Immunity to malaria using the rodent malaria parasite *Plasmodium chabaudi chabaudi* AS as a model of the human malaria *Plasmodium falciparum*

by

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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.

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ABBREVIATIONS

B cell	B lymphocyte
BCG	Bacille Calmette Guèrin
BSA	Bovine serum albumin
ConA	Concanavalin A
cpm	Counts per minute
CSP	Circumsporozoite protein
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
Fab	Antigen-binding fragment of immunoglobulin
Fc	Crystallisble fragment of immunoglobulin
FCS	Foetal calf serum
g	Acceleration in the earth's gravitational field
g	Gramme(s)
^{3}H-T	Tritiated thymidine
Hb	Haemoglobin
HLA	Human leukocyte antigens
hr(s)	Hour(s)
ICAM-1	Intercellular adhesion molecule-1
IFA	Indirect fluorescence antibody
IFAT	Indirect fluorescence antibody test
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneally
IU	International unit(s)
i.v.	Intravenously
kDa	Kilodalton(s)
l	Litre(s)
L-NMMA	L-N^{G}-monomethyl arginine
Log	Logarithm
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody

MHC	Major histocompatibility complex
mg	Milligramme(s)
µg	Microgramme(s)
mm	Millimetre(s)
µm	Micrometre(s)
µM	Micromolar
MOPS	Morpholinopropanesulphonic acid
MSP	Merozoite surface antigen
ng	Nanogramme(s)
NK cell	Natural Killer cell
nm	Nanometre(s)
nRBC(s)	Normal/uninfected red blood cell(s)
PABA	Para-aminobenzoic acid
PBS	Phosphate buffered saline
p.i.	post-infection
pRBC(s)	Parasitised/infected red blood cell(s)
RBC	Red blood cell
rIFNγ	Recombinant IFNγ
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RT	Room temperature
S.D.	Standard deviation
T cell	T lymphocyte
TCR	T cell receptor
TGFβ	Transforming growth factor-beta
TH	T helper lymphocyte
TMB	3,3',5,5'-Tetramethyl-benzidine
TNF	Tumour necrosis factor
v/v	Volume per volume
WHO	World Health Organisation
w/v	Weight per volume

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SUMMARY

The immune mechanisms leading to acquisition of immunity against blood stages of malaria parasites include both cell-mediated and humoral responses. Resistance to blood stages of *Plasmodium chabaudi chabaudi* AS is largely $CD4^+$ T lymphocytes dependent. Both TH1 and TH2 cell subsets produce soluble mediators that have been associated with protection or susceptibility. These include the cytokines Interferon-gamma (IFNγ), Tumour Necrosis Factor-alpha (TNFα), Transforming Growth Factor-beta (TGFβ) and Interleukin-4 (IL-4). The use of a live system for the delivery of cytokines *in vivo* constitutes an alternative to injection of recombinant cytokines, as a method of examining their role in induction, development and/or implementation of immunity against blood forms of *P. c. chabaudi* AS. Attenuated *S. typhimurium* expressing the genes for IFNγ (*S. typhimurium*/IFNγ), TNFα (*S. typhimurium*/TNFα), TGFβ (*S. typhimurium*/TGFβ) or IL-4 (*S. typhimurium*/IL-4) were used in mice to deliver these cytokines *in vivo*. The cytokines are secreted in organs colonised by the bacteria, i.e. mesenteric lymph nodes, liver, and spleen.

The role of IFNγ in acquisition of immunity against erythrocytic forms of *P.c.chabaudi AS* was studied. Inbred NIH mice given the construct 7 days before the malaria infection, showed a significant delay in the onset and in the level of the recrudescent parasitaemia when compared with controls. Administration of the construct 8 days before malaria infection resulted usually in a significantly decreased primary parasitaemia in comparison with controls. No differences, however, were observed in the recrudescent parasitaemia between the groups. NIH mice infected with malaria 3 days after or on the same day as the administration of the IFNγ construct, showed a primary peak of infection similar to controls, but the resolution of this patent parasitaemia occurred 1 or 2 days earlier in the experimental mice when compared with controls. In the same experiment, mice given the construct 10 days before malaria infection had a similar course of infection as controls. Simultaneous inoculation with two *S. typhimurium* constructs: IFNγ and TNFα, 8 days before malaria infection resulted in a course of parasitaemia similar to that observed in mice given the IFNγ construct alone. On the other hand, inoculation of 'susceptible' inbred A/J mice with *S. typhimurium*/IFNγ 3 days or 8 days before malaria infection had no effect on the course of the parasitaemia when compared with controls. The immune mechanisms involved in the better control of the malaria infection in NIH mice given *S. typhimurium*/IFNγ, seem to be independent of nitric oxide (NO) production, since increased levels of the molecule were demonstrable around the peak of the primary

parasitaemia in control groups but not in experimental mice. In the latter basal levels of serum NO were observed from the period after the *S. typhimurium*/IFNγ inoculation until up to three days after the peak of the parasitaemia. The role of macrophages on the enhanced control of the malaria infection in *S. typhimurium*/IFNγ recipient mice remains unexplored.

To assess further the role of TNFα in acquisition of immunity against *P. c. chabaudi* AS, 'resistant' NIH and 'susceptible' A/J mice were given TNFα using the *S. typhimurium* delivery system. NIH mice inoculated orally with the TNFα construct 5 and 3 days before, or simultaneously with the malaria infection, had a course of infection similar to controls. However, A/J mice given the construct 3 or 5 days before the malaria infection showed a significantly decreased peak parasitaemia when compared with controls.

Other *S. typhimurium* constructs were available and their effect on the course of *P.c.chabaudi* AS infection was studied. Preliminary experiments with a TGFβ construct included its inoculation into NIH mice 4 and 7 days before malaria infection. No significant differences were observed in the course of parasitaemia of experimental mice in comparison with control groups. Similarly, inoculation of this construct into A/J mice 3 days before malaria infection had no effect on the course of parasitaemia.

A *S. typhimurium*-IL-4 construct was given to NIH mice 4 or 7 days before malaria infection. This resulted in a similar course of infection in experimental and control mice. A/J mice infected with malaria 3 days after administration of the IL-4 construct showed a course of parasitaemia which was not significantly different from controls.

A role for NO in the control the infection with *P. c. chabaudi AS*, has been suggested by other authors. To investigate this further, inducible NO synthase (iNOS) deficient mice were infected with the parasite and the course of parasitaemia was followed. The mice had an increased peak parasitaemia when compared with controls. However, the significance of these results was dependent on inclusion in the experiments of a high number of mice in the experimental and control groups. *In vitro* proliferation of spleen cells taken 6 days after malaria infection and stimulated with ConA, showed a significantly increased response of cells from iNOS depleted mice in comparison with controls. However, proliferation of spleen cells from iNOS depleted mice obtained at days 0, 10, 14, and 18 was no different from controls. The supernatants from the spleen cell cultures showed that iNOS depleted mice had a higher production of IFNγ than controls, when cells were obtained at day 6 p.i., but not at day 10, 14 or 18 p.i. The pattern and degree of sequestration of pRBC observed at around the peak of infection, evaluated by taking peripheral blood smears, was the same in iNOS depleted and control mice. Furthermore, other

parameters such as anaemia and reticulocytaemia did not differ in the iNOS deficient mice in comparison with controls.

The work with the iNOS depleted model confirms that although not essential for the resolution of parasitaemia, NO probably exerts cytostatic and cytotoxic effects against blood forms of *P. c. chabaudi* AS which contribute with the control of the infection around peak parasitaemia. A scheme to indicate immune interactions in mice injected with *P. c. chabaudi* is drawn up.

The results using *S. typhimurium*/IFNγ and *S. typhimurium*/TNFα confirm that these two cytokines have a role in the control of *P. c. chabaudi* AS blood forms during early stages of infection, and that the degree of their effect depends on the strain of mice used. IFNγ seems to dominate immunity during early stages of infection in NIH 'resistant' mice, while TNFα is important in acquisition of immunity by A/J 'susceptible' mice. Furthermore, the work reported here suggests the feasibility of using *S. typhimurium* mutants to deliver cytokines *in vivo* in mice. The next step includes the use of these *S. typhimurium* mutants to study synergistic effects of cytokines and malarial antigens. This could be achieved by either creating a co-expression system to deliver cytokines and malarial antigens *in vivo* or by immunisation with malarial antigens of mice inoculated with *S. typhimurium* expressing cytokine genes.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1. Historical outline

Malaria is an ancient disease. For centuries it was attributed to the inhalation of noxious vapours, or miasms, from marshes, and hence the derivation of the word malaria from the Italian "Mala Aria" (bad air). Earliest references to the disease seem to come from inscriptions found at the temple of Denderah (Tentyra, upper Egypt) where accounts of an annually recurrent febrile disease have been found (Boyd, 1949). The Orphic poems written three thousand years ago talk about tertian and quartan fevers (Hoops, 1934 as cited by Boyd, 1949). Hippocrates (400 BC.) made a very satisfactory description of malaria (Jones, 1923 as cited by Boyd, 1949). In his writings he seemed familiar with the intermittent and periodic fevers of a disease he considered to be related to living in proximity to marshes. Later, Celsius (circa 50) and Galen (second century) contributed to characterise further clinical aspects of the disease (Hoops, 1934 as cited by Boyd, 1949).

It was not until the seventeenth century when research on malaria was extended further. Sydenham (1624-1689) studied the differences in the periodicity of the fever and made a clinical characterisation of complicated malaria (Boyd, 1949).

No important contributions were made towards the control of malaria until the middle of the seventeenth century, when the Peruvian Bark known by natives as "Quina-quina" was introduced to Europe. The earliest mention in Europe of the bark is by Heyden in 1643. It was first brought to England around 1660. Sydenham and Morton (1637-1698) experimented with it and confirmed the efficacy against malaria fevers. This bark was later called Cinchona by Carl von Linne (Linnaeus) who classified it as a new genus.

Meckel in 1847, discovered the presence of black granules in the blood of a patient dying from malaria. In 1880 Alphonse Laveran observed exflagellation and produced the theory that a parasite was the cause of the infection. Almost simultaneously Gerhardt reported successful inoculation of healthy persons with blood from infected

patients. Together these observations were responsible for discrediting the doctrine of miasms.

As early as 1883, American physicians implicated mosquitoes in the transmission of malaria (King, 1883). Manson in 1894 suggested that when the malaria parasites were ingested by a mosquito they underwent further development within it. Later, Ronald Ross started studies in India which allowed him to confirm Manson theory by infecting *Culex* and *Aedes* with blood of infected individuals and observed maturation of gametocytes in the mosquito stomach, but they did not develop further. He then (1897) carried on to demonstrate oocysts on the stomach of *Anopheles* that were fed on a crescent carrier. However, all developmental stages were demonstrated in *Culex* when studying malaria in birds (Ross, 1898 as cited by Boyd, 1949). Late in 1898 Grassi was the first to verify the life cycle of *P. falciparum* in anophelines completely.

The first demonstration of a cycle development outside the red blood cell was made in avian species of *Plasmodium*. James & Tate described in 1937, the exo-erythrocytic schizogony of *P. gallinaceum* in the reticulo-endothelial cells of chickens. However, a tissue phase for malaria in mammals was not described until after World War II. Shortt & Garnham (1948a) observed pre-erythrocytic forms of *P. cynomolgi* in the liver of rhesus monkeys, soon similar forms were found in the livers of human volunteers infected with *P. vivax* and *P. falciparum* (Shortt & Garnham, 1948b; Shortt, 1948)

1.2. Taxonomy

Malaria parasites are protozoans with the ability to form spores and therefore they fall into the Phylum Sporozoa (or Apicomplexa, due to the presence of a characteristic apical complex) (Cox, 1993). Since the spores, which develop at the end of a trophic cycle, lack polar capsules or filaments, they have been classified into the class Telosporea. Their zygotes are motile and their sporozoites devoid of resistant

membranes. The mature trophozoites are small and intracellular and are therefore assigned to the Sub-class Coccidia. Within this Sub-class the Order Eucoccidia comprises those which undergo sexual reproduction (sporogony) as well as asexual reproduction (schizogony). Being unable to pass any part of their life cycle outside the body of their host, they are in the Sub-order Haemosporina in which they constitute the Family Plasmodiidae. This Family includes parasites that undergo schizogony in the vertebrate host and sporogony in the mosquito. The Family contains a single Genus, *Plasmodium* (Baker as cited by Phillips, 1983).

There are about 120 species of *Plasmodium*, 22 of them infect primates and 19 rodents, bats and other mammals. Four species naturally infect humans: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. Of these *P. falciparum* is the most important in public health as it is the parasite responsible for the high mortality observed in malaria.

