEN, S. (1976) The effect of human immune IgG on the *in vitro* development of *Plasmodium falciparum*. *Parasitology*, **72**, 149.

MOGIL, R.J., PATTON, C.L. & GREEN, D.R. (1987) Cellular subsets involved in cell-mediated immunity to murine *Plasmodium yoelii* 17X malaria. *Journal of Immunology*, **138**, 1933.

MOMBAERTS, P., CLARK, A.R., RUDNICKI, M.A., IACOMINI, M.A., ITOHARA, S., LAFAILLE, J.J., WANG, L., ISHIKAWA, Y., JAENISCH, R., HOOPER, M.L. & TONEGAWA, S. (1992) Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature*, **360**, 225.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*, **43**, 109.

MONS, B. & SINDEN, R.E. (1990) Laboratory models for research *in vivo* and *in vitro* on malaria parasites of mammals: current status. *Parasitology Today*, **6**, 3.

MOORE, G.E., GERNER, R.E. & FRANKLIN, H.A. (1967) Culture of normal human leukocytes. *Journal of the American Medical Association*, **199**, 519.

MORENO, A. & PATARROYO, M.E. (1989) Development of an asexual blood stage malaria vaccine. *Blood*, **74**, 537.

MORTAZAVI-MILANI, S.M., BADAKERE, S.S. & HOLBOROW, E.J. (1984) Antibody to intermediate filaments of the cytoskeleton in the sera of patients with acute malaria. *Clinical and Experimental Immunology*, **55**, 177.

MOSMANN, T.R. & COFFMAN, R.L. (1987) Two types of mouse helper T-cell clone: implications for immune regulation. *Immunology Today*, **8**, 223.

MOSMANN, T.R. & COFFMAN, R.L. (1989) T_H1 and T_H2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology*, **7**, 145.

MSHANA, R.N., BOULANDI, J., MSHANA, N.M. (1991) Cytokines in the pathogenesis of malaria: levels of Il-1β, Il-4, Il-6, TNF-α and IFN-γ in plasma od healthy individuals and malaria patients in holoendemic area. *Journal of Clinical and Laboratory Immunology*, **34**, 131.

MUNOZ, E., BEUTNER, U., ZUBIAGA, A. & HUBER, B.T. (1990) Il-1 activated two separate transduction pathways in T helper type II cells. *Journal of Immunology*, **144**, 964.

MURPHY, J.R. (1979) Host defences in murine malaria: analysis of the mechanisms of immunity to *Plasmodium berghei* generated in response to immunisation with formalin killed blood stage parasites. *Infection and Immunity*, **24**, 707.

MURPHY, J.R. (1981) Host defences in murine malaria: non specific resistance to *Plasmodium berghei* generated in response to *Mycobacterium bovis* infection or *Corynebacterium parvum* stimulation. *Infection and Immunity*, **33**, 199.

MURRAY, M.J., MURRAY, A.B., MURRAY, N.J. & MURRAY, M.B. (1981) Diet and cerebral malaria. *American Journal of Clinical Nutrition*, **31**, 57.

MUTETWA, S.M. & JAMES, E.R. (1984a) Cryopreservation of *Plasmodium chabaudi* I: Protection by glycerol and dimethylsulfoxide during cooling and by glucose following thawing. *Cryobiology*, **21**, 329.

MUTETWA, S.M. & JAMES, E.R. (1984b) Cryopreservation of *Plasmodium chabaudi* II: Cooling and warming rates. *Cryobiology*, **21**, 552.

NAGEL, R.L. (1990) Innate resistance to malaria: the intraerythrocytic cycle. *Blood Cells*, **16**, 321.

NARDIN, E.H., NUSSENZWEIG, R.S., McGREGOR, I.A. & BRYAN, J.H. (1979) Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. *Science*, **206**, 597.

NARDIN, E.H., NUSSENZWEIG, V., NUSSENZWEIG, R.S., COLLINS, W.E., HARINASUTA, K.T., TAPCHAISRI, P. & CHOMCHARN, Y. (1982) Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. *Journal of Experimental Medicine*, **156**, 20.

NASH, A.A., JAYASURIYA, A., PHELAN, J., COBBOLD, S.P., WALDMANN, H. & PROSPERO, T. (1987) Different roles for L3T4$^+$ and Lyt-2$^+$ T cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system. *Journal of General Virology*, **68**, 825.

NAYSMITH, J.D. & JAMES, K. (1968) Effect of F(ab')2 from rabbit anti-mouse lymphocyte IgG on the graft-versus host reaction in F_1 hybrid mice. *Nature*, **217**, 260.

NEVA, F.A., HOWARD, W.A., GLEW, R.H., KROTOSKI, W.A., GAM, A.A., COLLINS, W.E., ATKINSON, J.P. & FRANK, M.M. (1974) Relationship of serum complement levels to events of the malaria paroxysm. *Journal of Clinical Investigation*, **54**, 451.

NEWTON, C.R.J.C., KIRKHAM, F.J., WINSTANLEY, P.A., PASVOL, G., PESHU, N., WARRELL, D.A. & MARSH, K. (1991) Intracranial pressure in African children with cerebral malaria. *Lancet*, **337**, 573.

NOGEUR, A., WERNSDORFER, W., KOWNETSOV, R., HEMPEL, J. (1978) The malaria situation in 1976. *WHO Chronicles*, **32**, 9

NOSSAL, G.J.V. & PIKE, B.L. (1973) Studies on the differentiation of B lymphocytes in the mouse. *Immunology*, **25**, 33.

NUSSENZWEIG, R.S. & NUSSENZWEIG, V. (1986) Development of a malaria sporozoite vaccine. *American Journal of Tropical Medicine and Hygiene*, **35**, 678.

NUSSENZWEIG, R.S. (1967) Increased non specific resistance to malaria produced by administration of killed *Corynebacterium parvum*. *Parasitology*, **21**, 224.

NUSSENZWEIG, R.S., VANDERBERG, J. & MOST, H. (1969) Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. *Military Medicine*, **134** (Suppl), 1176.

NUSSENZWEIG, R.S., VANDERBERG, J., SANABRIA, Y. & MOST, H. (1972b) *Plasmodium berghei*: accelerated clearance of sporozoites from blood as part of the immune mechanism in

mice. *Experimental Parasitology*, **31**, 88.

NUSSENZWEIG, R.S., VANDERBERG, J., SPITALNY, G.L., RIVERA, C.I.O., ORTON, C. & MOST, H. (1972a) Sporozoite induced immunity in mammalian malaria. *American Journal of Tropical Medicine and Hygiene*, **21**, 722.

NUSSLER, A., DRAPIER, J-C., RENIA, L., PIED, S., MILTGEN, F., GENTILINI, M. & MAZIER, D. (1991b) L-arginine dependent destruction of intrahepatic malaria parasites in response to TNF and/or Il-6 stimulation. *European Journal of Immunology*, **21**, 227.

NUSSLER, A., PIED, S., GOMA, J., RENIA, L., MILTGEN, F., GRAU, G.E. & MAZIER, D. (1991a) TNF inhibits malaria hepatic stages *in vitro* via Il-6 liver synthesis. *International Immunology*, **3**, 317.

O'NEILL, P. & JOHNSON, G.D. (1970) Multispot immunofluorescence: a simple method of processing large numbers of tests. *Journal of Clinical Pathology*, **23**, 185.

OCKENHOUSE, C.F. & SHEAR, H.L. (1983) Malaria-induced lymphokines: stimulation of macrophages for enhanced phagocytosis. *Infection and Immunity*, **42**, 733.

OCKENHOUSE, C.F. & SHEAR, H.L. (1984) Oxidative killing of the intraerythrocytic malaria parasite *Plasmodium yoelii* by activated macrophages. *Journal of Immunology*, **132**, 424.

OMATA, Y., YAGAMI, K., TAKEI, Y., SUSUKI, N. & NAKABAYASHI, T. (1981) Protective reaction against malaria infection in mice sensitised with frozen and thawed *Toxoplasma* tachyzoites. *Z. Bakteriol. Microbiol. Hyg. Med.*, **250**, 223.

OO, M.M., AIKAWA, M., THAN, T., AYE, T.M., MYINT, P.T., IGARISHI, I. & SCHOENE, W.C. (1987) Human cerebral malaria: a pathological study. *Journal of Neuropathology and Experimental Neurology*, **46**, 223.

OQUENDO, P., HUNDT, E., LAWLER, J. & SEED, B. (1989) CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitised erythrocytes. *Cell*, **58**, 95.

ORJIH, A.U. & NUSSENZWEIG, R.S. (1979) *Plasmodium berghei*: suppression of antibody response to sporozoites by acute blood stage infection. *Clinical and Experimental Immunology*, **38**, 1.

OSORIO, L.M., FONTE, L. & FINLAY, C.M. (1992) Inhibition of human monocyte function by prophylactic doses of chloroquine. *American Journal of Tropical Medicine and Hygiene*, **46**, 165.