1.3. Life cycle

For mammalian malaria parasites infection of the host is initiated by the bite of an infected female anopheline mosquito. During a blood meal, motile sporozoites contained in the mosquito's saliva are inoculated into the host's bloodstream. A mean of 15 sporozoites are inoculated (Rosenberg *et al.*, 1990). Sporozoites remain in the circulation for 15 to 60 minutes (Fairley, 1947; Sinden & Smith, 1982) before entering the hepatocytes where they undergo asexual division (pre-erythrocytic or exo-erythrocytic schizogony) to develop into exo-erythrocytic schizonts (Garnham, 1966). The mature schizonts disrupt the hepatic cell and thousands of haploid merozoites are discharged into the bloodstream. In human malaria this exo-erythrocytic schizogony takes between 5.5 and 15 days, depending on the species, and is thought to occur only once in the case of mammalian malaria parasites.

In *P. falciparum* and *P. malariae*, all sporozoites immediately undergo tissue schizogony, while in *P. vivax* and, probably, *P. ovale* infections, a proportion of sporozoites develop into latent forms known as hypnozoites which resume development some time later. The presence of hypnozoites explains the relapses seen in *P. vivax* (Krotoski *et al.*, 1982).

In avian malaria, the exo-erythrocytic development of the parasite occurs in the fixed cells of the reticulo-endothelial system rather then in the liver. In addition, merozoites coming from exo-erythrocytic and erythrocytic schizont can originate further cycles of secondary exo-erythrocytic schizogony (Phillips, 1983).

Once released into the circulation, the merozoites rapidly invade the red blood cells where they begin the part of the cycle known as erythrocytic schizogony. The invasion of red blood cells is probably due to the presence of a species-specific receptor in/on the erythrocyte membrane which enables the union of the cell membrane with the apical complex of the merozoite. For the human malarias *P. vivax* and *P. falciparum*, these receptors are known to be associated with the Duffy blood

group antigen (Miller *et al.*, 1975b) and glycophorin (Miller *et al.*, 1977a; Perkins, 1981) respectively. This complex contains factors that cause invagination of the erythrocyte membrane adjacent to the merozoite and the parasite is enclosed in a parasitophorous vacuole (Dvorak *et al.*, 1975, Aikawa *et al.*, 1978). It has been known for many years that some species of malaria preferentially parasitise young erythrocytes (reviewed by Bray & Garnham, 1982). *In vivo* studies showed that *P. berghei* preferentially infects reticulocytes. Similarly *P. vivax* has a preference for young red cells (reviewed by Phillips, 1983). On the other hand, *P. knowlesi*, for instance shows no preference for reticulocytes, while *P. malariae* prefer mature red cells. Increased susceptibility of younger erythrocytes could be related to differences in cell surface antigens affecting attachment and penetration (Pasvol *et al.*, 1980).

Within the red blood cell, the merozoite develops into ring and trophozoite stages and undergoes asexual multiplication to form a schizont containing between 8-32 merozoites. Schizogony occurs by a process of budding which occurs just below the surface of the schizont (reviewed by Phillips, 1983). Finally, the erythrocytic schizont bursts releasing merozoites which invade further red blood cells (Dvorak *et al.*, 1975).

The erythrocytic schizogony takes 48 hours in *P. vivax*, *P. falciparum* and *P. ovale*, while in *P. malariae* it lasts 72 hours. This cycle is relatively synchronous in the natural host thus explaining the clinical symptoms of alternating fever and chills characteristic of the disease (Hawking *et al.*, 1968). The periodicity of the paroxysms depends upon the time of occurrence of the schizogony, and fever coincides with the bursting of infected red blood cells and reinvasion of new red blood cells by the merozoites.

Most of the merozoites released after schizogony invade red blood cells and undergo asexual replication, but a small proportion may differentiate into sexual forms or gametocytes. The stimulus which makes a merozoite transform into macrogametocyte (female) or microgametocyte (male) is unknown. However, *in vitro* gametogony of *P. falciparum* has been induced by the addition of antimalarial antibodies, hypoxanthine,

lymphocytes and serum from infected children, corticosteroids or N-acetylglucosamine (reviewed by Cornelissen & Waliker, 1985) Gametocytes complete their sexual cycle in the female mosquito after being taken up from the circulation in a blood meal. Once in the midgut of the vector, the microgametocyte undergoes a process of mitotic division and exflagellation before fertilisation of the macrogamete. The resultant diploid zygote undergoes meiotic division and transforms into a motile ookinete, crosses the gut wall and develops into a haploid oocyst between the basal lamina and the epithelial layer (Sinden & Strong, 1978). During the next 10-16 days the oocyst divides and liberates up to 10,000 motile sporozoites into the mosquito's haemocoel (reviewed by Russell *et al.*, 1963). The sporozoites migrate to the mosquito's salivary gland ready to be injected into the host during the next blood meal (Vanderberg, 1975) (Figure 1).

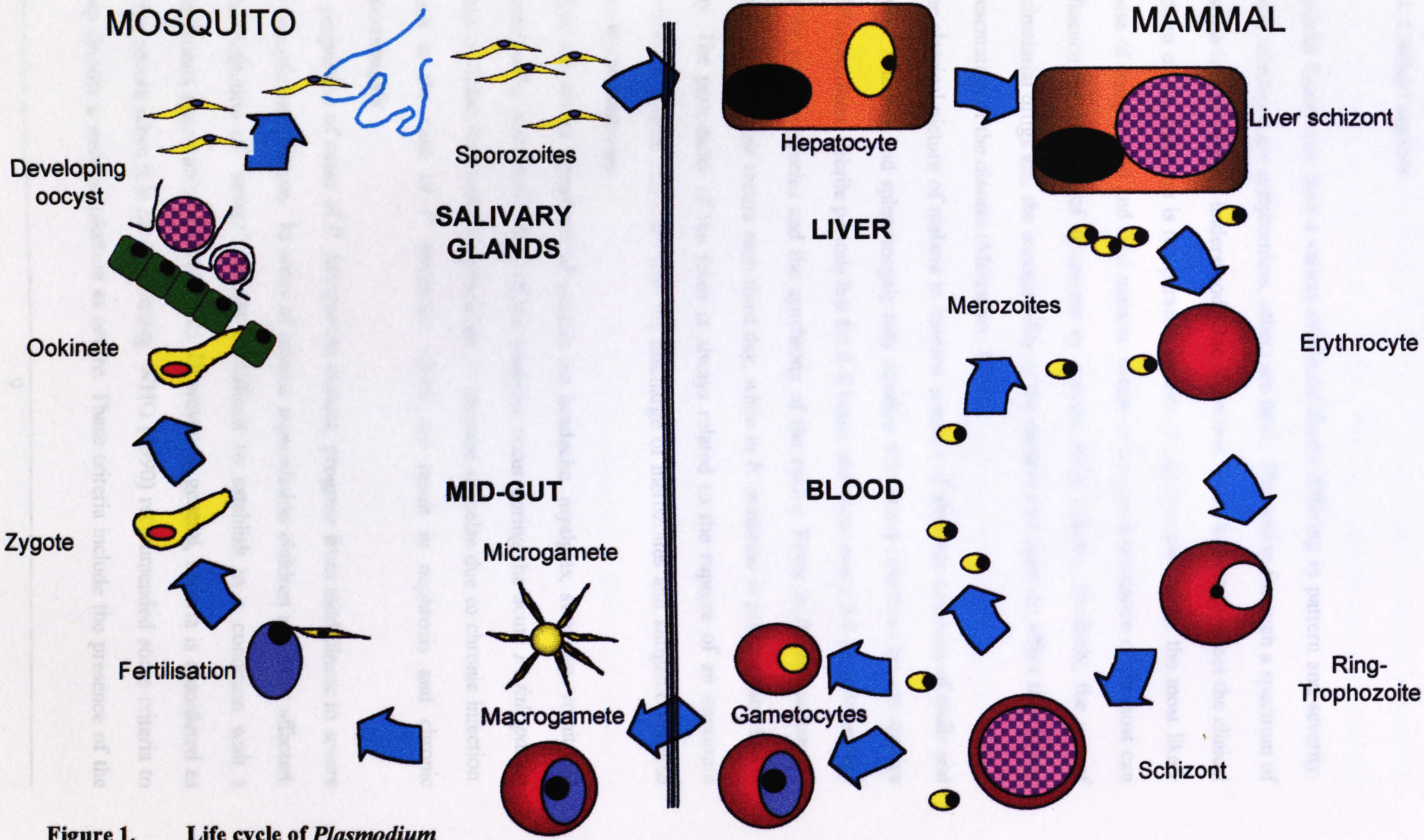


Figure 1. **Life cycle of *Plasmodium***

1.4. Clinical aspects

Malarial illness may take a variety of clinical forms differing in pattern and severity. Some infections are symptomless, others are fatal. The reason for such a spectrum of disease is only partially understood. One important factor known to affect the clinical picture of the infection is the parasite species, *P. falciparum* being the most likely cause of complicated and fatal malaria. Innate or acquired resistance of the host can influence the severity of symptoms or prevent them entirely. Similarly, the use of antimalarial drugs and the susceptibility of the parasites to them do affect the clinical presentation of the disease (Molyneux, 1995).

The classical picture of malaria in humans consists of alternate episodes of chills and fever. Anaemia and splenomegaly may develop with heavy infections by any species of *Plasmodium*. Febrile periods last for 6-8 hours and recur every 2-3 days depending on the parasite species and the synchrony of the cycles. Fever in *P. falciparum*, *P. vivax* and *P. ovale* recurs each third day, while in *P. malariae* is present each fourth day. The periodicity of the fever is always related to the rupture of an important number of mature schizonts with the discharge of merozoites and antigenic material into the bloodstream.

Other associated symptoms of malaria are headache, myalgias, sweating, vomit and occasionally, diarrhoea. Most of the fatalities occur during the acute *P. falciparum* malaria. Some, however, are secondary to immune sequelae due to chronic infection. Such is the case of *P. malariae* which can result in nephrosis and chronic splenomegaly.

A proportion of cases of *P. falciparum* malaria, progress from mild illness to severe or complicated disease. In areas of intense transmission children are most affected. The definition of "severe" disease is difficult to establish in a condition with a continuous spectrum of clinical forms. However, in general, malaria is considered as being severe when it is life-threatening. WHO (1990) recommended some criteria to help classify a malaria infection as severe. These criteria include the presence of the

following complications: coma, renal failure, severe anaemia, acidosis, respiratory distress syndrome, hypoglycaemia, bleeding, shock, intravascular haemolysis, altered consciousness, prostration, convulsions, jaundice and hyperparasitaemia.

During pregnancy, the clinical impact of malaria differs according to the level of endemicity. In holoendemic areas of *P. falciparum* infection, pregnant women are more likely to be parasitaemic and at higher levels than non-pregnant women. However, there is no convincing evidence that pregnant women suffer more symptomatic malaria that non-pregnant women. Maternal and placental malaria are associated with low birth weight in the case of primigravidae (Brabin, 1983; McGregor *et al.*, 1983). In areas of low or unstable transmission, pregnant women suffer more severe disease than non-pregnant women when infected with *P. falciparum* (Subramanian *et al.*, 1992; Nosten *et al.*, 1994). In these, there is an increased risk of foetal loss, intrauterine or neonatal death, and increased maternal mortality (Molyneux, 1995). Congenital cases of malaria in holoendemic areas are rare, but in areas of low transmission, malaria in the mother at term may be followed by congenital malaria. Symptoms begin to appear between 2 weeks and 2 months and include fever, anorexia, hepatosplenomegaly and anaemia (Molyneux, 1995).

Chronic complications can develop in populations subjected to intense transmission of malaria. Although rare, some individuals may develop a hyperactive splenomegaly, nephrotic syndrome (secondary to *P. malariae* infection) or, when co-infection with Epstein-Barr virus exists, Burkitt's lymphoma (Molyneux, 1995).

1.5. Diagnostic methods

The cornerstone of diagnosis and still the basis of clinical practice is the stained peripheral blood film. Morphological examination of the parasite can be done through evaluation of thin blood films. A thick blood film is a more appropriate screening test as it allows diagnosis of low-density parasitaemias.

A dipstick antigen detection test constitutes an attractive alternative for the diagnosis of the infection in field, where staining and microscopy equipment are not easily available. Sensitivity and specificity of this method for the diagnosis of *P. falciparum* infection, are between 80% and 100% when compared with blood films (Shiff *et al.*, 1994).

Other methods for the detection of malaria parasites include the quantitative buffy coat method, which uses acridine orange (Spielman & Perrone, 1989) and polymerase chain reaction (PCR) (Snounou *et al.*, 1993). The latter detects low numbers of parasites in small sample volumes and has been reported of particular utility in epidemiological studies as it allows genotyping of different *P. falciparum* isolates (Viriyakosol et al, 1995). Although these methods have high sensitivity and specificity, they are impractical for field work.