OSTER, C.N., KOONTZ, L.C. & WYLER, D.J. (1988) Malaria in asplenic mice: effects of splenectomy, congenital asplenia and splenic reconstitution on the course of infection. *American Journal of Tropical Medicine and Hygiene*, **29**, 1138.

OSWALD, I.P., GAZZINELLI, R.T., SHER, A. & JAMES, S.L. (1992) Il-10 synergises with Il-4 and transforming growth factor-b to inhibit macrophage cytotoxic activity. *Journal of Immunology*, **148**, 3578.

OTT, K.J. (1968) Influence of reticulocytes on the course of infection of *Plasmodium chabaudi* and *P. berghei*. *Journal of Protozoology*, **15**, 365.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524.

PANUSH, R.S. (1975) Effects of certain antirheumatic drugs upon normal human peripheral blood lymphocytes. Inhibition of mitogen- and antigen-stimulated incorporation of tritiated thymidine. *Arthritis and Rheumatology*, **18**, 418.

PASVOL, G. & WILSON, R.J.M. (1982) The interaction of malaria parasites with red blood cells. *British Medical Bulletin*, **38**, 133.

PASVOL, G., WEATHERALL, D.J. & WILSON, R.J.M. (1978) Cellular mechanism for the

protective effect of haemoglobin S against *Plasmodium falciparum* malaria. *Nature*, **274**, 701.

PATARROYO, M.E., AMADOR, R., CLAVIJO, P., MORENO, A., GUZMAN, F., ROMERO, P., TASCON, R., FRANCO, A., MURILLO, L.A., PONTON, G. & TRUJILLO, G. (1988) A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. *Nature*, **332**, 158.

PATARROYO, M.E., ROMERO, P., TORRES, M.L., CLAVIJO, P., MORENO, A., MARTINEZ, A., RODRIEGUEZ, R., GUZMAN, F. & CABEZAS, E. (1987) Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature*, **328**, 629.

PEIRIS, J.S.M., PREMAWANSA, S., RANAWAKA, M.B.R., UDAGANA, P.V., MUNASINGHE, Y.D., NANAYAKKARA, M.V., GAMAGE, C.P., CARTER, R., DAVID, P.H. & MENDIS, K.N. (1988) Monoclonal and polyclonal antibodies block and enhance transmission of human *Plasmodium vivax* malaria. *American Journal of Tropical Medicine and Hygiene*, **39**, 26.

PEIRIS, J.S.M., PREMAWANSA, S., RANAWAKA, M.B.R., UDAGANA, P.V., MUNASINGHE, Y.D., NANAYAKKARA, M.V., GAMAGE, C.P., CARTER, R., DAVID, P.H. & MENDIS, K.N. (1988) Monoclonal and polyclonal antibodies block and enhance transmission of human *Plasmodium vivax* malaria. *American Journal of Tropical Medicine*, **39**, 26.

PERERA, M.K., CARTER, R., GOONEWARDENE, R. & MENDIS, K.M. (1994) Transient increase in circulating γδ T cells during *Plasmodium vivax* malarial paroxysyms. *Journal of Experimental Medicine*, **179**, 311.

PERKINS, M.E. (1981) Surface proteins of *Plasmodium falciparum* merozoites binding to the erythrocyte receptor, glycophorin. *Journal of Experimental Medicine*, **160**, 788.

PHANUPHAK, P., TIRAWATNPONG, S., HANVANICH, M., PANMUONG, W., MOOLLAOR, P., VEJJAJIVA, S., SITPRIJA, V., INTRAPRASERT, R. & PHANTHUMKOSOL, D. (1983) Autoantibodies in falciparum malaria: a sequential study in 183 Thai patients. *Clinical and Experimental Immunology*, **53**, 627.

PHILLIPS, R.S. & JONES, V.E. (1972) Immunity to *Plasmodium berghei* in rats: maximum levels of protective antibody are associated with eradication of infection. *Parasitology*, **64**, 117.

PHILLIPS, R.S. & WILSON, R.J.M. (1978) Cryopreservation of *Plasmodium falciparum* in liquid nitrogen. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **72**, 643.

PHILLIPS, R.S. (1970) *Plasmodium berghei*: passive transfer of immunity by antisera and cells. *Experimental Parasitology*, **27**, 479.

PHILLIPS, R.S. (1983) *Malaria*. Edward Arnold, London.

PHILLIPS, R.S. (1992) Vaccination against malaria. *Immunobiology*, **184**, 240.

PHILLIPS, R.S., TRIGG, P.I., SCOTT-FINNIGAN, T.J., BARTHOLOMEW, R.K. (1972) Culture of *Plasmodium falciparum in vitro*: a subculture technique used for demonstrating antiplasmodial activity in serum from some Gambians resident in an endemic malarious area. *Parasitology*, **65**, 525.

PHILPOTT, K.L., VINEY, J.L., KAY, G., RASTAN, S., GARDINER, E.M., CHAE, S., HAYDAY, A.C. & OWEN, M.J. (1992) Lymphoid development in mice congenitally lacking T cell receptor αβ–expressing cells. *Science*, **256**, 1448.

PICOT, S., PEYRON, F., DONADILLE, A., VUILLEZ, J-P., BARBE, G. & AMROISE-THOMAS, P. (1993) Chloroquine-induced inhibition of the production of TNF, but not of Il-6, is affected by disruption of iron metabolism. *Immunology*, **80**, 127.

PICOT, S., PEYRON, F., VUILLEZ, J.P., BARBE, G. & AMBROISE-THOMAS, P. (1991) Chloroquine inhibits TNF production by human macrophages *in vitro*. *Journal of Infectious Diseases*, **164**, 830.

PIED, S., CIVAS, A., BERLOT-PICARD, F., RENIA, L., MILTGEN, F., GENTILINI, M., DOLY, J. & MAZIER, D. (1992) Il-6 induced by Il-1 inhibits malaria pre-erythrocytic stages but its secretion is down-regulated by the parasite. *Journal of Immunology*, **148**, 197.

PIED, S., RENIA, L., NUSSLER, A., MILTGEN, F. & MAZIER, D. (1991) Inhibitory activity of Il-6 on malaria hepatic stages. *Parasite Immunology*, **13**, 211.

PILARSKI, L.M. & CUNNINGHAM, A.J. (1974) Host derived antibody-forming cells in lethally irradiated mice. *Journal of Immunology*, **114**, 138.

PLAYFAIR, J.H.L. (1982) Immunity to malaria. *British Medical Bulletin*, **38**, 153.

PLAYFAIR, J.H.L. (1990) Tumor necrosis factor and malaria. Beneficial and harmful aspects. *Diagnostic Microbiology and Infectious Disease*, **13**, 435.

PLAYFAIR, J.H.L., DeSOUZA, J.B. & COTTRELL, B.J. (1977) Protection of mice against malaria by a killed vaccine: differences in effectiveness against *Plasmodium yoelii* and *P. berghei*. *Immunology*, **33**, 507.

PLAYFAIR, J.H.L., TAVERNE, J., BATE, C.A.W. & DE SOUZA, J.B. (1990) The malaria vaccine: anti-parasite or anti-disease? *Immunology Today*, **11**, 25.

PODOBA, J.E. & STEVENSON, M.M. (1991) $CD4^+$ and $CD8^+$ T lymphocytes both contribute to acquired immunity to blood stage *Plasmodium chabaudi* AS. *Infection and Immunity*, **59**, 51.

POLDER, T., JERUSALEM, C. & ELING, W. (1983) Topographical distribution of the cerebral lesions in mice infected with *Plasmodium berghei*. *Tropenmed. Parasitol*, **34**, 235.

POLOGE, L.G. & RAVETCH, J.V. (1986) A chromosomal rearrangement in a *Plasmodium falciparum* histidine-rich protein gene is associated with the knobless phenotype. *Nature*, **322**, 474.

PONGPONRATN, E., RIGANTI, M., BUNNAG, D. & HARINASUTA, T. (1987) Spleen in falciparum malaria: ultrastructural study. *Southeast Asian Journal of Tropical Medicine and Public Health*, **18**, 491.

POTOCNJAK, P., YOSHIDA, N., NUSSENZWEIG, R.S. & NUSSENZWEIG, V. (1980) Monovalent fragments (F_{ab}) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *Journal of Experimental Medicine*, **151**, 1504.

POTTER, M., PUMPHREY, J.G. & WATTERS, J.L. (1972) Growth of primary plasmacytomas in the mineral oil-conditioned peritoneal environment. *Journal for National Cancer Institute*, **49**, 305.

QUAKYI, I.A., CARTER, R., RENER, J., KUMAR, N., GOOD, M.F. & MILLER, L.H. (1987) The 230 kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmission-blocking antibodies. *Journal of Immunology*, **139**, 4213.

QUAKYI, I.A., OTOO, L.N., POMBO, D., SUGARS, L.Y., MENON, A., DeGROOT, A.S., JOHNSON, A., ALLING, D., MILLER, L.H. & GOOD, M.F. (1989) Differnetial non-responsiveness in humans of candidate *Plasmodium falciparum* vaccine antigens. *American Journal of Tropical Medicine and Hygiene*, **41**, 125.

QUINN, T.C. & WYLER, D.J. (1979a) Mechanisms of action of hyperimmune serum in mediating protective immunity to rodent malaria (*Plasmodium berghei*). *Journal of Immunology*, **123**, 2245.

QUINN, T.C. & WYLER, D.J. (1979b) Intravascular clearance of parasitised erythrocytes in rodent malaria. *Journal of Clinical Investigation*, **63**, 1187.

QUINN, T.C. & WYLER, D.J. (1980) Resolution of acute malaria (*Plasmodium berghei* in the rat): reversibility and spleen dependence. *American Journal of Tropical Medicine and Hygiene*, **29**, 1.

RABIN, E.M., MOND, J.J., OHARA, J. & PAUL, W.E. (1986) Interferon-γ inhibits the action of B

cell stimulatory factor (BSF)-1 on resting B cells. *Journal of Immunology*, **137**, 1573.

RANAWAKA, M.B., MUNESINGHE, Y.D., DeSILVA, D.M.R., CARTER, R. & MENDIS, K.N. (1988) Boosting of transmission blocking immunity during natural *Plasmodium vivax* infections in humans depends upon frequent reinfection. *Infection and Immunity*, **56**, 1820.

RANK, R.G. & WEIDANZ, W.P. (1976) Nonsterilising immunity in avian malaria: an antibody-independent phenomenon. *Proceedings of the Society of Experimental Biology and Medicine*, **151**, 257.

REESE, R.T. & MOTYL, M.R. (1979) Inhibition of *in vitro* growth of *Plasmodium falciparum*. I. Effects of immune serum and purified immunoglobulin from owl monkeys. *Journal of Immunology*, **123**, 1894.

RENER, J., GRAVES, P.M., CARTER, R., WILLIAMS, J.L. & BARKOT, T.R. (1983) Target antigens of transmission-blocking immunity on gametes of *Plasmodium falciparum*. *Journal of Experimental Medicine*, **158**, 976.

REST, J.R. (1982) Cerebral malaria in inbred mice. I. A new model and its pathology. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **76**, 410.

REST, J.R. (1983) Pathogenesis of cerebral malaria in golden hamsters and inbred mice. *Contributions in Microbiology and Immunology*, **7**, 139.

RHODES-FEUILLETTE, A., BELLOSGUARDO, M., DRUILHE, P., BALLET, J.J., CHOUSTERMAN, S., CANIVET, M. & PERIES, J. (1985b) The interferon compartment of the immune response in human malaria. II. Presence of serum-interferon gamma following the acute attack. *Journal of Interferon Research*, **5**, 169.

RHODES-FEUILLETTE, A., JAUREGUIBERRY, G., BALLET, J.J., ANDRIEU, B., DRUILHE, P., LeBRAS, J., GALIBERT, F. & PERIES, J. (1985a) The interferon compartment of the immune response in human malaria. I. Interferon inducers in *Plasmodium falciparum* cultures. *Journal of Interferon Research*, **5**, 159.

RIDLEY, R.G., TAKACS, B., ETLINGER, H. & SCAIFE, J.A. (1990) A rhoptry antigen of *Plasmodium falciparum* is protective in *Saimiri* monkeys. *Parasitology*, **101**, 187.

RIECKMANN, K.H., BEAUDOIN, R.L., CASSELLS, J.S. & SELL, K.W. (1979) Use of attenuated sporozoites in the immunisation of human volunteers against falciparum malaria. *Bulletin WHO*, **57** (Suppl. 1), 261.

RIECKMANN, K.H., CARSON, P.E., BEAUDOIN, R.L., CASSELLS, J.S. & SELL, K.W. (1974) Sporozoite induced immunity in man against an Ethiopian strain of *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **68**, 258.

RILEY, E.M., ANDERSSON, G., OTOO, L.N., JEPSEN, S. & GREENWOOD, B.M. (1988a) Cell-mediated immune responses to *Plasmodium falciparum* antigens in adult Gambians. *Clinical and Experimental Immunology*, **71**, 337.

RILEY, E.M., ANDERSSON, G., OTOO, L.N., JEPSEN, S. & GREENWOOD, B.M. (1988b) Cellular immune responses to *Plasmodium falciparum* antigens in Gambian children during and after an acute attack of falciparum malaria. *Clinical and Experimental Immunology*, **73**, 17.

RILEY, E.M., JOBE, D. & WHITTLE, H.C. (1989a) $CD8^+$ T cells inhibit *Plasmodium falciparum*-induced lymphoproliferation and gamma interferon production in cell preparations from some malaria-immune individuals. *Infection and Immunity*, **57**, 1281.

RILEY, E.M., JOBE, O., BLACKMAN, M., WHITTLE, H.C. & GREENWOOD, B.M. (1989b) *Plasmodium falciparum* schizont sonic extracts suppress lymphoproliferative responses to mitogen and antigens in malaria-immune adults. *Infection and Immunity*, **57**, 3181.

ROBERTS, D.J, CRAIG, A.G., BERENDT, A.R., PINCHES, R., NASH, G., MARSH, K. & NEWBOLD, C.I. (1992) Rapid switching to multiple antigenic and adhesive phenotypes in

malaria. *Nature*, **357**, 689.

ROBERTS, D.W. & WEIDANZ, W.P. (1979) T-cell immunity to malaria in the B-cell deficient mouse. *American Journal of Tropical Medicine and Hygiene*, **28**, 1.

ROBERTS, D.W., RANK, R.G., WEIDANZ, W.P. & FINERTY, J.F. (1977) Prevention of recrudescnet malaria in nude mice by thymic grafting or treatment with hyperimmune serum. *Infection and Immunity*, **16**, 821.

ROBERTS, D.W., SHERWOOD, J.A., SPITALNIK, S.L., PANTON, L.J., HOWARD, R.J., DIXIT, V.M., FRAZIER, W.A., MILLER, L.H. & GINSBERG, V. (1985) Thrombospondin binds falciparum malaria parasitised erythrocytes and may mediate cytoadherence. *Nature*, **318**, 64.

ROBERTS, J.A. & TRACEY-PATTE, P. (1969) Adoptive transfer of immunity to *Plasmodium berghei*. *Journal of Protozoology*, **16**, 728.

ROCKETT, K.A., AWBURN, M.M., AGGARWAL, B.B., COWDEN, W.B. & CLARK, I.A. (1992) *In vivo* induction of nitrite and nitrate by tumor necrosis factor, lymphotoxin, and interleukin-1: possible roles in malaria. *Infection and Immunity*, **60**, 3725.

ROCKETT, K.A., AWBURN, M.M., COWDEN, W.B. & CLARK, I.A. (1991) Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. *Infection and Immunity*, **59**, 3280.

ROCKETT, K.A., TARGETT, G.A.T. & PLAYFAIR, J.H.L. (1988) Killing of blood stage *Plasmodium falciparum* by lipid peroxides from tumor necrosis serum. *Infection and Immunity*, **56**, 3180.

RONAI, Z., AVRAHAM, H. & SULITZEANU, D. (1981) Autoantibodies to red blood cells in rats infected with *Plasmodium berghei*. *Journal of Parasitology*, **67**, 351.

ROSENBERG, E.B., STRICKLAND, G.T., YONG, S. & WHALEN, G.E. (1973) IgM antibodies to red cells and autoimmune anemia in patients with malaria. *American Journal of Tropical Medicine and Hygiene*, **22**, 146.

ROSENBERG, R., WIRTZ, R.A., SCHNEIDER, I. & BURGE, R. (1990) An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 209.

ROTH, E.F. Jr., FRIEDMAN, M., UEDA, Y., TELLEZ, I., TRAGER, W. & NAGEL, R.L. (1978) Sickle rates of human AS red cells infected *in vitro* with *Plasmodium falciparum* malaria. *Science*, **202**, 650.

ROTHLEIN, R., CZARJKOWSKI, M., O'NEILL, M.M., MARLIN, S.D., MAINOLFI, E. & MERLUZZI, V.J. (1988) Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by proinflammatory cytokines. *Journal of Immunology*, **141**, 1665.

ROUSILHON, C., AGARPART, M., BALLET, J-J. & BENSUSSAN, A. (1990) T lymphocytes bearing the γδ T cell receptor in patients with acute *Plasmodium falciparum* malaria. *Journal of Infectious Diseases*, **162**, 283.

RUSSEL, P.F., WEST, L.S., MANWELL, R.D. & McDONALD, G. (1963) *Practical Malariology*, 2nd edition, Oxford University Press, Oxford & London.

SALMERON, G. & LIPSKY, P.E. (1983) Immunosuppressive potential of antimalarials. *American Journal of Medicine*, **75** (Suppl.), 19.