1.6. Chemotherapy

For nearly two centuries following its introduction, cinchona was largely dispensed as a powder prepared from the bark of the tree. By 1810 Gomez, a Portuguese chemist, had extracted an impure alkaloid called cinchoquine, which did not come into wide use. In 1820 the French chemists Pelletier and Caventou, found two alkaloids which were named quinine and cinchonine (Scott, 1939 as cited by Boyd, 1949). Quinine was soon found to be a more reliable therapeutic agent than the bark (Kremers, 1931 as cited by Boyd, 1949). By the middle of this century, Ledger collected a species of cinchona which was found to have a high quinine content. This was subsequently named *Cinchona ledgeriana* and soon constituted the main source of quinine. In all more than twenty crystallizable and amorphous alkaloids have been isolated from cinchona. Quinine remained the standard treatment for malaria until synthetic substitutes were developed. Schuleman (1927), synthesised plasmochin and atabrine (Boyd, 1949). During the 1930s-1940s, quinine was largely replaced by less toxic and more efficacious synthetic compounds (Geary & Jensen, 1983).

Chloroquine is the prototype of 4-aminoquinolines, which also includes amodiaquine (reviewed by McChesney, 1983). They are rapid blood schizonticides which by binding to DNA, block synthesis of nucleic acid (reviewed by Cox, 1993). Primaquine, an 8-aminoquinoline, is useful in the control of tissue schizonts. In addition, it is effective against sexual forms of *P. falciparum*. Primaquine is metabolised to a quinoline diquinone structure which blocks electron transport (reviewed by Cox, 1993) disrupting the parasite's energy metabolism.

Antifolates drugs include pyrimethamine and sulphadoxine. They act by blocking synthesis of tetrahydrofolate, an important cofactor in the parasite's metabolism. Pyrimethamine is a competitive inhibitor of dihydrofolate reductase, while sulphadoxine blocks dihydropteroate synthetase (reviewed by Cox, 1993). They are useful blood schizonticides and are used in combination in the case of chloroquine resistant *P. falciparum* infections. Another antifolate, proguanil, is a biguanide which has a similar mechanism of action as pyrimethamine, and is effective as a blood schizonticide. A biguanide, chlorproguanil, in combination with dapsone (an anti-leprosy compound), have been used as an alternative treatment in cases of non-severe *P. falciparum* malaria (reviewed by Watkins, 1995) .

Mefloquine and halofantrine, both arylaminoalcohol compounds have potent action against blood schizonts and are highly effective in the treatment of acute malaria.

Artemisin (Quinghaosu), a sesquiterpenelactone, is the antimalarial principle isolated from *Artemisia annua.* Artemether, artesunate and dihydroartemisin have been produced from artemisin. They all have a more potent malarial activity than the parent compound and appear to be the most rapidly acting of all the antimalarials developed so far. Their action is mainly against schizonts but their mechanism of action is not completely understood.

Pyronaridine and atovaquone are two new antimalarials. The former exhibits a marked blood schizonticidal activity and it may have potential as replacement for oral formulations of chloroquine. Atovaquone, a hydroxynaphthoquinone, has a broad-spectrum antiprotozoan activity. It has a novel mode of action by inhibiting electron

transport with a high intrinsic activity *in vitro* against erythrocytic stages of *P. falciparum* . It has also been found effective against the liver stages (reviewed by Olliaro & Trigg, 1995).

Finally, benflumetol, a fluoromethanol, is an active blood schizonticide. In preclinical studies synergy between this and artemether has been observed (reviewed by Olliaro &Trigg, 1995).

Drug resistant strains may arise as a result of mutation which are selected after frequent use of the drug or incomplete treatments. Dissemination of resistance results after genetic recombination between strains (reviewed by Phillips, 1983). Increasing drug resistance is particularly observed in countries where *P. falciparum* is endemic. The parasite has developed resistance to chloroquine, sulpha/pyrimethamine combinations and, to some extent, quinine. Chloroquine resistance is widespread in Africa, particularly in eastern Africa. Multidrug resistance of *P. falciparum* is has been observed in certain countries of East Asia and Western Pacific and constitutes a serious problem as it may spread to African countries (reviewed by Olliaro & Trigg, 1995).

1.7. Host resistance to malaria

1.7.1. Innate resistance

Innate resistance is observed in some hosts that have not been exposed to the parasite. Some of the mechanisms so far identified involved in this type of resistance are genetically controlled whilst others may simply be incidental, reflecting changing environmental factors.

Incompatibility between the conditions found within the host and the biochemical or physiological requirements of the parasite may affect survival and/or reproduction. Biochemical incompatibility can take different forms. For instance, this may exist when essential molecular structures, such as those mediating attachment to and penetration of the parasite into cells, are missing or altered. Physiological incompatibility may occur when parameters such as pH, oxygen tension or the concentration of various metabolites are unfavourable for parasite survival. Furthermore, a host which could offer an acceptable biochemical environment to the parasite may be resistant to infection due to the occurrence of natural immune mechanisms different from those evoked secondary to infection.

Malaria merozoites have to invade red blood cells as an integral part of the life cycle. In order to invade these cells, malaria parasites must make specific attachment through specific ligands to chemical groupings (receptors) present on the cell. In *P. knowlesi* , specific attachment leading to invasion does not occur when receptors on the erythrocyte surface are removed with chymotrypsin (Miller *et al.*, 1975a). Miller *et al.*, (1975b) provided evidence that the Duffy antigen on human erythrocytes was the receptor for *P. knowlesi* . Their *in vitro* studies showed that *P. knowlesi* failed to invade Duffy-negative red blood cells. This was later suggested in *P. vivax* infection (Miller *et al.*, 1976). Duffy-negative homozygous enjoy almost complete protection whereas heterozygous are susceptible.

Red blood cell surface glycophorins have also been implicated as major determinants of invasion by merozoites (Pasvol *et al.*, 1982). Glycophorins are sialic acid-rich glycoproteins comprising four subgroups (a, b, c and d). Cells lacking glycophorin a (En(a-)- cells) or b (S-s-U-cells) are significantly resistant to *P. falciparum* merozoite invasion (reviewed by Cox, 1993).

The susceptibility of the erythrocytes to invasion by malaria parasites is dependent upon the age of the cell (reviewed by Bray & Garnham, 1982). Of the human malarias, *P. vivax* and *P. ovale* are predominantly found in either reticulocytes or slightly older normocytes. *P. falciparum* is thought to have a preference for metabolically young red blood cells (Phillips, 1983). In murine malarias, *P. berghei* has shown preference for invasion of reticulocytes.

Ring forms of the parasite feed on host cell cytoplasm, mainly haemoglobin. Thus growth of the parasite is dependent on the catabolism of haemoglobin. Mutations in or near the globin genes which alter the structure (amino acid sequence) of or the rate of synthesis of a particular globin chain, have been associated with protection against malaria. Most of the pathologic genetic variants result from a single amino acid substitution in one of the normal globin chains (HbA). The most representative of these haemoglobinopathies is sickle-cell anaemia, where a single gene mutation determines that the sixth amino acid from the N-terminal end of the β-globin chain substitutes valine for glutamic acid. As a result the abnormal haemoglobin (HbS) causes the erythrocyte to adopt the shape of a sickle when oxygen tension is low. Innate resistance to *P. falciparum* infection in individuals with HbS has long been recognised (Allison, 1954; Luzzato, 1979). *In vitro* experiments have shown that potassium loss and not the HbS related sickling is responsible for killing of the parasite, since abrogation of potassium loss prevents parasitic death even in sickled HbAS cells. In addition, HbS polymer has being shown to impair parasite growth in spite of aerobic conditions. In the case of homozygous cells (HbSS) needle-like aggregates of deoxyhaemoglobin formed when the cell is under low oxygen tension, disrupt the parasite (reviewed by Phillips, 1983).

Other haemoglobins have also been associated with protection against malaria these include: α and β thalassaemias; C, E and foetal haemoglobin.

Glucose 6-phosphate dehydrogenase (G-6-PD) is another red blood cell defect which has been suggested to give some protection against *P. falciparum* malaria (reviewed by Weatherall, 1987; Martin, 1994). Although questionable, oxidative stress is the most plausible explanation for reduced susceptibility observed in female heterozygous (Roth *et al.*, 1988). Support for this theory comes from the fact that isouramil, an oxidant isolated from fava beans, has been found to be more damaging to parasites in G-6-PD-deficient cells (Golenser *et al.*, 1983).

Structural membrane defects of the red blood cell seem to exert a protective effect against infection. Individuals with ovalocytosis have a lower parasitaemia than normal when infected with *P. falciparum*, *P. vivax*, or *P. malariae* (Serjeantson *et al.*, 1977). Similarly, eliptocytosis has been reported to promote resistance to invasion by both *P. knowlesi* and *P. falciparum* (Hadley & Miller, 1988).

Diet has been shown to affect the course of malaria infection. Erythrocytic stages, and probably pre-erythrocytic, of the parasite appear to be suppressed by a diet with low para-amino benzoic acid (PABA) (Hawking, 1954; Gilks, 1988). Similarly, low protein intake has been reported to depress rodent *P. berghei* (Gilks *et al.*, 1989) infections. This may in part explain why famine relief in humans is sometimes accompanied by outbreaks of malaria (Murray *et al.*, 1981).

The genetic background of the host has also been shown to affect predisposition to infection. An association between certain HLA class I and II haplotypes and protection from severe malaria has been observed in the Gambia (Hill *et al.*, 1991). Molecular analysis of the HLA B53 association has been studied by sequencing of peptides eluted from the B53 molecule followed by screening of canditate epitopes from pre-erythrocytic stage antigens of *P. falciparum* (Hill *et al.*, 1992). Among malaria-immune Africans, HLA-B53-restricted cytotoxic T lymphocytes recognised a conserved peptide from liver-stage-specific antigen-1 (LSA-1).

In the 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.*, 1989). Susceptible mouse strains, such as A/J, develop a fulminating parasitaemia and succumb to infection. Resistant hosts, such as C57BL/6 and NIH mice, develop a moderate level of peak parasitaemia, eliminate the acute infection in five weeks and are immune to reinfection. The ability to produce high amounts of NO early during *P. chabaudi* infection has been correlated with resistance to infection . Resistant C57BL/6 mice had an increased expression of iNOS mRNA during early stages of the infection when compared with susceptible A/J mice (Jacobs *et al.*, 1995). Furthermore, high levels of nitrate, an oxidised form of NO, have been observed in infected mice. during primary parasitaemia and a sharp peak of nitrate production has been associated with the peak of infection (Taylor-Robinson *et al.*, 1993). Others have demonstrated increased levels of nitrate in plasma of individuals infected with *P. falciparum* or *P. vivax* (Cot *et al.*, 1994; Nüssler *et al.*, 1994). The mechanisms mediating the NO-dependent control of the blood forms of the malaria parasites are not clear. In *P. falciparum* a direct cytostatic effect has been observed *in vitro* in cultures treated with SNAP (an NO producer) (Balmer *et al.*, 1995), while NO derivatives had a cytostatic effect *in vitro*, on the parasite.

The cytokines IFNγ and TNFα are critical in the regulation of the production of NO. Resistant C57BL/6 mice treated with anti-IFNγ and anti-TNFα mAb had a reduction of both iNOS mRNA in the spleen and serum NO_3^- and an increased peak parasitaemia during infection with blood forms of *P. chabaudi* (Jacobs *et al.*, 1996a). IFNγ production peaks in resistant mice 2-3 days before the peak of infection with *P. chabaudi* AS (Slade & Langhorne, 1989). *In vitro* production of the cytokine by spleen cells from resistant mice has been observed 1-2 days before peak parasitaemia (Stevenson *et al.*, 1990a, Taylor-Robinson & Phillips, 1994a). Furthermore, neutralisation of IFNγ *in vivo* results in exacerbation of infection and injection of rIFNγ depress parasitaemia (Stevenson *et al.*, 1990a). Tsuji *et al.* (1995)

have reported a slight delay (2-3 days) in the clearance of a blood infection with *P. chabaudi adami* in IFNγ-receptor β-chain deficient mice in comparison with controls. Infection of IFNγ-deficient mice with blood forms of *P. chabaudi adami*, resulted in a higher peak parasitaemia and prolonged infection when compared with controls (van der Heyde *et al.*, 1997).

Stevenson & Ghadirian (1989) showed that treatment of resistant C57BL/6 mice with human recombinant TNFα has no effect on the course or outcome of *P. chabaudi* AS infection. On the other hand, similar treatment of susceptible A/J mice resulted in survival from a *P. chabaudi* AS infection and a significant decrease in the peak parasitaemia (Stevenson & Ghadirian, 1989) compared with controls. More recent work into the role of TNFα in the genetically dependent resistant to *P. chabaudi* infection has shown that resistant C57BL/6 mice have higher levels of TNFα messenger RNA (mRNA) in spleen and liver, than susceptible mice (Jacobs *et al.*, 1996b). Furthermore, increased levels of TNFα in serum and of TNFα mRNA in liver later during infection, were found in susceptible A/J mice. This suggests that TNFα has a protective role during a *P. chabaudi* infection when it is produced in the spleen and the liver early during infection and has a deleterious effect when it is found in high levels only in the liver. Jacobs *et al.* (1996b) proposed that the paradoxical role of protection (or resistance) versus pathology (or susceptibility) of TNFα in malaria infection may be dependent on the amount of TNFα released and the timing and site of its expression. In humans, a genetic propensity of the host to produce high levels of TNFα during *P. falciparum* infections has been observed (McGuire *et al.*, 1994), as a result of changes in the TNFα gene promoter region. Therefore, children who are homozygous for the TNF-2 allele have a significantly increased risk of suffer neurological sequelae due to cerebral malaria.

1.7.2. Immunity to malaria

1.7.2.1. Innate immunity

Non-specific acquisition of resistance against malaria has been observed after administration of unrelated agents. In murine malarias previous exposure to *Mycobacterium bovis* (BCG) resulted in resistance to infection (Clark *et al.*, 1976, Murphy, 1981), Similarly, inoculation with killed *Propionibacterium acnes* (formerly known as *Corynebacterium parvum*) have conferred a degree of protection against subsequent challenge with *P. chabaudi* (Clark *et al.*, 1977), *P. vinckei* (Lucia & Nussenzweig, 1969; Cottrell *et al.*, 1977) and *P. berghei* (Nussenzweig, 1967; Murphy, 1981).

Exposure to a range of products can result in protection against malaria. These include Concavalin A (ConA), lipopolysaccharide (LPS) (Cottrell *et al.*, 1977), *Coxiella burnetti* extract (Clark, 1979), freeze-thawed *Toxoplasma gondii* tachyzoites (Omata *et al.*, 1981), endotoxin (Martin *et al.*, 1967), and magnesium silicate in calcium phosphate gel (Michel *et al.*, 1982).

The mechanisms which mediate such non-specific immune response to malaria have not been completely elucidated. These may include, amongst others, macrophage activation (Nussenzweig, 1967) (with or without the production of macrophage autocrine factor, TNFα), and increase of natural killer (NK) cells levels. In separate observations TNFα was implicated in parasite death through the release of nitric oxide (Green *et al.*, 1990) and superoxide ions (Allison & Eugui, 1982).

Endogenous environmental factors such as hormones play an important role in the modulation of the immune response (reviewed by Schuurs & Verheul, 1990). In murine infection with *P. chabaudi*, a spleen-dependent mechanism has been implicated in the suppression of acquisition of immunity to blood forms of the parasite (Benten *et al.*, 1991).

The processes involved in naturally acquired immunity to malaria are not well understood. The fact that many different antigens are presented to the host's immune system throughout a malaria infection, results in a complex immune response to the disease. As a result the immune responses generated tend to be stage specific as well as species and strain specific.

The development of malarial immunity is related to the level of endemicity (Bjorkman *et al.*, 1986). Evidence of acquired immunity to malaria infection in humans starts to appear at the beginning of the second week of patent parasitaemia and is manifested as a reduction in the parasite's reproduction rate and in the number of pRBC in the blood. After a variable period, the immunity decreases the parasitaemia to low or undetectable levels (McGregor, 1956). The immune response can diminish the clinical symptoms of infection. However, there is no direct correlation between parasitaemia and the presence of symptoms. "Anti-disease" immunity, results after development of an immune response against certain parasite antigens, probably different form those conferring anti-parasite immunity (reviewed by Playfair, 1991). As acquired immunity is short-lasting, constant exposure to the parasite is required in order to be maintained. The presence of sterile immunity has been described in rodents infected with *P. berghei* (Cox, 1962), but epidemiological studies indicate that it does not exist in humans.

Infants born to *P. falciparum* immune mothers are protected from infection up to the age of 6 months. This protection might be explained by the effect of maternal antibodies and the less favourable intracellular environment created by the presence of foetal haemoglobin (reviewed by Bruce-Chwatt, 1979), in addition to PABA deficit (milk is deficient in PABA) (Hawking, 1954). During the following 2 years of age, they suffer from severe infections, after which high parasite densities persist, but less pronounced illness is observed ((Wilson *et al.*, 1950; McGregor, 1960, Playfair, 1990).

1.7.2.2. Acquired immunity against asexual erythrocytic stages

Although asexual forms of *Plasmodium* are not the initial stage of the parasite to be encountered by the host, they are responsible for essentially all of the pathology associated with malaria. In natural infection, the synchronous rupture of infected erythrocytes allows massive antigen release. Subsequently, on invasion of new RBC, different antigens are expressed on the surface of the cell. Therefore, most of the parasite antigens that are exposed to the immune system derived from the asexual blood stage. However, elucidation of the nature of the effector immune mechanisms to the asexual blood stages have been difficult, with many host as well as parasite factors affecting the outcome (Hviid *et al.*, 1992).

a). Humoral immunity

In human malaria, *in vivo* evidence for the importance of antibodies in protection against infection comes from studies of passive transference of IgG (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991). In addition, *in vitro* studies have shown that antibodies are involved in the inhibition of red cell invasion by merozoites of *P. knowlesi* (Miller *et al.*, 1975a) or *P. falciparum* (Phillips *et al.*, 1972; Mitchell *et al.*, 1976). Purified IgG anti-*P. falciparum* antibodies promote *in vitro* phagocytosis of merozoites and schizont-infected erythrocytes by monocytes (Bouharoun-Tayoun *et al.*, 1990). The observation that some individuals with high levels of antimalarial antibodies may evidence clinical disease can be explained by functional differences existing among antibodies of the same antigenic specificity (Bouharoun-Tayoun & Druilhe, 1992). Opsonization by cytophilic antibodies may be inhibited by IgG_2 and IgM, which in addition are unable to arm monocytes (Ho & Sexton, 1995). It has been observed that in a *P. falciparum* endemic population, IgG_2 and IgM level are increased in susceptible groups, while IgG_1 and IgG_3 increase with age was correlated with clinical immunity (Bouharoun-Tayoun & Druilhe, 1992).

b). Cellular immunity

Development of immunity to malaria requires an intact and functioning T cell system. This has been demonstrated in thymectomised hosts, which had higher and more persistent parasitaemias when compared with controls. Experiments of this type have been carried out in *P. berghei* infected rats (Brown *et al.*, 1968); in *P. chabaudi* infected mice (McDonald & Phillips, 1978) and *P. yoelii* infected mice (Jayawardena *et al.*, 1977). Furthermore, B-cell-depleted mice are able to control infection with *P. chabaudi adami* (Grun & Weidanz, 1981) or *P. vinckei* (Kumar *et al.*, 1989) probably through T-cell mediation. In *P. falciparum* infection, both humoral and cellular immune responses have been implicated in mediation of anti-parasite immunity (reviewed by Ho & Sexton, 1995).

(i). T cells general features

The majority of mature T cells express either CD4 or CD8 surface glycoproteins. The main, but not exclusive, role of $CD8^+$ T cells is antigen-specific cytoxicity (Bloom *et al.*, 1992; Salgame *et al.*, 1991), which results in the destruction of autologous infected cells, in order to prevent spread of pathogens. $CD4^+$ T cells are involved in a variety of functions but their major role is the regulation of the immune response necessary for both the induction of cellular effector mechanisms and for production of antibodies to foreign antigens (reviewed by Troye-Blomberg *et al.*, 1994).

T cells recognise antigens via their T cell receptors (TCR) as peptides in the context of major histocompatibility complex (MHC) molecules of either class I or class II. MHC class I molecules present mainly endogenously derived antigenic peptides and are required for the TCR-mediated response of $CD8^+$ T cells (Townsend & Bodmer, 1989). On the other hand, exogenously derived peptides are presented by MHC class

II molecules and are responsible for induction of $CD4^+$ T cells (Rammensee *et al.*, 1993).

The TCR on the majority of peripheral T cells is a disulphide-linked heterodimer of polypeptides designated α and β chains. A smaller subset of $CD3^+$ cells carries an alternative TCR composed of γ and δ chains (Haas *et al.*, 1993). γ/δ TCR repertoire appears to be more limited than that of the α/β TCR (Haas *et al.*, 1993). The predominance of γ/δ T cells in epithelial tissue has led to the suggestion that these cells may preferentially see antigen presented by non-conventional antigen presenting cells (APC), such as epithelial cells or keratinocytes (Band *et al.*, 1991). The significance of γ/δ T cells in infection is under investigation but accumulating evidence suggests that they play a role in the defence against micro-organisms (Band *et al.*, 1991). Human and murine γ/δ T cells showed strong proliferative responses to mycobacterial extracts in the presence of APC (Haregewoin *et al.*, 1989; Janis *et al.*, 1989). Human γ/δ cells also showed cytotoxicity toward lymphoma cells (Fisch *et al.*, 1990). Activation of these T cells results in proliferation, cytokine production and induction of cytolytic effector functions (Kabelitz, 1991)

A major advance in the understanding of immune response to infection was the identification in mice of regulatory $CD4^+$ T helper (TH) cell termed TH1 and TH2 based on their production of either IL-2 and IFN γ (TH1 cells); or IL-4, IL-5, IL-6, and IL-10 (TH2 cells) (Mosmann *et al.*, 1986; Mosmann & Coffman, 1989; Mosmann & Moore, 1991). $CD4^+$ T cells that are capable of producing several or all of these cytokines have been designated TH0 cells (Swain *et al.*, 1991; Mosmann *et al.*, 1991). Furthermore, although cytokines synergise with each other and may duplicate the activities of others, some of those derived from TH1 and TH2 cells act antagonistically (Mosmann & Moore, 1991; Fernandez-Botran *et al.*, 1988; Gajewski *et al.*, 1989; Fiorentino *et al.*, 1989). Initially, it was stated that the distinction between TH1 and TH2 cells does not hold up in humans. These two functional populations of $CD4^+$ T cells have been demonstrated in humans, however, in response to a variety of antigens (Maggi *et al.*, 1992; Parronchi *et al.*, 1991; Modlin

& Nutman, 1993). In human infectious diseases, the TH1 pattern of cytokine production has been associated with immunity or resistance to infection. However the critical question of which factors regulate the TH1/TH2 balance in the course of an immune response is largely unresolved. One early host factor that has recently been identified to be responsible for development of adaptive immunity, involving TH cells, cytotoxic T cells, and B cells, is the cytokine IL-12 (Trinchieri, 1993; Chehimi & Trinchieri, 1994). IL-12 was originally identified as NK cell stimulatory factor and has been shown to have pleiotropic effects on both NK and T cells (Kobayashi *et al.*, 1989). This cytokine induces production of IFN γ as well as TNFα and GM-CSF (Stevenson *et al.*, 1995). Because of this ability to induce IFN γ production, IL-12 promotes the differentiation of $CD4^+$ T cells *in vitro* (Hsieh *et al.*, 1993; Manetti *et al.*, 1993; Seder *et al.*, 1993)

In parasitic infections, TH-dependent immuno-regulation is often associated with a phenomenon called cross-regulation. This concept is based on a mechanism of down-regulation exerted by cytokines. Thus, IFN γ inhibits the proliferation of TH2 cells and Interleukin (IL)-4 secretion, while IL-10 inhibits both the production and the activity of IFN γ (Taverne, 1993). Cytokine production by normal T cells, especially from non-immunised mice, showed that many normal T cells, when first stimulated, produce large amounts of IL-2 but no other T-cell cytokines (Street *et al.*, 1990). Thus the resting T cells that have not recently been stimulated may produce only IL-2 on that first contact with the antigen, and T cells producing multiple cytokines may present later and in more differentiated phenotypes.

(ii). Role of $CD4^+$ T cells

The role of $CD4^+$ T cells in protection against blood stage *P. chabaudi adami* malaria in mice has been established by using antigen-specific cell lines and clones (Brake *et al.*, 1988), and, in *P. chabaudi chabaudi* infection, by *in vivo* T-cell depletion (Langhorne *et al.*, 1990) and adoptive transfer of T cells or T-cell clones to

thymectomised or T-cell-deficient mice (McDonald & Phillips, 1978 & 1980; Süss *et al.*, 1988; Cavacini *et al.*, 1986; Brake *et al.*, 1986; Meding & Langhorne, 1991). In *P. chabaudi* AS murine malaria cells of TH1 type were activated during the early acute phase, once this phase was under control, the activated $CD4^+$ T cells were shown to be mainly of the TH2 type, providing antibody help (Langhorne *et al.*, 1989; Langhorne 1989; Langhorne & Simon 1989). *P. chabaudi* AS-specific $CD4^+$ T cell clones derived from early infection were mainly TH1 type, while the clones derived from reinfected mice were TH2 type (Taylor-Robinson & Phillips, 1992). In addition, adoptive transfer of these clones into naive mice resulted in resistance to *P. chabaudi* AS challenge (Taylor-Robinson & Phillips, 1994a). This involvement of TH1 and TH2 T cell in a sequential order has also been observed by Stevenson & Tam (1993). They have found, however, that induction of a strong TH2 response early in infection may result in a severe and lethal course of infection.