SAUL, A., COOPER, J., INGRAM, L., ANDERS, R.F. & BROWN, G.V. (1985) Invasion of erythrocytes *in vitro* by *Plasmodium falciparum* can be inhibited by monoclonal antibody directed against an S antigen. *Parasite Immunology*, **7**, 587.

SAUL, A., MYLER, P., SCHOFIELD, L. & KIDSON, C. (1984) A high molecular weight antigen in *Plasmodium falciparum* recognised by inhibitory monoclonal antibodies. *Parasite Immunology*, **6**, 39.

SAUVAGER, F. & FAUCONNIER, B. (1978) The protective effect of endogenous interferon in mouse malaria, as demonstrated by the use of anti-interferon globulins. *Biomedicine*, **29**, 184.

SCHMIDT-ULRICH, R., BROWN, J., WHITTLE, H. & LIN, P. (1986) Human-human hybridomas secreting monoclonal antibodies to the M_r 195,000 *Plasmodium falciparum* blood stage antigen. *Journal of Experimental Medicine*, **163**, 179.

SCHMITZ, J.M., ASSENMACHER, M. & RADBRUCH, A. (1993) Regulation of T helper cytokine expression: functional dichotomy of antigen presenting cells. *European Journal of Immunology*, **23**, 191.

SCHNITZER, B., SODEMAN, T., MEAD, M.L. & CONTACOS, P.G. (1972) Pitting function of the spleen in malaria. Ultrastructural observations. *Science*, **177**, 175.

SCHOFIELD, L. (1989) T cell immunity to malaria sporozoites. *Experimental Parasitology*, **68**, 357.

SCHOFIELD, L., FERREIRA, A., ALTSZULAR, R., NUSSENZWEIG, V. & NUSSENZWEIG, R.S. (1987a) Interferon-gamma inhibits the intrahepatocytic development of malaria exoerythrocytic forms *in vitro*. *Journal of Immunology*, **139**, 2020.

SCHOFIELD, L., VILLAQUIREN, J., FERREIRA, A., SCHELLEKENS, H., NUSSENZWEIG, R.S. & NUSSENZWEIG, V. (1987b) Gamma-interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. *Nature*, **330**, 664.

SCHWARTZ, R.H., YANO, A. & PAUL, W.E. (1978) Interaction between antigen-presenting cells and primed T lymphocytes: an assessment of Ir gene expression in the antigen-presenting cell. *Immunology Reviews*, **40**, 153.

SCUDERI, P., LAM, K.S., RYAN, K.J., PETERSON, E., STERLING, K.E., FINLEY, P.R., RAY, C.G., SLYMEN, D.J. & SOLOMON, S.E. (1986) Raised serum levels of tumour necrosis factor in parasite infections. *Lancet*, **ii**, 1364.

SEEGER, R.C. & OPPENHEIM, J.J. (1970) Synergistic interaction of macrophages and lymphocytes in antigen-induced transformation of lymphocytes. *Journal of Experimental Medicine*, **132**, 44.

SEMPERTEQUI, F., ESTRELLA, B., MOSCOSO, J., PIEDRAHITA, L., HERNANDEZ, D., GAYBOR, J., NARANJO, P., MANCERO, O., ARIAS, S., BERNAL, R., CORDOVA, M.E., SUAREZ, J. & ZICKER, F. (1994) Safety, immunogenicity and protective effect of the SPf66 malaria synthetic vaccine against *Plasmodium falciparum* infection in a randomised double-blind placebo-controlled field trial in an endemic area of Ecuador. *Vaccine*, **12**, 337.

SENALDI, G., KREMSNER, P.G. & GRAU, G.E. (1992) Nitric oxide and cerebral malaria. *Lancet*, **340**, 1554.

SETHI, K.K. (1983) Contribution of macrophage arginase in the intrinsic restriction of herpes simplex virus replication in permissive macrophage cultures induced by gamma-interferon containing products of activated spleen cells. *Immunobiology*, **165**, 459.

SHAPER, A.G., KAPLAN, M.I., MODY, N.J. & McINTYRE, P.A. (1968) Malarial antibodies and autoantibodies to heart and other tissues in the immigrant and indigenous peoples of Uganda. *Lancet*, **i**, 1342.

SHEAGREN, J.N., TOBIE, J.E., FOX, L.M. & WOLFF, S.M. (1970) Reticuloendothelial system phagocytic function in naturally acquired human malaria. *Journal of Laboratory and Clinical Medicine*, **75**, 481.

SHEAR, H.L. (1984) Murine malaria: immune complexes inhibit Fc receptor-mediated phagocytosis. *Infection and Immunity*, **44**, 130.

SHEAR, H.L., NUSSENZWEIG, R.S. & BIANCO, C. (1979) Immune phagocytosis in murine malaria. *Journal of Experimental Medicine*, **149**, 1288.

SHEAR, H.L., SRINIVASAN, R., NOLAN, T. & NG, C. (1989) Role of IFN-γ in lethal and non-lethal malaria in susceptible and resistant murine hosts. *Journal of Immunology*, **143**, 2038.

SHER, A. & COFFMAN, R.L. (1992) Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annual Review of Immunology*, **10**, 385.

SHERMAN, I.W. & GREENAN, J.R.T. (1986) *Plasmodium falciparum*: regional differences in lectin and cationised ferritin binding to the surface of the malaria-infected human erythrocyte. *Parasitology*, **93**, 17.

SHERMAN, I.W. & WINOGRAD, E. (1990) *Plasmodium* and the infected erythrocyte: antigens on the *Plasmodium falciparum* infected erythrocyte are not parasite derived. *Parasitology Today*, **6**, 317.

SHERMAN, I.W., GREENAN, J.R.T. & DE LA VEGA, P. (1988) Immunofluorescent and immunoelectron microscopic localisation of protein antigens in red cells infected with the human malaria *Plasmodium falciparum*. *Annals of Tropical Medicine and Parasitology*, **82**, 531.

SHIN, S.C.J., VANDERBERG, J.F. & TERZAKIS, J.A. (1982) Direct infection of hepatocytes by sporozoites of *Plasmodium berghei*. *Journal of Protozoology*, **29**, 448.

SHORTT, H.E. & GARNHAM, P.C.C. (1948a) Demonstration of a persisting exoerythrocytic cycle in *Plasmodium cynomolgi* and its bearing on the production of relapses. *British Medical Journal*, **i**, 1225.

SHORTT, H.E. & GARNHAM, P.C.C. (1948b) The pre-erythrocytic development of *Plasmodium cynomolgi* and *Plasmodium vivax*. *Transactions of the Royal Society of Tropical Medicine and hygiene*, **41**, 785.

SHORTT, H.E. (1948) The life cycle of *Plasmodium cynomolgi* in its insect and mammalian hosts. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **42**, 227.

SHUNGU, D.M. & ARNOLD, J.D. (1972) Induction of growth and division synchrony in *Plasmodium vinckei chabaudi* by photoperiodic rhythm. *Journal of Parasitology*, **58**, 142.

SIDDIQUI, W.A., TAM, L.Q., KRAMER, K.J., HUI, G.S.N., YAMAGA, K.M., CHANG, S.P., CHAN, E.B.T. & KAN, S-C. (1987) Merozoite surface coat precursor protects *Aotus* monkeys against *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Science* USA, **84**, 3014.

SINDEN, R.E. & SMALLEY, M. (1976) Gametocytes of *Plasmodium falciparum*: phagocytosis by leucocytes *in vivo* and *in vitro*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **70**, 344.

SINDEN, R.E. & SMITH, J.E. (1982) The role of the Kuppfer cell in the infection of rodents by sporozoites of *Plasmodium*: uptake of sporozoites by perfused liver and establishment of infection *in vivo*. *Acta tropica*, **39**, 11.

SINDEN, R.E. & STRONG, K. (1978) An ultrastructural study of the sporogonic development of *Plasmodium falciparum* in *Anopheles gambiae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **72**, 477.

SINDEN, R.E. (1981) Sexual development of malarial parasites in their mosquito vectors. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **75**, 171.

SINGER, I. (1954) The cellular reactions to infections with *Plasmodium berghei* in the white mouse. *Journal of Infectious Diseases*, **94**, 241.

SINIGAGLIA, F. & PINK, J.R.L. (1985) Human T lymphocyte clones specific for malaria (*Plasmodium falciparum*) antigens. *EMBO*, **13B**, 3819.

SLADE, S.J. & LANGHORNE, J. (1989) Production of interferon-gamma during infection of mice with *Plasmodium chabaudi chabaudi*. *Immunobiology*, **179**, 353.

SLATER, A.F.G. & CERAMI, A. (1992) Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. *Nature*, **355**, 167.

SLUITER, W., HULSING-HESSELINK, E., ELZENGA-CLAASEN, I. & VAN FURTH, R. (1985) Method to select mice in the steady state for biological studies. *Journal of Immunological Methods*, **760**, 135.