The generation of TH2 cells late during the primary infection appears to be dependent on the presence of B cells (Meding & Langhorne, 1991; von der Weid & Langhorne, 1993b; Taylor-Robinson & Phillips, 1996). Most likely, these B cells may act as APC and produce cytokines or specific costimulatory signals that promote TH2 development (Troye-Blomberg *et al.*, 1994). Such is the case observed in other experimental systems in which different types of APC have been shown to activate $CD4^+$ T cells in a selective form (reviewed by Troye-Blomberg *et al.*, 1994)

Infection of mice with *P. berghei*- parasitised erythrocytes leads to activation of T cells, thereby enhancing IFNγ production (Grau *et al.*, 1989). IFNγ exerts a priming effect on monocytes/macrophages for the production of TNFα as was demonstrated by Gifford *et al.*, (1987). IFNγ primed monocytes, activated by plasmodia antigens, readily produce increased amounts of TNFα (Bate *et al.*, 1988), as well as reactive oxygen intermediates (ROI) (reviewed by Weidanz*et al.*, 1990) and NO (Kremsner *et al.*, 1993). This process almost certainly occurs in the spleen, where parasites are filtered through a network of T cells as well as monocyte-macrophages. Thus, both the presence of a spleen and an intact splenic architecture are required for a

successful achievement of antibody-independent immunity (Kumar *et al.*, 1989, Weiss, 1990).

TNFα is an important cytokine that, along with ROI and NO, generates a delayed-type hypersensitivity immune response modulating macrophage populations granulocytes and eosinophils. Moreover, it contributes to the activation of B cells. Clark *et al.* (1976) suggested that blood stage parasites were killed by an unknown mechanism that resulted in the appearance of "crisis" forms. Subsequent studies reported that, in rodents, recombinant TNF (Taverne *et al.*, 1981) reduced parasitaemias in acute malaria infections. Clark *et al.*, (1987b) noted that clinical findings observed in malaria infection were similar to side effects observed in cancer patients under treatment with TNFα infusions. The amounts of circulating TNF in *P. falciparum* infections can be correlated with many of the complications of the disease, including death from cerebral malaria, perhaps through the local production of noxious amounts of NO. These findings corroborate the view that cytokines may mediate the clinical symptoms of malaria and that clinical acquired immunity in adults living in endemic regions might be achieved by controlling the production of high amounts of these cytokines.

Stevenson *et al.* (1995), have observed IL-12-regulation of development of resistance to *P. chabaudi* AS via a TH1 response that involves $CD4^+$ T cells as well as cytokines, IFN γ and TNF α' and NO. They concluded that the treatment with recombinant IL-12 in the appropriate dose, may be useful in the induction of protective immunity against this blood-stage malaria.

Langhorne *et al.* (1990), showed that there were increased levels of IL-2 early in murine malaria. IL-2 may act, initially, in the activation of natural killer (NK) cells to induce a cytotoxic immune response and could also participate in the activation of macrophages in order to produce a delayed-type hypersensitivity immune response.

(iii). Role of $CD8^+$ T cells

The role of $CD8^+$ T lymphocytes during the erythrocytic stage of malaria is far from clear. They may contribute to the control of the parasite in early stages of infection as exacerbation and prolongation of *P. chabaudi* primary parasitaemia was observed in $CD8^+$ T cells depleted mice (Süss *et al.*, 1988). In a similar model of blood-stage *P. chabaudi* AS, protection against infection was related to presence of both $CD4^+$ and $CD8^+$ cells (Podoba & Stevenson, 1991). Mogil *et al.* (1987), demonstrated that adoptive transfer of $CD8^+$ T cells from *P. yoelii*-immune animals into naive mice accelerated recovery. However, *in vivo* observations confirmed that only $CD4^+$ T cells are crucial for protection when using adoptive transfer of immune T-cell subsets or T-cell depletion of mice by antibody (Vinetz *et al.*, 1990). $CD8^+$ T cells themselves were neither protective nor did they enhance immunity. In addition, $CD8^+$ cells were suggested to be involved in pathogenesis in *P. berghei* by inducing TNF α secretion from macrophages (Waki *et al.*, 1993)

(iv). Role of γ/δ T cells

γ/δ T cells may contribute to parasite killing via their cytolytic activity (Langhorne *et al.*, 1995). It has been shown that human γ/δ T cells can inhibit the growth of *P. falciparum in vitro* and that their activity is directed primarily against the extracellular merozoite (Elloso *et al.*, 1994). However, in a murine *P. chabaudi* infection, γ/δ T cell were not able control a primary erythrocytic infection in absence of $\alpha\beta$ T cells, and were inefficient as helper cells for B cell production of malaria-specific IgG antibodies *in vivo* (Langhorne *et al.*, 1995).

Due to the magnitude of the proliferative response of γ/δ T cells in malaria infection and the predominance of inflammatory cytokines secreted by them, it has been suggested that γ/δ cells may have a role in the pathology associated with the infection (Langhorne, 1996)

1.8. Development of a malaria vaccine

Antigens for vaccine development are being identified from sporozoites, pre-erythrocytic forms, asexual and sexual stages. Attenuated (Clyde *et al.*, 1973; Weinbaum *et al.*, 1976; Rieckman *et al.*, 1979; Waki *et al.*, 1986) and killed (Desowitz & Miller, 1980) malaria parasites have 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 of *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. At present, the goal is to stimulate protective immunity to the whole parasite from only one or 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. *Salmonella* mutants have been successfully used to deliver heterologous antigens to the immune system in mice. This system may also be taken further by generating constructs that contain cytokines alongside malarial antigens. Thus it would be possible to use the adjuvant effect of a specific cytokine to enhance the immunogenicity of a particular malarial antigen. A brief review of the malarial antigens which may be important to consider for inclusion in a Salmonella system follows.

1.8.1. Sporozoite/pre-erythrocytic stage antigens

A fully protective effective sporozoite vaccine is expected to induce immunity which either stops invasion of liver cells, or if invasion occurs it should prevent the parasite of completing its pre-erythrocytic cycle.

The major sporozoite protein is a coat protein (circumsporozoite/CS protein) which has the same general makeup in human, simian and murine malarias. The central third of the protein consists of multiple repeating immunogenic sequences which are unique for each species. In *P. falciparum* the major central region of the protein is a repeating sequence (about 40 times) of asparagine-alanine-asparagine-proline (NANP). This region is an 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 human trials were carried out (Ballou*et al.*, 1987, Herrington *et al.*, 1987). However, failure of CSP subunit vaccines to protect humans fully (Ballou *et al.*, 1987; Herrington *et al.*, 1987; Fries *et al.*, 1992), highlighted the important role of T cells responses in development of protective immunity (Krzych *et al.*, 1995). Protective cell-mediated immunity to sporozoites by immunisation with attenuated *S. typhimurium* transformed with the *P. berghei* CS gene has been observed in mice in the absence of antisporozoite antibodies (Sadoff *et al.*, 1988).

Several non-CSP sporozoite surface molecules have been identified (Galey *et al.*, 1990). These include among other: sporozoite threonine- and asparagine-rich protein (STARP), thrombospondin-related anonymous protein (TRAP) (Robson *et al.*, 1988) and sporozoite- and liver- stage antigen (SALSA) (Bottius *et al.*, 1996). However, their precise contribution on acquisition of immunity against sporozoite challenge is subject of intense research.

1.8.2. Asexual blood stage antigens

A large number of antigens derived from asexual blood-stages of malaria parasites have now been described and conserved and/or antigenically conserved portions of many of the target antigens for vaccine development have now been identified.

Antigens currently considered for inclusion in an anti-*P. falciparum* asexual blood stage vaccine are briefly described below.

An antigen which has been examined in great detail as a potential malaria vaccine component is the major merozoite surface protein (MSP-1 or MSA). *P. falciparum* MSP-1 is on the surface of the merozoite as a complex of polypeptides after the proteolytic cleavage of a precursor. The protein is held on the membrane surface by a 42kDa fragment that has a GPI anchor. At the moment of red cell invasion (or immediately before), the complex is released from the surface by protease cleavage within the 42kDa fragment. A 19 kDa anchored domain, which consists of two epidermal growth factor-like motifs, is produced after this cleavage and is retained on the intracellular parasite (reviewed by Egan *et al.*, 1996). Antibodies raised against the MSP-1 protein have shown to block merozoite invasion *in vitro* (Howard *et al.*, 1993), possibly by inhibiting the protease cleavage (Blackman *et al.*, 1994). Similarly, *in vivo* immunisation of monkeys with the native protein resulted in protection against *P. falciparum* challenge (Etlinger *et al.*, 1991). The successful expression of a C-terminal fragment of MSP-1 in yeast of a baculovirus recombinant system elicited antibodies that blocked the *in vitro* growth of the parasite (Chang *et al.*, 1992). A synthetic peptide, SPf66, which contains a sequence of MSP-1 protein, has partially protected humans and monkeys (Patarroyo *et al.*, 1987, Patarroyo *et al.*, 1988) against natural and experimental challenges. This vaccine is now undergoing trial in humans and initial results showed protection in 38.8% of subjects resident in a endemic region of Colombia (Patarroyo *et al.*, 1987; Patarroyo *et al.*, 1988), in 66.8% in a similar population in Ecuador (Sempertegui *et al.*, 1994) and 31% in an endemic region in Tanzania (Alonso *et al.*, 1994). However, conflicting results have been obtained in different populations. Recent trials carried out in The Gambia and Thailand found no co-relation between protection and vaccination in children under 15 year old (Nosten *et al.*, 1996).

MSP-2 is another *P. falciparum* merozoite surface protein, contains T-cell epitopes, both in the constant and variant parts of the antigen (Rzepczyk *et al.*, 1992).

Immunisation with this *P. falciparum* protein has led to protection of mice against an otherwise mortal challenge with *P. chabaudi* (Saul *et al.*, 1992).

The antigen *P. falciparum* (Pf) 155/RESA was first detected by a modified immunofluorescence assay on erythrocytes infected with ring forms of *P. falciparum* (Holder *et al.*, 1988; Foley *et al.*, 1983). Pf155/RESA is a merozoite-derived polypeptide deposited in the erythrocyte membrane during parasite invasion. Antibodies against Pf155/RESA have shown to inhibit invasion of *P. falciparum* merozoites *in vitro* (Berzins *et al.*, 1985, Ruangjiraporn *et al.*, 1988). The majority of antibody responses against this protein are directed to conserved repeat regions of the molecule (Hviid *et al.*, 1992). A specific T cell response has been described against this Pf155/RESA antigen in malaria-exposed donors. The strongest and most frequent of the T cell responses are also directed towards conserved repeat regions of the molecule (Troye-Blomberg *et al.*, 1988).

PfEMP-1 (Leech *et al.*, 1984, Van Schravendijk *et al.*, 1991), is a protein which mediates cytoadherence of *P. falciparum* parasitised red blood cells to the vascular endothelium and is an attractive vaccine candidate since induction of immunity against this molecule could result in a prevention of cerebral malaria. Antibodies to PfEMP1 prevented cytoadherence and therefore sequestration. Previously, David *et al.* (1983) and more recently, Goldring *et al.*, (1992), have demonstrated reversal of sequestration after inoculation of polyspecific IgG . In addition, antibodies that prevent rosetting (Carlson *et al.*, 1990) have been associated with the absence of cerebral malaria. The importance of these findings relates not only to the abolishment of pathology in the main organs, but such treatment could lead to an increase in parasite circulation which enables the immune system to make better contact with it.

Two P. *falciparum* histidine rich proteins (PfHRP-1 and PfHRP-2) (Howard *et al.*, 1986; Rock *et al.* 1987), and Ag332 (Mattei & Scherf, 1992; Mattei *et al.*, 1992) are other membrane surface antigens which have been studied as potential vaccines.

The rupture of parasitised red blood cells permits the release of large amounts of antigen derived both from the parasite and the parasitised cell. Immunisation with

phosphatidyl-inositol and phosphatidylserine has resulted in production of high and prolonged IgG titres against toxic malarial antigens (Bate *et al.*, 1993) as measured by reduction in ability of peritoneal macrophages to produce TNFα *in vitro*. The effect of these antibodies on induction of TNFα *in vivo* is yet to be studied (reviewed by Clark & Rockett, 1994). In 1988 Riley *et al.* evaluated cellular immune responses in Gambian adults finding that 50% of immune patients produced IFN γ when cells were stimulated with soluble antigens. Later, proliferative immune responses have been found against identified soluble antigens such as Ag1 which was associated with trophozoites and schizonts but not with merozoites, and Ag7 was found associated with schizont only. Both antigens, Ag1 and Ag7, have been reported as inducers of T cell responses with production of IFN γ but have not been associated with humoral responses.

1.8.3. Sexual stage antigens

Immunity to sexual stages of the parasite, though of no immediate benefit to the host, can prevent transmission of the disease by blocking the parasite life cycle within the mosquito. Such immunity is known as transmission blocking.