SMALLEY, M.E., ABDALLA, S. & BROWN, J. (1980) The distribution of *Plasmodium falciparum* in the peripheral blood and bone marrow of Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **75**, 103.

SMITH, J.E., SINDEN, R.E., BEADLE, J. & HARTLEY, R.H. (1981) The effect of silica treatment on the uptake and infectivity of *Plasmodium yoelii* sporozoites *in vitro* and *in vivo*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **75**, 605.

SMITH, K.A. (1984) Interleukin-2. *Annual Review of Immunology*, **2**, 319.

SMITH, P.K., KROHN, R.I., HERMANSON, G.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, **150**, 76.

SMRKOVSKI, L.L. (1981) Effect of route of *Mycobacterium bovis* BCG administration on induced suppression of sporozoite immunity in rodent malaria. *Infection and Immunity*, **31**, 408.

SMYTHE, J., COPPEL, R.L., BROWN, G.V., RAMASAMY, R., KEMP, D.J. & ANDERS, R.F. (1988) Identification of two integral membrane proteins of *Plasmodium falciparum*. *Proceedings of the National Academy of Science* USA, **85**, 5195.

SNAPPER, C.M. & PAUL, W.E. (1987) Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science*, **236**, 944.

SNAPPER, C.M., FINKELMAN, F.D. & PAUL, W.E. (1988) Regulation of IgG_1 and IgE production by interleukin 4. *Immunology Reviews*, **102**, 51.

SNICK, J. VAN. (1990) Interleukin 6: an overview. *Annual Review of Immunol*, **8**, 253.

SOLOWAY, P., FISH, S., PASSMORE, H., GEFTER, M., COFFEE, R. & MANSER, T. (1991) Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *Journal Of Experimental Medicine*, **174**, 847.

SPENCER, H.C., MILLER, L.H., COLLINS, W.E., KNUD-HANSEN, C., McGINNIS, M.H., SHIROISHI, T., LOBAS, R.A. & FELDMAN, R.A. (1978) The Duffy blood group and resistance to *Plasmodium vivax* in Honduras. *American Journal of Tropical Medicine and Hygiene*, **27**, 664.

SPIRA, D.T., SILVERMAN, P.H. & GAINES, C. (1970) Anti-thymocyte serum effects on *Plasmodium berghei* infection in rats. *Immunology*, **19**, 759.

SPITALNY, G.L. & NUSSENZWEIG, R.S. (1973) *Plasmodium berghei*: relationship between protective immunity and anti-sporozoite (CSP) antibody in mice. *Experimental Parasitology*, **33**, 168.

SPITALNY, G.L., RIVERA-ORTIZ, C.I. & NUSSENZWEIG, R.S. (1976) *Plasmodium berghei*: the spleen in sporozoite induced immunity to mouse malaria. *Experimental Parasitology*, **40**, 179.

SPITALNY, G.L., VERHAVE, J.P., MEUWISSEN, J.H.F. & NUSSENZWEIG, R.S. (1977) *Plasmodium berghei*: T cell-dependence of sporozoite-induced immunity in rodents. *Experimental Parasitology*, **42**, 73.

SPITZ, S. (1946) Pathology of acute falciparum malaria. *Military Medicine*, **99**, 555.

SRICHAIKUL, T., PUWASATIEN, P., KAMJANAJETANEE, J. & BOKISH, V.A. (1975) Complement changes and disseminated intravascular coagulation in *Plasmodium falciparum* malaria. *Lancet*, **i**, 770.

STAUNTON, D., MARLIN, S.D., STRATOWA, C., DUSTIN, M.L. & SPRINGER, S.D. (1988) Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell*, **52**, 925.

STECHSCHULTE, D.J. (1969) Cell-mediated immunity in rats infected with *Plasmodium berghei*. *Military Medicine*, **134** (Suppl), 1147.

STECHSCHULTE, D.J., BRIGGS, N.T. & WELLDE, B.T. (1969) Characterisation of protective antibodies produced in *Plasmodium berghei* infected rats. *Military Medicine*, **134** (Suppl), 1140.

STEVENSON, M.M. & GHADIRIAN, E. (1989) Human recombinant TNF-α protects susceptible A/J mice against lethal *Plasmodium chabaudi* AS infection. *Infection and Immunity*, **57**, 3936.

STEVENSON, M.M. & TAM, M.F. (1993) Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice. *Clinical and Experimental Immunology*, **92**, 77.

STEVENSON, M.M., FONG TAM, M., BELOSEVIC, M., VAN DER MEIDE, P.H. & PODOBA, J.E. (1990a) Role of endogenous gamma interferon in host response to infection with blood-stage *Plasmodium chabaudi* AS. *Infection and Immunity*, **58**, 3225.

STEVENSON, M.M., HUANG, D.Y., PODOBA, J.E. & NOWOTARSKI, M.E. (1992) Macrophage activation during *Plasmodium chabaudi*AS infection in resistant C57Bl/6 and susceptible A/J mice. *Infection and Immunity*, **60**, 1193.

STEVENSON, M.M., LYANGA, J.J. & SKAMENE, E. S (1982) Murine malaria: genetic control of resistance for *Plasmodium chabaudi*. *Infection and Immunity*, **38**, 80.

STEVENSON, M.M., NESBITT, M.N. & SKAMENE, (1988) Chromosomal location of the gene determining resistance to *Plasmodium chabaudi* AS. *Current Topics in Microbiology and Immunology*, **37**, 325.

STEVENSON, M.M., NOWOTARSKI, M. & YAP, G. (1990b) Review: cytokines and malaria. *Clinical and Investigative Medicine*, **13**, 353.

STEVENSON, M.M., TAM, M.F. & NOWOTARSKI, M. (1990c) Role of interferon-γ and tumor necrosis factor in host resistance to *Plasmodium chabaudi* AS. *Immunology Letters*, **25**, 115.

STUEHR, D.J. & MARLETTA, M.A. (1987) Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *Journal of Immunology*, **139**, 518.

STUEHR, D.J. & NATHAN, C.F. (1989) Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumot target cells. *Journal of Experimental Medicine*, **169**, 1543.

STURCHLER, D. (1989) How much malaria is there worldwide? *Parasitology Today*, **5**, 39.

SUSS, G., EICHMANN, K., KURY, E., LINKE, A. & LANGHORNE, J. (1988) Roles of CD4- and CD8- bearing T lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi*. *Infection and Immunity*, **56**, 3081.

SWAIN, S.L., BRADLEY, L.M., CROFT, M., TONKONOGY, S., ATKINS, G., WEINBERG, A.D., DUNCAN, D.D., HEDRICK, S.M., DUTTON, R.W. & HUSTON, G. (1991) Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunology Reviews*, **123**, 115.

TALIAFERRO, W.H. & CANNON, P.R. (1936) The cellular reactions during primary infections and superinfections of *Plasmodium brasilianum* in Panamanian monkeys. *Journal of Infectious Diseases*, **59**, 72.

TALIAFERRO, W.H. & MULLIGAN, H.W. (1937) The histopathology of malaria with special reference to the function and origin of the macrophages in defense. *Indian Medical Research Memoirs*, **29**, 1.

TALIAFERRO, W.H. & TALIAFERRO, L.G. (1944) The effect of immunity on the asexual reproduction of *Plasmodium brasilianum*. *Journal of Infectious Diseases*, **75**, 1.

TALIAFERRO, W.H. & TALIAFERRO, L.G. (1951) The effect of X-rays on immunity: a review. *Journal of Immunology*, **66**, 181.

TALIAFERRO, W.H. (1929) *The Immunology of Parasitic Infections*. Century, New York.

TANABE, K., IZUMO, A. & KAGEYAMA, K. (1986) Growth of *Plasmodium falciparum* in sodium-enriched erythrocyte. *American Journal of Tropical Medicine and Hygiene*, **35**, 476.

TARGETT, G.A.T. & VOLLER, A. (1965) Studies on antibody levels during vaccination of Rhesus monkeys against *Plasmodium knowlesi*. *British Medical Journal*, **ii**, 1104.

TARGETT, G.A.T. (1988) *Plasmodium falciparum*: natural and experimental transmission-blocking immunity. *Immunology Letters*, **19**, 235.

TARGETT, G.A.T. (1990) Immunity to sexual stages of human malaria parasites: immune modulation during natural infections, antigenic determinants, and the induction of transmission-blocking immunity. *Scandinavian Journal of Infectious Diseases*, **76** (Suppl.), 79.

TAVERNE, J., BARE, C.A.W., SARKAR, D.A., MEAGER, A., ROOK, G.A.W. & PLAYFAIR, J.H.L. (1990) human and murine macrophages produce TNF in response to soluble antigens of *Plasmodium falciparum*. *Parasite Immunology*, **12**, 33.

TAVERNE, J., DOCKRELL, H.M. & PLAYFAIR, J.H.L. (1981) Endotoxin induced serum factor kills malaria parasites *in vitro*. *Infection and Immunity*, **33**, 83.

TAVERNE, J., TREAGUST, J.D. & PLAYFAIR, J.H.L. (1986) Mcrophage activity in lethal and non-lethal murine malaria and the effect of vaccination. *Clinical and Experimental Immunology*, **66**, 44.

TAYLOR, P.M., THOMAS, D.B. & MILLS, K.H.G. (1987) *In vitro* culture of T cell lines and clones. In: *Lymphocytes: a Practical Approach* (ed. G.C.B. Klaus), pp 133. IRL Press, Oxford.

TAYLOR-ROBINSON, A.W. & PHILLIPS, R.S. (1992) Functional characterisation of protective $CD4^+$ T-cell clones reactive to the murine malaria parasite *Plasmodium chabaudi*. *Immunology*, **77**, 99.

TAYLOR-ROBINSON, A.W. & PHILLIPS, R.S. (1993) Protective $CD4^+$ T-cell lines raised against *Plasmodium chabaudi* show characteristics of either T_H1 or T_H2 cells. *Parasite Immunology*, **15**, 301.

TAYLOR-ROBINSON, A.W. & PHILLIPS, R.S. (1994a) T_H1 and T_H2 $CD4^+$ T cell clones specific for *Plasmodium chabaudi* but not for an unrelated antigen protect against blood stage *Plasmodium chabaudi* infection. *European Journal of Immunology*, **24**, 158.

TAYLOR-ROBINSON, A.W. & PHILLIPS, R.S. (1994b) B cells are required for the switch from T_H1- to T_H2-regulated immune responses to *Plasmodium chabaudi chabaudi* infection. *Infection and Immunity*, **62**, in press.

TAYLOR-ROBINSON, A.W. & PHILLIPS, R.S. (1994c) Expression of the Il-1 receptor discriminates T_H2 from T_H1 cloned $CD4^+$ T cells specific for *Plasmodium chabaudi*. *Immunology*, **81**, 216.

TAYLOR-ROBINSON, A.W. (1991) The Ly-4^+ T lymphocyte subset in.the host immune response to the asexual stages of *Plasmodium chabaudi chabaudi*. PhD Thesis, University of Glasgow.

TAYLOR-ROBINSON, A.W., LIEW, F.Y., SEVERN, A., XU, D., McSORLEY, S.J., GARSIDE, P., PADRO, J. & PHILLIPS, R.S. (1994) Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *European Journal of Immunology*, **24**, 980.

TAYLOR-ROBINSON, A.W., PHILLIPS, R.S., SEVERN, A., MONCADA, S. & LIEW, F.Y. (1993) The role of T_H1 and T_H2 cells in a rodent malaria infection. *Science*, **260**, 1931.

TEUSCHER, T., ARMSTRONG SCHELLENBERG, J.R.M., BASTOS DE AZEVEDO, I., HURT, N., SMITH, T., HAYES, R., MASANJA, H., SILVA, Y., LOPEZ, M.C., KITUA, A., KILAMA, W., TANNER, M. & ALONSO, P.L. (1994) SPf66, a chemically synthesised subunit malaria vaccine, is safe and immunogenic in Tanzanians exposed to intense malaria transmission. *Vaccine*, **12**, 328.

THAITHONG, S., BEALE, G.H., FENTON, B., McBRIDE, J., ROSARIO, V., WALKER, A. & WALLIKER, D. (1984) Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **78**, 242.

THEANDER, T.G., BYGBERG, I.C., JACOBSEN, L., LARSEN, P.B. & KHARAZMI, A. (1986a) Low parasite specific T-cell response in clinically immune individuals with low grade *Plasmodium falciparum* parasitaemia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 1000.

THEANDER, T.G., BYGBJERG, I.C., ANDERSON, B.J., JEPSEN, S., KHARSAZMI, A. & ODUM, N. (1986b) Suppression of parasite-specific responses in *Plasmodium falciparum* malaria. A longitudinal study of blood mononuclear cell proliferation and subset composition. *Scandinavian Journal of Immunology*, *24*, *73*.

TIETZ, P., YAMAZAKI, K. & LARUSSO, N.F. (1990) Time-dependent effect of chloroquine on pH of hepatocyte lysosomes. *Biochemical Pharmacology*, **6**, 1419.

TITUS, R.G., SHERRY, B. & CERAMI, A. (1991) The involvement of TNF, Il-1 and Il-6 in the immune response to protozoan parasites. *Immunology Today*, **12**, A13.

TRAGER, W. & JENSON, J.B. (1976) Human malaria parasites in continuous culture. *Science*, **193**, 673.

TRAGER, W. (1987) The cultivation of *Plasmodium falciparum*: applications in basic and applied research on malaria. *Annals of Tropical Medicine and Parasitology*, **81**, 511.

TRIST, D.G. & WEATHERALL, M. (1981) Inhibition of lymphocyte transformation by mepacrine and chloroquine. *Journal of Pharmacy and Pharmacology*, **33**, 434.

TRIZIO, D. & CUDKOWICZ, G. (1974) Separation of T and B lymphocytes by nylon wool columns: Evaluation of efficacy by functional assays in vivo. *Journal of Immunology*, **113**, 1093.

TROPICAL DISEASES REPORT (1989) Progress in International Research 1987-1988. *Ninth Programme Report of the UNDP/World Bank/WHO Special Report for Research and Training in Tropical Diseases*. WHO, Geneva.

TROYE-BLOMBERG, M. & PERLMANN, P. (1988) T cell functions in *Plasmodium falciparum* and other malarias. *Progress in Allergy*, **41**, 253.

TROYE-BLOMBERG, M., ANDERSSON, G., STOCZKOWSKA, M., SHABO, R., ROMERO, P., PATARROYO, M.E., WIGZELL, H. & PERLMANN, P. (1985) Production of Il-2 and IFN-γ by T cells from malaria patients in response to *Plasmodium* or erythrocyte antigens *in vitro*. *Journal of Immunology*, **135**, 3498.

TROYE-BLOMBERG, M., KABILAN, L., ANDERSSON, G., PATARROYO, M.E. & PERLMANN, P. (1987) Cellular regulation of the immune response in *Plasmodium falciparum* malaria *in vitro*. In: *Immune Regulation by Characterised Polypeptides* Vol.7 (eds. G. Goldstein, J-F. Bach & H. Wigzell), p.699. Alan R. Liss Inc., New York.

TROYE-BLOMBERG, M., PERLMANN, H., PATARROYO, M.E. & PERLMANN, P. (1983b) Regulation of the immune response in *Plasmodium falciparum* malaria. II. Antigen specific proliferative responses *in vitro*. *Clinical and Experimental Immunology*, **53**, 345.

TROYE-BLOMBERG, M., SJOBERG, K., OLERUP, O., RILEY, E.M., KABILAN, L., PERLMANN, H., MARBIAH, N.T. & PERLMANN, P. (1990) Characterisation of regulatory T cell responses to defined immunodominant T cell epitopes of the *Plasmodium falciparum* antigen Pf155/RESA. *Immunology Letters*, **25**, 129.

TROYE-BLOMBERG, M., SJOHOLM, P-E., PERLMANN, H., PATARROYO, M.E. & PERLMANN, P. (1983a) Regulation of the immune response in *Plasmodium falciparum* malaria. I. Non-specific proliferative responses *in vitro* and characterisation of lymphocytes. *Clinical and Experimental Immunology*, **53**, 235.

TSUJI, M., MOMBAERTS, P., LEFRANCOIS, L., NUSSENZWEIG, R.S., ZAVALA, F. & TONEGAWA, S. (1994) γδ T cells contribute to immunity against the liver stages of malaria in αβ-T cell-deficient mice. *Proceedings of the National Academy of Science* USA, **91**, 345.

TSUJI, M., ROMERO, P., NUSSENZWEIG, R.S. & ZAVALA, F. (1990) $CD4^+$ cytolytic T cell clone confers protection against murine malaria. *Journal of Experimental Medicine*, **172**, 1353.

UDEINYA, I.J., SCHMIDT, J.A., AIKAWA, M., MILLER, L.H. & GREEN, I. (1981) Falciparum malaria-infected erythrocytes specifically bind to cultured human endothelial cells. *Science*, **213**, 555.

UDOMSANGPETCH, R., LUNDGREN, K., BERZINS, K., WAHLIN, B., PERLMANN, H., TROYE-BLOMBERG, M., CARLSSON, J., WAHLGREN, M., PERLMANN, P. & BJORKMAN, A. (1986) Human monoclonal antibodies to Pf155, a major antigen of malaria parasite *Plasmodium falciparum*. *Science*, **231**, 57.

UNANUE, E.R. (1989) Macrophages, antigen presenting cells, and the phenomena of antigen handling and presentation, pp. 95-115. In: *Fundamental Immunology*, 2nd edition (ed. W.E. Paul), Raven Press, New York.