A humoral response against sexual form of rodent malaria parasites has been described by Harte *et al.* (1985). Antibodies against macro- and micro-gametocytes may disrupt the transmission cycle of the disease by inhibiting the fertilisation and development of the zygote (reviewed by Kaslow, 1993). Expression of recombinant Pfs25, a protein normally found on the surface of P. falciparum gametocytes/gametes and ookinetes, has been used successfully to induce transmission blocking immunity antibodies (Kaslow *et al.*, 1991).

The vaccine candidate antigens which have been examined in some detail are *P. falciparum* 230 and *P. falciparum* 48/45 (Howard *et al.*, 1993). Riley *et al.* (1990), demonstrated T-cell responses against *P. falciparum* 48/45 in 40% of immune donors and association with IFN γ production *in vitro*.

A large product of *P. falciparum* coded by the Pf11-1 gene, has been recently described in the cytoplasm of gametocytes and in the membrane of lysed erythrocytes, suggesting a role for Pf11-1 in erythrocyte rupture within the mosquito gut (Sherf *et al.*, 1993). After studying the immune response against this protein in mouse and humans exposed to malaria, it was concluded that the Pf11-1 molecule might induce an unusually heterogeneous B and T cell response during natural infection in man.

1.9. *Salmonella* vectors

1.9.1. Attenuated *Salmonella*

Current developments in recombinant DNA technology have resulted in the production of highly effective live attenuated vaccines (Hone *et al.*, 1994), which offer major advantages over killed preparations since induction of immunity via live systems (e.g. *Escherichia coli, S. typhi and S. typhimurium*) allows the generation of stronger, cellular and longer-lasting immune responses (Chatfield *et al.*, 1995). This probably occurs because some of them have the ability to replicate within the host in antigen presenting cells (MacKaness, 1971). Genetically engineered live vaccines possess stable and well defined non-reverting mutations of genes essential for survival in the host and therefore they establish self-limiting infections that mimic early stages of the natural infection (Chatfield *et al.*, 1995). These characteristics make recombinant live vaccines more suitable for immunogenicity studies when compared with other attenuated vaccines (i.e. irradiated vaccines). Agents studied as live vaccine vectors include vaccinia virus, avipoxviruses, adenovirus, polioviruses, herpesviruses, *E. coli*, *Salmonella* and *Mycobacterium bovis* (BCG).

Several promising *Salmonella* vaccines candidates have been investigated (Chatfield *et al.*, 1992). One group of the *Salmonella* mutants that has been extensively studied includes those strains harbouring defined mutations in gene encoding enzymes in the pre-chorismate biosynthetic pathway (aro mutants) (Chatfield *et al.*, 1995). The

explanation of the attenuation of aro mutants is presumed to lie in their *in vivo* inability to scavenge essential aromatic metabolites, para-aminobenzoic acid among others (Chatfield *et al.*, 1995). Since vertebrates lack the pre-chorismate pathway, sustained bacterial replication is impossible (Chatfield *et al.*, 1995). An important advantage of these mutants is that their reduced virulence results from starvation of essential nutrients and not from their inability to resist the host's immune response (Chatfield *et al.*, 1995). Hence, they are safe to use in immunosuppressed populations.

1.9.2. *Salmonella* life cycle and pathogenesis

Orally administered *S. typhimurium* penetrate the wall of the small intestine through the Peyer's patches (WHO, 1989), where antigen-sampling cells known as microfold or M cells (Walker, 1994) reside. M cells transport antigens or whole micro-organisms from the intestinal lumen to the follicle underneath, leave the Peyer's patches via the efferent lymphatics and enter the systemic circulation via the thoracic duct. What follows involves the return of lymphocytes to the effector site (*i.e.* the lamina propria of the gastrointestinal tract) and the production of secretory IgA in external secretions (Williamson *et al.*, 1995).

In mice, *S. typhimurium* delivered orally can also reach other organs such as liver and spleen, after invading the lamina propria and the draining mesenteric lymph nodes (O'Callaghan *et al.*, 1988), and hence allowing the production of specific systemic immune responses after colonisation of macrophages within the spleen and liver.

1.9.3. Immunity to *Salmonella* infection

Mice infected with *S. typhimurium* divide into susceptible and resistant according to the presence of different alleles of the *Lsh/Ity/Bcg* gene. BALB/c mice cannot control the infection with virulent salmonellae and succumb to fatal infection. However, when they are immunised with live attenuated strains of *S. typhimurium*, protective immunity to further virulent challenge can be induced (Hosieth & Stocker, 1991). A period of non-specific immunity has been observed as the initial replication of the attenuated organisms reaches a plateau, during which resistance to infection with *Listeria monocytogenes* as well as virulent *Salmonella* has been reported (Hormaeche *et al.*, 1991). NK cells and macrophages are primary mediators of this non-specific protection (Schafer & Eisenstein, 1992).

Resistance to virulent infection after the initial phase is antigen specific and requires the presence of T cells (Mastroeni *et al.*, 1992). In order to transfer this immunity to naive susceptible animals, T cells in addition to serum from immune animals are required (Mastroeni *et al.*, 1992). Antibodies are though to contribute with killing of extracellular organisms and so help to contain infection. Macrophage activation and increase production of IFNγ and TNFα are critical in the killing of intracellular micro-organisms (Mastroeni *et al.*, 1992) and can cause *S. typhimurium* killing if administered *in vivo* during infection (Nakanu *et al.*, 1990). Since infection with salmonella elicits a strong $CD8^+$ CTL response (Aggarwal *et al.*, 1990), lysis of infected cells by cytotoxic T cells is also suggested to contribute to bacteria clearance (Conlan & Norh, 1992).

1.9.4. Attenuated *Salmonella* as heterologous carriers

The first reports of the use of a live vaccinia virus expressing foreign antigens were made in 1982 (Panicalli & Paoletti, 1982, Mackett *et al.*, 1982). Since then other vectors have been shown to be useful for the transport of antigens. Among them are *E. coli*, *S. typhimurium*, *S. typhi*, BCG, poxvirus, adenovirus, herpesvirus and

poliovirus. Of particular interests is the potential of attenuated *S. typhimurium* and *S. typhi* strains as carriers of diverse antigens from a number of other pathogens. *Salmonella* mutants can induce strong humoral and cellular immune responses and therefore can be manipulated to induce such immune responses to foreign antigens. Antigens of bacterial, viral and parasite origin have been expressed in *S. typhimurium* as prospective vaccines against a number of diseases.

S. typhimurium have been engineered to express *Leishmania* antigens. The gene encoding *L. major* gp63 was expressed in a aroA mutant of *S. typhimurium* and mice were immunised with this construct. Susceptible BALB/c mice had reduced lesion development after oral immunisation with the vaccine probably through acquisition of a protective TH1- like response to *L. major* (McSorley, 1995).

This technology has been applied to research in malaria achieving the expression of malarial antigens such as *P. falciparum* serine-rich protein (SERP) and *P. falciparum* histidine rich protein-2 (PfHRP-2) by *S. typhimurium* (Schorr *et al.*, 1991). More significantly, recombinant *Salmonella* expressing the circumsporozoite protein (CSP) has shown to protect mice against sporozoite challenge (Sadoff *et al.*, 1988, Flynn *et al.*, 1990). A CSP expressing *Salmonella* vaccine was used in humans which later developed significant humoral responses against the protein (Gonzalez *et al.*, 1994).

In mice, *S. typhimurium* mutants have also been engineered to deliver biologically active cytokines. *S. typhimurium* expressing murine IL-1β confer protection against lethal gamma irradiation due to functional IL-1β activity (Carrier *et al.*, 1992). Murine IL-4 has also been expressed in *S. typhimurium* and shown to have activity in vivo (McSorley, 1995). Similarly, administration of a *S. typhimurium* mutant expressing murine TGFβ had a significant effect in reducing induced oedema (Ianaro *et al.*, 1995).

Attenuated *S. typhimurium* can be used as carriers of heterologous antigens to the immune system in animal models and future progression from a single attenuated typhoid fever vaccine to a multivalent vaccine against diverse pathogens may be possible.

1.10. Immune evasion by malaria parasites

1.10.1. Sequestration

In order to kill malaria parasites, the host immune system must have access to the parasite and an opportunity to interact with other cells of the system (reviewed by Allred, 1995). Because asexual forms of malaria parasites are intracellular, they are not accessible to immune system during most of their reproductive cycle. Alterations in the red cell membrane may result in changes in cytoadhesion when infected with some *Plasmodium* species (*i.e. P. falciparum*). Cytoadherence of infected RBCs to post-capillary venous endothelium results in withdrawal from the peripheral circulation (sequestration) of the trophozoite and schizont-stage parasite.

Regardless of the host's immune state, only very immature sexual forms or mature gametocytes are usually found circulating in the blood of humans infected with *P. falciparum*. The more mature stages localise to the post-capillary venular endothelium of the brain, placenta or gut. Host factors seem to influence sequestration. In humans, *P. falciparum* schizont sequestration is almost complete. In other non-human primates this phenomena does not occur to the same extent and the main sites are different (Miller, 1969; David *et al.*, 1983). *P. vivax* undergoes sequestration to some degree (Garnham, 1966).

In other primate and rodent malarias, some degree of sequestration has been demonstrated. These include *P. coatneyi*, *P. fragile* (Desowitz *et al.*, 1969; Fremount & Miller, 1975), *P. knowlesi* (Miller *et al.*, 1971) and *P. berghei* (Alger, 1963; Weiss, 1983; Mackey *et al., 1980*; Rest, 1982; Warrell, 1987). Similarly, markedly sequestration of pRBC occurs in the liver during *P. chabaudi* infection.

Cytoadherence of infected red cells to endothelial cells represents the acquisition of specific properties on the membrane of the infected cell. These binding properties of *P. falciparum* are directed towards individual ligands. However, they are not constant

and vary with the isolate and previous cytoadherence history (Marsh *et al.*, 1988; Magowan *et al.*, 1988; Biggs *et al.*, 1991 &1992; Roberts *et al.*, 1992; Ockenhouse *et al.*, 1992). Parasitised RBC can adhere to five different endothelial cell surface molecules: thrombospodin (Roberts *et al.*, 1985), CD36 (Ockenhouse *et al.*, 1988 & 1991; Oquendo *et al.*, 1989; Barnwell *et al.*, 1989), intercellular adhesion molecule 1 (ICAM-1) (Berendt *et al.*, 1989), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (or endothelial cell adhesion molecule 1, ELAM-1) (Ockenhouse *et al.*, 1992). Upregulation of ICAM-1 expression can mediated by cytokines (Staunton *et al.*, 1988). IL-1, IL-2, IFNγ and TNFα can all induce ICAM-1 expression on diverse cell types (Dustin *et al.*, 1986; Rothlein *et al.*, 1988; Asarnow *et al.*, 1989). TNFα dependent upregulation of ICAM-1 is of particular importance since elevated TNFα expression has been associated with cerebral malaria (Udomsangpetch *et al.*, 1989; Kwiatkowski *et al.*, 1990).

The identity of the components on the surface of the *P. falciparum* infected red blood cell mediating cytoadherence, is under debate. For some authors the receptor involved in the expression of the cytoadherent phenotype is PfEMP1, which is a large, parasite derived molecule expressed on the surface of the infected erythrocyte (Biggs *et al.*, 1992; Leech *et al.*, 1984). Others have demonstrated that sequestrin, a large molecule which is also expressed on the pRBC (Ockenhouse *et al.*, 1991) and a modified erythrocyte endogenous component derived from band 3 (Winograd & Sherman, 1989; Crandall & Sherman, 1994), selectively bind CD36 (Ockenhouse *et al.*, 1991; Crandall *et al.*, 1994).

Cytoadherence of pRBC to the endothelium may prevent their opsonisation and phagocytosis both by physically blocking circulating phagocytic cells engulfing infected cells and by preventing passage through the spleen. However, the pRBC still be accessible to action of monocytes, neutrophils, NK cells or other effector cells (reviewed by Allred, 1995).

1.10.2. Antigenic variation

Cox (1962) was the first to suggest that malaria parasites undergo antigenic variation, when he studied mice infected with blood forms of *P. berghei.* Later, conclusive studies were made by Brown & Brown (1965) when working with *P. knowlesi.* They studied rhesus monkeys infected with the parasite and treated with antimalarials at subcurative doses which develop recrudescent parasitaemias. This allowed them to demonstrate that each recrudescence was characterised by a specific antigen which elicited a specific antibody response. Parasites collected from later relapse populations in the same host did not react significantly with earlier sera. These findings have also been confirmed by other authors using clone parasite lines of *P. knowlesi* (Howard *et al.,* 1983).

Antigenic variation in *P. falciparum* has been observed *in vivo,* when using a cloned line to infect Saimiri monkeys (Hommel *et al.,* 1983) and *in vitro* (Biggs *et al.,* 1991; Roberts *et al.,* 1992). Antigenic variation of *P. falciparum* can occur very rapidly *in vitro,* 2% per generation, when no immune pressure is being exerted (Roberts *et al.,* 1992), indicating that could be a non-induced event (reviewed by Allred, 1995). During the period of a single infection, asexual parasites repeatedly change antigens presented to the host. The new antigenic variants are little affected by immunity to previous variants, and by the time the response to the new antigens reaches effective levels, a newer variant is being produced. Such mechanism allows parasite's survival regardless of the effect of the specific acquired immunity.