VALERO, M.V., AMADOR, L.R., GALINDO, C., FIGUEROA, J., BELLO, M.S., MURILLO, L.A., MORA, A.L., PATARROYO, G., ROCHA, C.L., ROJAS, M., APONTE, J.J., SARMIENTO, L.E., LOZADO, D.M., CORONELL, C.G., ORTEGA, N.M., ROSAS, J.E., ALONSO, P.L. & PATARROYO, M.E. (1993) Vaccination with SPf66, a chemically synthesised vaccine, against *Plasmodium falciparum* malaria in Colombia. *Lancet*, **341**, 705.

VAN DER HEYDE, H.C., MANNING, D.D., ROOPENIAN, D.C. & WEIDANZ, W.P. (1993a) Resolution of blood-stage malarial infections in $CD8^+$ cell-deficent $\beta 2\text{-}m^{0/0}$ mice. *Journal of Immunology*, **151**, 3187.

VAN DER HEYDE, MANNING, D.D. & WEIDANZ, W.P. (1993b) Role of $CD4^+$ T cells in the expansion of $CD4^-CD8^-$ γδ T cell subset in the spleens of mice during blood-stage malaria. *Journal of Immunology*, **151**, 6311.

VAN DER MEID, J.W.M., ENDRES, S., LONNEMAN, G., CANNON, J.G., IKEJIMA, T., OKUSAWA, S., GELFAND, J.A. & DINARELLO, C.A. (1988) Concentrations of immunoreactive human tumor necrosis factor alpha produced by human mononuclear cells *in vitro*. *Journal of Leucocyte Biology*, **43**, 216.

VAN MEIRVENNE, N., JANSSENS, P.G. & MAGNUS, E. (1975) Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei* I. Rationalization of the experimental approach. *Ann. Soc. Belge Méd.Trop.*, **55**, 1.

VAN SCHRAVENDIJK, M-R. ROCK, E.P., MARSH, K., ITO, Y., AIKAWA, M., NEEQUAYE, J., OFORIADJEI, D., RODRIQUEZ, R., PATARROYO, M.E. & HOWARD, R.J. (1991) Characterisation and localisation of *Plasmodium falciparum* surface antigens on infected erythrocytes from West African patients *Blood*, **78**, 226.

VAN WAUWE, J., DE MAY, J.R. & GOOSSENS, J.G. (1980) OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. *Journal of Immunology*, **124**, 2708.

VANDERBERG, J.P. (1975) Development of infectivity by the *Plasmodium berghei* sporozoite. *Journal of Parasitology*, **61**, 43.

VERDRAGER, J. (1964) Observations on the longevity of *Plasmodium falciparum*: with special reference to findings in Mauritius. *Bulletin WHO*, **54**, 685.

VERHAVE, J.P., STRICKLAND, G.T., JAFFE, H.A. & AHMAD, A. (1978) Studies on the transfer of protective immunity with lymphoid cells from mice immune to malaria sporozoites. *Journal of Immunology*, **121**, 1031.

VERMEULEN, A.N., PONNUDURAI, T., BECKERS, P.J.A., VERHAVE, J.P., SMITS, M.A. & MEUWISSEN, J.H.E.T. (1985) Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. *Journal of Experimental Medicine*, **162**, 1460.

VICKERMAN, K. & COX, F.E.G. (1967) *The Protozoa*, London, John Murray.

VINCKE, I.H. & LIPS, M. (1948) Un nouveau *Plasmodium* d'un rongeur sauvage du Congo, *Plasmodium berghei* n.sp.. *Ann. Soc. Belge Méd. Trop*, **28**, 97.

VINCENDEAU, P., DAULOUEDE, S., VEYRET, B., DARDE, M.L., BOUTEILLE, B. & LEMESRE, J.L. (1992) Nitric oxide-mediated cytostatic activity on *Trypanosoma brucei gambiense* and *Trypanosoma brucei brucei*. *Experimental Parasitology*, **75**, 353.

VINETZ, J.M., KUMAR, S., GOOD, M.F., FOWLKES, B.J., BERZOFSKY, J.A. & MILLER, L.H. (1990) Adoptive transfer of $CD8^+$ T cells from immune animals does not transfer immunity to blood stage *Plasmodium yoelii* malaria. *Journal of Immunology*, **144**, 1069.

VOLLER, A. & ROSSAN, R.N. (1969) Immunological studies with simian malarias. I. Antigenic variants of *Plasmodium cynomolgi bastianelli*. *Transactions of the Royal Society of Medicine and Hygiene*, **63**, 46.

VOLLER, A. (1964) Fluorescent antibody methods and their use in malaria research. *Bulletin of the World Health Organisation*, **30**, 343.

VON DER WEID, T. & LANGHORNE, J. (1993a) The roles of cytokines produced in the immune response to the erythrocytic stages of mouse malarias. *Immunobiology*, **189**, 397.

VON DER WEID, T. & LANGHORNE, J. (1993b) Altered response of $CD4^+$ T cell subsets to *Plasmodium chabaudi chabaudi* in B cell-deficient mice. *International Immunology*, **5**, 1343.

VREDEN, S.G., VAN DEN BROEK, M.F., OETTINGER, M.C., VERHAVE, J.P., MEUWISSEN, J.H., SAUERWEIN, R.W. (1992) Cytokines inhibit the development of liver schizonts of the malaria parasite *Plasmodium berghei in vivo*. *European Journal of Immunology*, **22**, 2271.

WAHLGREN, M., BERZINS, K., PERLMANN, P. & BJORKMAN, A. (1983) Characterisation of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of micro ELISA. *Clinical and Experimental Immunology*, **54**, 127.

WAKI, S., YONOME, I. & SUSUKI, M. (1986) *Plasmodium yoelii*: induction of attenuated mutants by irradiation. *Experimental Parasitology*, **62**, 316.

WALDMANN, H. & MILSTEIN, C. (1982) Monoclonal antibodies. In: *Clinical Aspects of Immunology* (eds. P.J. Lachmann & K. Peters), pp.476. Blackwell Scientific Press, Oxford.

WALLACE, W.J. & CAUGHEY, W.S. (1975) Mechanism for the autoxidation of hemoglobin by phenols, nitrite and "oxidant" drugs. Peroxide formation by one electron donation to bound dioxygen. *Biochemical and Biophysical Research Communications*, **62**, 561.

WALLIKER, D. (1983) The genetic basis of diversity in malaria parasites. *Advances in Parasitology*, **22**, 217.

WALLIKER, D., CARTER, R. & MORGAN, S. (1971) Genetic recombination in malaria parasites. *Nature*, **232**, 561.

WALLIKER, D., CARTER, R. & SANDERSON, A. (1975) Genetic studies on *Plasmodium chabaudi*: recombination between enzyme markers. *Parasitology*, **70**, 19.

WALLIKER, D., QUAKYI, I.A., WELLEMS, T.E., McCUTCHAN, T.F., SZARFMAN, A., LONDON, W.T., CORCORAN, L.M., BURKOT, T.R. & CARTER, R. (1987) Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science*, **236**, 1661.

WARD, K.N., WARRELL, M.J., RHODES, J., LOOAREESUWAN, S. & WHITE, N.J. (1984) Altered expression of human monocyte Fc receptors in *Plasmodium falciparum* malaria. *Infection and Immunity*, **44**, 623.

WARHURST, D.C. (1966) Bioassay of *Plasmodium berghei*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **60**, 6.

WARRELL, D.A. (1987) Pathophysiology of severe falciparum malaria in man. *Parasitology*, **94**, 553.

WASH, A.C. (1979) Cerebral malaria. *Archives of Neurology Chicago*, **36**, 119.

WASSERMAN, G.M., KUMAR, S., AHLERS, J., RAMSDELL, F., BERZOFSKY, J.A. & MILLER, L.H. (1993) An approach to development of specific T-lymphocyte lines by use of pre-processed antigens in *Plasmodium vinckei vinckei* murine malaria. *Infection and Immunity*, **61**, 1958.

WEATHERALL, D.J. (1987) Common genetic disorders of the red cell and the 'malaria hypothesis'. *Annals of Tropical Medicine and Parasitology*, **81**, 539.

WEATHERALL, D.J. (1990) Mice, men and sickle cells. *Nature*, **343**, 121.

WEBER, J.L. & HOCKMEYER, W.T. (1985) Structure of the circumsporozoite protein gene in 18 strains of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **15**, 305.

WEIDANZ, W.P. & LONG, C.A. (1988) The role of T cells in immunity to malaria. *Progress in Allergy*, **41**, 215.

WEIDANZ, W.P. (1982) Malaria and alterations in immune reactivity. *British Medical Bulletin*, **38**, 167.

WEINBAUM, F.I., EVANS, C.B. & TIGELAAR, R.E. (1976a) An *in vitro* assay for T cell immunity to malaria in mice. *Journal of Immunology*, **116**, 1280.

WEINBAUM, F.I., EVANS, C.B. & TIGELAAR, R.E. (1976b) Immunity to *Plasmodium berghei yoelii* in mice. I. The course of infection in T-cell and B-cell deficient mice. *Journal of Immunology*, **117**, 1280.

WEISS, L. (1979) The mechanics of blood flow through the spleen. In: *Role of the Spleen in the Immunology of Parasitic Diseases*. Tropical Diseases Research Series 1, p.6. Schwabe & Co., Basel.

WEISS, L. (1983a) The red pulp of the spleen. Structural basis of blood flow. In: *Clinics in Haematology*, **12**, 373. W.B. Saunders, London, Philadelphia & Toronto.

WEISS, L. (1983b) Hematopoietic tissue in malaria: facilitation of erythrocytic recycling by bone marrow in *Plasmodium berghei*-infected mice. *Journal of Parasitology*, **69**, 307.

WEISS, L. (1989) Mechanisms of splenic control of splenic control of murine malaria: cellular reactions of the spleen in lethal (strain 17XL) *Plasmodium yoelii* malaria in BALB/c mice, and the consequences of pre-infective splenectomy. *American Journal of Tropical Medicine and*

Hygiene, **41**, 144.

WEISS, L. (1990) The spleen in malaria: the role of barrier cells. *Immunology Letters*, **25**, 165.

WEISS, L., GEDULDIG, U. & WEIDANZ, W. (1986) Mechanisms of splenic control of murine malaria: reticular cell activation and the development of a blood-spleen barrier. *American Journal of Anatomy*, **176**, 251.

WEISS, W.R. (1990) Host-parasite interactions and immunity to irradiated sporozoites. *Immunology Letters*, **25**, 39.

WEISS, W.R., GOOD, .M.F., HOLLINGDALE, M.R., MILLER, L.H. & BERZOFSKY, J.A. (1989) Genetic control of immunity to *Plasmodium yoelii* sporozoites. *Journal of Immunology*, **143**, 4263.

WEISS, W.R., SEDEGAH, M., BEAUDOIN, R.L., MILLER, L.H. & GOOD, M.F. (1988) $CD8^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunised with malaria sporozoites. *Proceedings of the National Academy of Science*USA, **85**, 573.

WELCH, S.G. (1977) The Duffy blood group and malaria prevalence in Gambian West Africans. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **71**, 295.

WELLS, R.A. & DIGGS, C.L. (1976) Protective activity in sera from mice immunised against *Plasmodium berghei*. *Journal of Parasitology*, **62**, 638.

WELLS, R.A., PAVANAND, K., XZOLYOMI, S., PERMPANICH, B. & McDERMOTT, R.P. (1980) Anti-lymphocyte antibodies in sera of Thai adults infected with *Plasmodium falciparum* or *P. vivax*. *Clinical and Experimental Immunology*, **39**, 663.

WESTENBERGER, U., THANNER, S., RUF, H., GERSONDE, K., SUTTER, G. & TRENTZ, O. (1990) Formation of free radicals and nitric oxide derivative of hemoglobin in rats during toxic shock. *Free Radical Research Communications*, **11**, 167.

WHITTLE, H.C., BROWN, J., MARSH, K., GREENWOOD, B.M., SEIDELIN, P., TIGHE, H. & WEDDERBURN, L. (1984) T-cell control of Epstein-Barr virus-infected B cells is lost during *Plasmodium falciparum* malaria. *Nature*, **312**, 449.

WHO (1990) Practical chemotherapy of malaria. *Technical Report Series*, **805**. WHO, Geneva.

WHO (1993) World malaria situation in 1991. *Weekly Epidemiological Record*, **68**, 245. WHO, Geneva.

WILLIAMSON, W.A. & GREENWOOD, B.M. (1978) Impairment of the immune response to vaccination after acute malaria. *Lancet*, **i**, 1328.

WILSON, D.B., GARNHAM, P.C.C. & SWELLINGREBEL, N. (1950) A review of hyperendemic malaria. *Tropical Disease Bulletin*, **47**, 677.

WILSON, R.J.M. (1980) Serotyping *Plasmodium falciparum* malaria with S-antigens. *Nature*, **284**, 451.

WINOGRAD, E. & SHERMAN, I.W. (1989) Characterisation of a modified red cell membrane protein expressed on erythrocytes infected with the human malaria parasite *Plasmodium falciparum*: possible role as a cytoadherent mediating protein. *Journal of Cell Biology*, **108**, 23.

WINOGRAD, E., GREENAN, J.R.T. & SHERMAN, I.W. (1987) Expression of senescent antigen on erythrocytes infected with a knobby variant of the human malaria parasite *Plasmodium falciparum*. *Proceedings of the National Academy of Science* USA, **84**, 1931.

WOOD, P.R. & CLARK, I.A. (1982) Apparent irrelevance of NK cells to resolution of infections with *Babesia microti* and *Plasmodium vinckei petteri* in mice. *Parasite Immunology*, **4**, 319.

WRIGHT, D.H. (1968) The effect of neonatal thymectomy on the survival of golden hamsters infected

with *Plasmodium berghei*. *British Journal of Experimental Pathology*, **49**, 379.

WRIGHT, D.H., MASEMBE, R.M. & BAZIRA, E.R. (1971) The effect of anti-thymocyte serum on golden hamsters and rats infected with *Plasmodium berghei*. *British Journal of Experimental Pathology*, **55**, 465.

WYLER, D.J. & GALLIN, J.I. (1977) Spleen derived mononuclear cell chemotactic factor in malaria infections: a possible mechanism for splenic macrophage accumulation. *Journal of Immunology*, **118**, 478.

WYLER, D.J. (1983) The spleen in malaria. In: *Malaria and the Red Cell. Ciba Foundation Symposium*, **94**, 98.

WYLER, D.J., OSTER, C.N. & QUINN, T.C. (1979) The role of the spleen in malarious infections. In: *Role of the Spleen in the Immunology of Parasitic Diseases*. Tropical Diseases Research Series 1, p.183. Schwabe & Co., Basel.

WYLER, D.J., QUINN, T.C. & CHEN, L-T. (1981) Relationship of alterations in splenic clearance function and microcirculation to host defence in acute rodent malaria. *Journal of Clinical Investigation*, **67**, 1400.

YAP, G.S. & STEVENSON, M.M. (1994) Inhibition of *in vitro* erythropoiesis by soluble mediators in *Plasmodium chabaudi* AS malaria: lack of a major role for interleukin-1, tumor necrosis factor alpha, and gamma-interferon. *Infection and Immunity*, **62**, 357.

YOELI, M. & HARGREAVES, B.J. (1974) Brain capillary blockage produced by a virulent strain of rodent malaria. *Science*, **184**, 572.

YOSHIDA, N., NUSSENZWEIG, R.S., POTOCNJAK, P., NUSSENZWEIG, V. & AIKAWA, M. (1980) Hybridoma produces protective antibodies against the sporozoite stage of malaria parasites. *Science*, **207**, 71.

YOUNG, M.D., EYLES, D.E. & BURGESS, R.W. (1958) Experimental testing of Negroes to *Plasmodium vivax*. *Journal of Parasitology*, **44**, 371.

ZAVALA, F., MASUDA, A., GRAVES, P.M., NUSSENZWEIG, V., & NUSSENZWEIG, R.S. (1985) Ubiquity of the repetitive epitope of the CS protein in differnet isolates of human malaria parasites. *Journal of Immunology*, **135**, 2790.

ZIJLSTRA, M., BIX, M., SIMISTER, N.E., LORING, J.M., RAULET, D.H. & JAENISCH, R. (1990) β_2-microglobulin deficient mice lack $CD4^-8^+$ cytolytic T cells. *Nature*, **344**, 742.

ZLOTNIK, A., SHIMONKAVITZ, R.P., GEFTER, M.L., KAPPLER, J. & MARRACK, P. (1983) Characterisation of the γ-interferon-mediated induction of antigen-presenting ability in P33DI cells. *Journal of Immunology*, **131**, 2814.

ZOUALI, M., DRUILHE, P., GENTILINI, M. & EYQUEM, A. (1982) High titres of anti-T antibodies and other haemagglutins in human malaria. *Clinical and Experimental Immunology*, **50**, 83.

ZUCKERMAN, A. & GOLENZER, Y. (1970) The passive transfer of protection against *Plasmodium berghei* in rats. *Journal of Parasitology*, **56**, 379.

ZUCKERMAN, A. & JACOBSEN, R.L. (1976) Adoptive transfer of immunity to *Plasmodium berghei* by a population of immune rat spleen cells resistant to cyclophosphamide. *International Journal of Parasitology*, **6**, 103.

ZUCKERMAN, A. & YOELI, M. (1954) Age and sex as factors influencing *Plasmodium berghei* infections in intact and splenectomised rats. *Journal of Infectious Diseases*, **94**, 225.

ZUCKERMAN, A. (1945) *In vitro* opsonic tests with *Plasmodium gallinaceum* and *Plasmodium lophurae*. *Journal of Infectios Diseases*, **77**, 28.

ZUCKERMAN, A. (1960) Auto-antibody in rats with *Plasmodium berghei*. *Nature*, **185**, 189.