The first evidence that *P. chabaudi* undergoes antigenic variation was observed in experiments using NIH mice. Immune sera proved to be significantly less protective against recrudescent parasites than against their parent parasite (Brannan *et al.,* 1993). Infection with recrudescent clones of *P. chabaudi* has resulted in important differences in the course of parasitaemia, with some clones producing higher peak infections than other (Phillips *et al.,* 1997, in press). It has been suggested that this *in vivo* difference may reflect alterations in immunogenicity and functional characteristics associated with expression of antigenically variant antigens on the

surface of the infected red blood cell (Phillips *et al.*, 1997, in press). In *P. falciparum*, the PfEMP1 antigen is involved in antigenic variation (Biggs *et al.*, 1991), and undergoes changes *in vitro* in both apparent size and immunoreactivity (Magowan *et al.*, 1988; Biggs *et al.*, 1992; Roberts *et al.*, 1992). The significance of these changes is not clear, but altered sequestration of these antigenically variant parasites may result in increased pathological sequelae and could explain some of the binding diversity observed in field isolates (Goldring *et al.*, 1992), and why some young children develop cerebral malaria in the absence of other significant clinical signs. (reviewed by Allred, 1995).

1.10.3. Antigenic diversity

Antigenic diversity is the expression of antigenically different forms of an antigen by different malaria parasites. Differences in geographical location, individuals within the same location, or bouts of malaria within the one individual; may result in antigenic diversity of the parasite. Furthermore, diversity may also be recognisable in pRBCs taken at various times from a non-cloned parasite isolated cultivated *in vitro*. Antigenic diversity has been suggested to confer, in some occasions, selective advantage to the parasite by mechanisms that may be antibody-independent. Significant antigenic diversity in *P.falciparum* in humans, has been observed when volunteers infected with the parasite showed more resistance when they were subsequently challenged with a homologous strain than with a heterologous one (e.g. Jeffrey, 1966).

For many years, geographical variation in drug resistance and transmissibility through mosquitoes has been demonstrated (Shute *et al.*, 1976. McGregor *et al.*, (1963) have shown that immune serum from West African patients was less effective that East African serum on disease in East Africa.

Numerous malarial antigens exhibit antigenic diversity in different *P. falciparum* isolates, e.g. the major glycoprotein on the surface of the asexual stage pRBC

(reviewed by McBride *et al.*, 1982) and a soluble antigen released into the plasma during rupture of schizont-infected RBCs (Wilson, 1980).

Antigenic diversity is thought to be the result of the expression of different allelic forms of a single gene. The diverse forms are generated either by genetic re-arrangements during meiosis or by accumulation of mutations. Heterogeneity is maintained by genetic recombination in addition to the mutation, as has been shown in *P. chabaudi chabaudi* (Walliker *et al.*, 1975) and in human malaria (Walliker *et al.*, 1987).

1.11. Immunopathology

1.11.1. Non-specific cell activation

Polyclonal B lymphocyte activation occurred during malaria infection leads to IgG hyperproduction (McGregor *et al.*, 1956, Cohen & Butcher, 1969). In *P. falciparum* infection IgG produced is non-specific and has been demonstrated against a wide range of self-antigens, auto-antibodies recognising heart, thyroid and gastric parietal cells (Shaper *et al.*, 1968), lymphocytes (Wells *et al.*, 1980), and erythrocytes (Rosenberg *et al.*, 1973; Ronai *et al.*, 1981; Zoulai *et al.*, 1982; Wahlgren *et al.*, 1983). High titres of antibodies to nuclear components (Greenwood *et al.*, 1970; Adu *et al.*, 1982), single strand DNA (Ribeiro *et al.*, 1984), mitochondria (Boonpucknavig & Ekapanyakul, 1984), and smooth muscle (Phanuphak *et al.*, 1983) have also been found in association with malaria infections.

Hypergammaglobulinaemia have shown to be a T cell-mediated event (Weidanz, 1982), probably due to production of B cell-activating cytokines (Ballet *et al.*, 1987, Kabilan *et al.*, 1987).

1.11.2. Immunosuppression

Antigen-specific unresponsiveness is often observed in association with clinical disease among residents of *P. falciparum* endemic areas (Ho *et al.*, 1986, Riley *et al.*, 1988b). Children affected with malaria have been shown to be deficient in their ability to mount primary immune responses against tetanus toxoid (McGregor & Barr, 1962), The O Ag of *S. typhi* (Greenwood *et al.*, 1972; Greenwood, 1984) and Group C meningococcal vaccine (Williamson & Greenwood, 1978).

Immunosuppression during malaria infection can be demonstrated *in vitro* as a lowered lymphocyte response to mitogenic or antigenic stimuli. It has been suggested that NO may be responsible for this malaria-induced immunosuppression (reviewed by Mendis & Carter, 1995). Spleen cells of *P. vinckei*-infected mice, which showed lowered responsiveness to mitogen stimulation *in vitro*, restored to normal levels of responsiveness when cultured in presence of N^{G}L-monomethyl arginine acetate (LNMMA) (reviewed by Mendis & Carter, 1995). During *P. chabaudi* infection, the immunosuppressed state was reversed when LNMMA was introduced into their drinking water before and during infection (Rockett *et al.*, 1994)

1.11.3. Cerebral Malaria

Cerebral malaria (CM) is a serious complication of *P. falciparum* infection and constitutes the most common cause of death due to malaria. Pathological changes during CM include: 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).

Two types of phenomenon have been studied in attempts to understand the underlying pathogenic mechanisms of CM. The first involves the adherence of pRBCs to host cells in the post-capillary venules of the brain. The second, relates induction of host cytokines with effects on brain tissues through production of secondary mediators, such as NO and free oxygen radicals.

The most obvious cell to cell attachment involved in human CM is that of pRBC with the endothelial cell lining of blood vessels (Trager *et al.*, 1966; Luse & Miller, 1971; MacPherson *et al.*, 1985). Cellular adhesion between pRBCs and other host cells , including nRBC (Handunnetti *et al.*, 1987;1989) may also be important in sequestration and pathology of acute *P. falciparum* malaria.

The molecules involved in the adhesion of pRBC to the endothelial cell surface are: vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) (Berendt *et al.*, 1989), endothelial adhesion molecule 1 (ELAM-1) or E-selectin, CD36 (Oquendo *et al.*, 1989), and thrombospondin (Robert *et al.*, 1985) (reviewed by Mendis & Carter, 1995). Induction of ICAM-1 expression is regulated by cytokines (Staunton *et al.*, 1988), indeed, TNFα, IL-1, IL-2 and IFNγ 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.* (1992) 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 the molecule. To support this theory they performed *in vitro* studies of cytoadherence of *P. falciparum* pRBCs comparing isolates form patients with uncomplicated malaria, severe malaria or cerebral forms of the disease. They showed that high binding of the pRBCs to ICAM-1 was correlated with cerebral involvement in the patients from whom the parasites derived (Berendt *et al.*, 1992).

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 antibody exerted a protective effect on the *P. berghe*i-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.

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

TNF induces activation of an inducible NO synthase (iNOS) to produce NO using arginine as initial substrate. iNOS activation due to cytokines results in a rise of up to 1000-fold of NO concentrations (reviewed by Mendis & Carter, 1995) which can shut down important aspects of synaptic transmission (Schuma & Madison, 1994). However, the importance of this NO-theory as a basis of pathology in malaria remains controversial (reviewed by Mendis & Carter, 1995).

1.12. Inducible nitric oxide synthase defective mice

Mice lacking iNOS were first described by Wei *et al.*, (1995). Wild-type MF1 mice were manipulated and the iNOS gene disrupted in their embryonic stem cells. Mice heterozygous for the mutation were bred to obtain homozygous which were viable and fertile, with no evidence of histopathology within major organs. Peritoneal macrophages from wild-type or heterozygous mice produced substantial amounts of NO and expressed iNOS mRNA and iNOS protein when stimulated with IFNγ and LPS *in vitro*. On the other hand, homozygous mice produced an altered iNOS mRNA, had no detectable levels of iNOS protein, and produced background levels of nitrite (Wei *et al.*, 1995).

When challenged with *L. major*, wild-type and heterozygous mice showed high resistance to infection. In contrast, iNOS defective mice were highly susceptible to infection. Spleen cells of these mice had increased T-cell proliferative responses when

cultured with leishmanial antigens or ConA *in vitro*. Upon stimulation with leishmanial antigens, IFNγ production was higher and IL-4 lower by cells from homozygous mice in comparison with wild-type and heterozygous mice (Wei *et al.*, 1995).

The ability of these mice to develop local inflammation in response to carrageenin injection has also been studied. Homozygous mutant mice developed significantly less footpad swelling than wild-type mice. As when infected with *L. major*, spleen cells from carrageenin-treated mice produced more IFNγ than wild type mice. Mutant mice were also more resistant to LPS-induced death (Wei *et al.*, 1995).

1.13. History of *Plasmodium chabaudi chabaudi*

The parasite used in this study, *Plasmodium chabaudi chabaudi* AS, was isolated from the blood of thicket rats (*Thamnomys rutilans*) in the Central African Republic by Landau (1965). The parasite infects mainly mature erythrocytes (Landau, 1965) although it can also invade reticulocytes under conditions of anaemia (Carter & Walliker, 1975; Jarra & Brown, 1989). The parasite can produce multiple infection of red cells (Carter & Walliker, 1975)

Asexual stages of the parasite have a synchronous cycle of 24 hrs and, under normal light conditions, rings and trophozoites are observed in peripheral blood during the day, while schizonts appear around midnight. Peripheral withdrawal of schizonts to deep tissue capillaries occur in this species as schizont maturation takes place (Shungu & Arnold, 1972; McDonald, 1977; McDonald & Phillips, 1978; Gilks *et al.*, 1990).

Blood induced infection with *P. chabaudi* AS strain in inbred NIH mice usually last around 60 days. A primary patent parasitaemia is observed which is followed by one or two patent recrudescences (Figure 2). In its natural host, *P. chabaudi* follows a less acute course of infection during its early stages, persisting, afterwards, as a chronic infection. In contrast, Injection of 10^6 pRBC of *P. chabaudi chabaudi* AS

result in a fulminant parasitaemia in A/J mice, 100% of animals died with a mean survival time of 10 days (Stevenson *et al.*, 1990a).

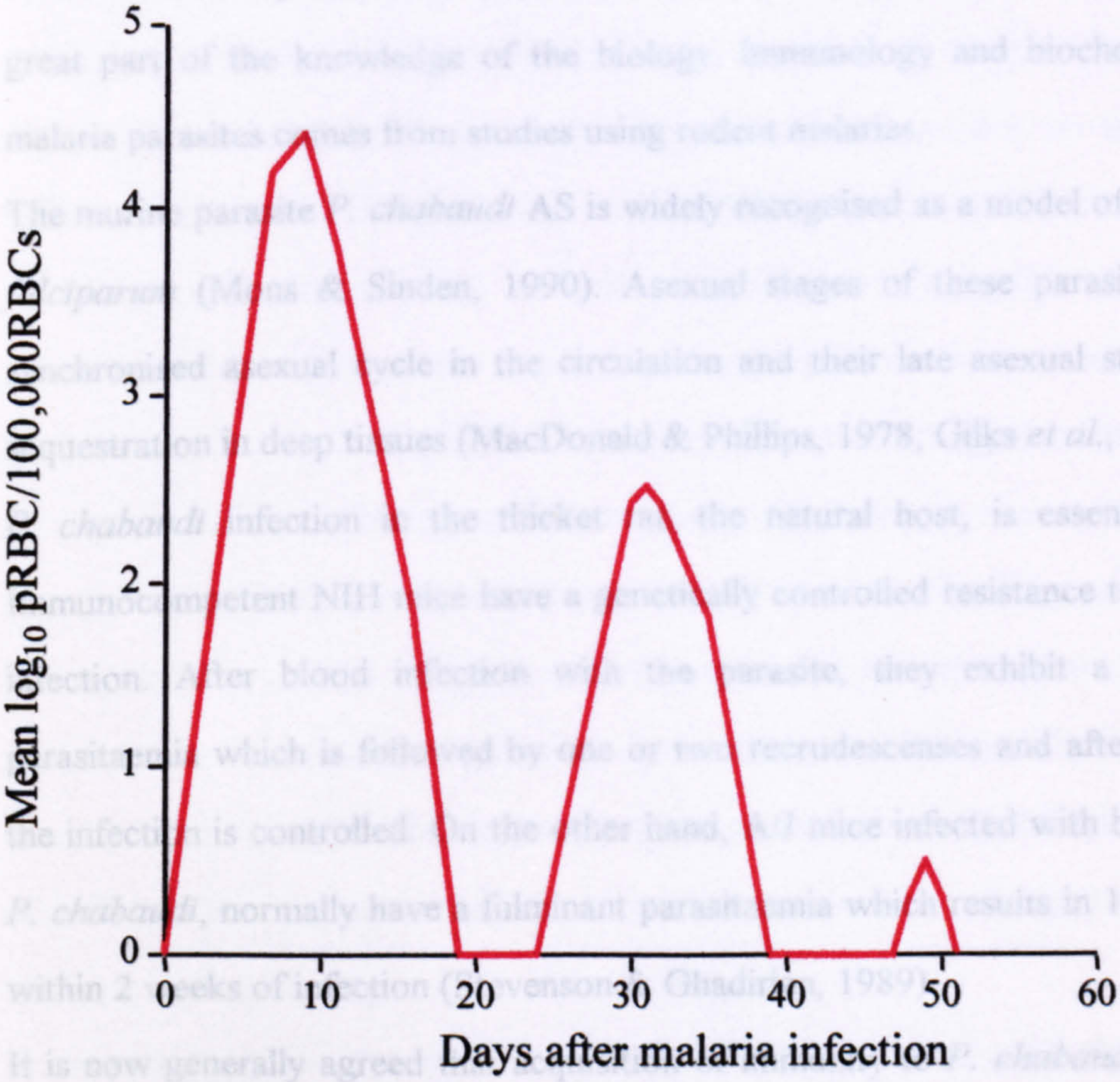


Figure 2. The course of *P. chabaudi chabaudi* AS infection usually observed in naive NIH.

1.14. Aim of the Project

The nature of the protective immune response against malaria is not completely understood. Murine models of malaria infection have been widely used to study some of the factors responsible for acquisition to immunity against the infection. Indeed a great part of the knowledge of the biology, immunology and biochemistry of the malaria parasites comes from studies using rodent malarias.

The murine parasite *P. chabaudi* AS is widely recognised as a model of the human *P. falciparum* (Mons & Sinden, 1990). Asexual stages of these parasites possess a synchronised asexual cycle in the circulation and their late asexual stages undergo sequestration in deep tissues (MacDonald & Phillips, 1978; Gilks *et al.*, 1990).

P. chabaudi infection in the thicket rat, the natural host, is essentially lifelong. Immunocompetent NIH mice have a genetically controlled resistance to *P. chabaudi* infection. After blood infection with the parasite, they exhibit a high primary parasitaemia which is followed by one or two recrudescenses and after 8-10 weeks, the infection is controlled. On the other hand, A/J mice infected with blood forms of *P. chabaudi*, normally have a fulminant parasitaemia which results in 100% mortality within 2 weeks of infection (Stevenson & Ghadirian, 1989).

It is now generally agreed that acquisition of immunity to *P. chabaudi* by 'resistant' strains (such as NIH) of mice, is dependent on $CD4^+$ T cells, with both TH1 and TH2 playing a crucial role. TH1 cells seem to be dominant in the control of the primary parasitaemia, while TH2 are predominant in the control of recrudescenses (reviewed by Phillips *et al.*, 1997, in press). However, unravelling the interactions of $CD4^+$ T cells, cytokines and effector mechanisms against *P. chabaudi*, has proved complex. Phillips *et al.* (1997, in press) have suggested that the initial control of the rising parasitaemia is mediated through non-specific effector mechanisms, which are largely driven by TH1 cells. Others have shown that treatment of mice with IFN γ has a protective affect against blood stage *P. chabaudi* infection in 'susceptible' mice

(Stevenson *et al.*, 1990), while injection of TNFα into susceptible mice resulted in protection against infection (Stevenson & Ghadirian, 1989).

The experiments described in this thesis aimed to assess further the role of T cells during the acquisition of immunity against *P. chabaudi* AS strain. Initial experiments were designed to define the feasibility of utilising a *S. typhimurium* mutant as a delivery vehicle for cytokines by studying the effects of their inoculation on the course of *P. chabaudi* infection. This was followed by studies on the role of the different TH1 and TH2 cytokines, such as IFNγ, TNFα, TGFβ or IL-4, in host defence against the infection in 'resistant' NIH and 'susceptible' A/J mice. The cytokines were delivered *in vivo* using the *S. typhimurium* recombinant system described by Carrier *et al.* (1992). The confirmation that a *S. typhimurium* mutant can be used in malaria infection to deliver murine cytokines *in vivo* would also be an important contribution in the study of a malaria vaccine. The long term intention of this work is to achieve coexpression of malarial antigens and cytokine genes by *S. typhimurium* and to obtain a synergistic action for acquisition of malaria immunity.

Another approach might be the vaccination against malaria of individuals inoculated with *S. typhimurium* expressing cytokines, in this case the cytokine(s) would enhance the immune response against the malarial antigens.

Previously, others have demonstrated that, in NIH mice, NO plays a significant role in host control of the primary patent parasitaemia but has no effect on the control of recrudescences (reviewed by Taylor-Robinson, 1996). Recently available inducible-NO-synthase(iNOS)-deficient mice might constitute a valuable model in understanding the *in vivo* role of NO in the control of a murine infection with *P. chabaudi*. To investigate this, the course of a blood infection in these mice was followed and changes in humoral and cellular responses were evaluated.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Mice

Three different strains of mice were used: inbred NIH and A/J, and outbred inducible nitric oxide synthase (iNOS) knockout mice.

Female NIH and A/J mice were purchased from Harlan Olac (Bicester, UK) and kept in the Wellcome Laboratories for Experimental Parasitology (WLEP) animal house breeding facility or in the Joint Animal Facility at Glasgow University. Rooms were maintained at 22 _C ± 2 _C and 50-60% relative humidity with 12 hours artificial light from 0800 to 2000 or 0700 to 1900 hr. Mice were fed pelleted CRM breeder diet (Labsure Ltd). Both water and food were administered on an *ad libitum* basis. All mice were used for experimental purposes when they were 6-12 weeks old and weighed approximately 25 g.

iNOS depleted mice were provided by the Department of Immunology of the University of Glasgow. These mice originated from the strain MF1 but due to a mutation in the iNOS gene they have a deficient production of the enzyme and therefore of the inducible form of NO (Wei *et al.*, 1995). Mice that are heterozygous for the mutation produce the enzyme normally and were used as controls.

2.2. Parasites

The AS strain of *Plasmodium chabaudi chabaudi* was originally isolated from adult thicket rats (*Thamnomys rutilans*) caught at La Maboke, Central African Republic, in March 1969 by Professor David Walliker (University of Edinburgh). The strain was established in laboratory mice and then cloned by limiting dilution (Walliker *et al.*, 1971). The parasites were supplied to the Department of Zoology, University of Glasgow, by Professor Walliker in 1973. Since then, the parent AS clone has been maintained by cryopreservation and subpassage through mice (see below) (Figure 3).

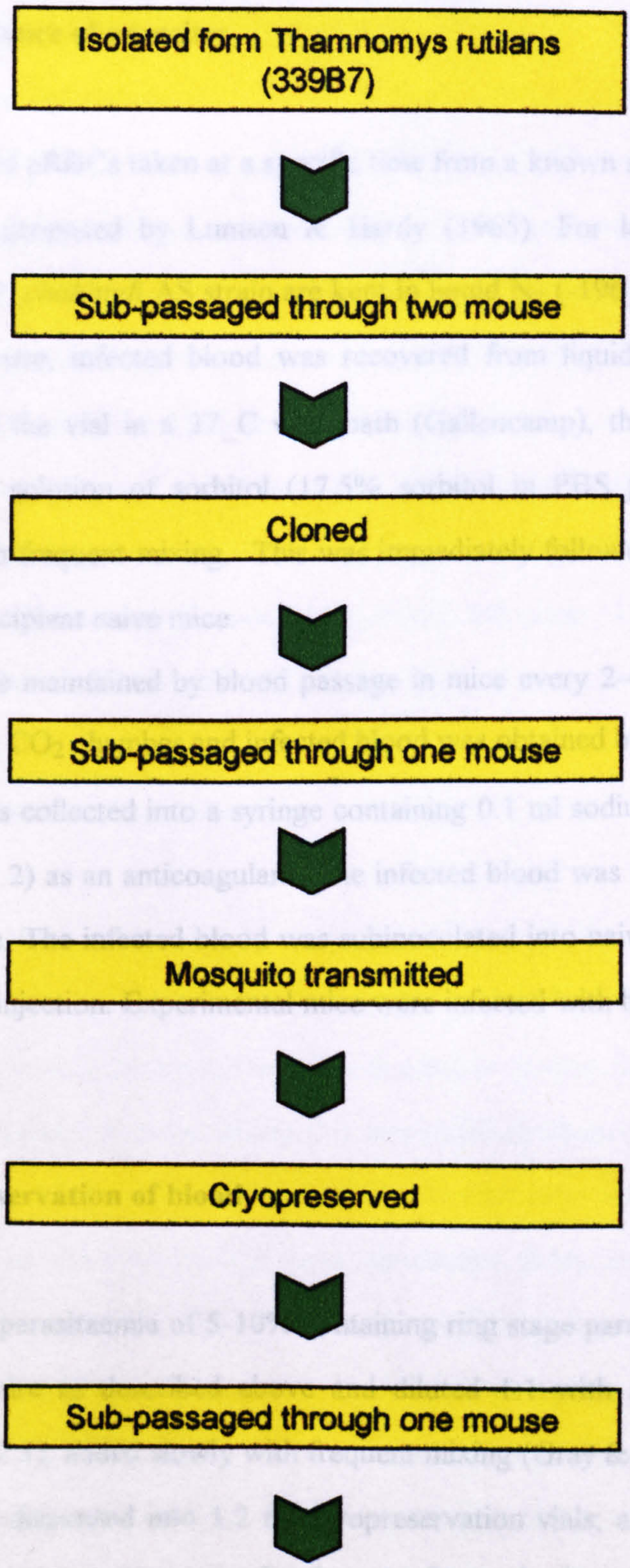


Figure 3. History of *Plasmodium chabaudi chabaudi* AS strain.

2.3. Maintenance of parasites

Suspensions of pRBCs taken at a specific time from a known source were designated stabilates as proposed by Lumsen & Hardy (1965). For long term preservation stabilates of *P. chabaudi* AS strain are kept in liquid N_2 (-196_C). When required for experimental use, infected blood was recovered from liquid N_2 and defrosted by immersion of the vial in a 37_C waterbath (Gallencamp), then diluted in an equal amount of a solution of sorbitol (17.5% sorbitol in PBS pH 7.2) added slowly dropwise with frequent mixing. This was immediately followed by i.p. injection into one or two recipient naive mice.

Parasites were maintained by blood passage in mice every 2-4 days. The mice were sacrificed in a CO_2 chamber and infected blood was obtained by cardiac puncture. For this blood was collected into a syringe containing 0.1 ml sodium heparin (10 IU/ml) in PBS (pH 7.2) as an anticoagulant. The infected blood was sub-inoculated i.v. into recipient mice. The infected blood was subinoculated into naive recipient mice either by i.v. or i.p. injection. Experimental mice were infected with blood one passage from stabilate.

2.4. Cryopreservation of blood

Blood with a parasitaemia of 5-10% containing ring stage parasites, was collected by cardiac puncture as described above and diluted 1:1 with a solution of sorbitol-glycerol (see 2.3), added slowly with frequent mixing (Gray & Phillips, 1981). 200 µl aliquots were dispensed into 1.2 ml cryopreservation vials; a code was assigned to each of them. These were immediately snap frozen by immersing in liquid N_2 and stored.

2.5. Challenge infections

Infected blood for challenge was obtained by cardiac puncture immediately after the mice were sacrificed in a CO_2 chamber. Giemsa's stained thin blood smears were used to evaluate the donor's parasitaemia and the blood was diluted to the required concentration of pRBCs/ml in RPMI 1640 medium (Moore *et al.*, 1967)

Before infection, the mice were placed in a warm box at 32_C for 10 min in order to allow vasodilatation and 200 µl (1 x 10^5 or 1 x 10^6 pRBC) of the suspension of pRBCs were administered via one of the lateral tail veins. For this purpose a 1 ml syringe fitted with a 26G needle was used.

2.6. Determination of parasitaemias

Parasitaemias were determined by daily examination of Giemsa's stained thin blood smears made from peripheral blood. Samples were taken between 0900-10:30 hr every day by piercing the tip of the tail with a lancet. A new lancet was used for each group of mice and within the group this was routinely dipped in alcohol in order to minimise the possibility of transfer of infected erythrocytes between animals. A drop of blood was placed onto washed glass microscope slides, smeared and air dried at room temperature (RT). Smears were fixed in 100% methanol and incubated for 20 min in a solution 1:10 of Giemsa stain in Giemsa's phosphate buffer (pH 7.2). They were then rinsed in tap water, air dried and examined under the microscope at x1000.

Parasitaemias were obtained by calculating the proportion of parasitised red blood cells (pRBC) from a total number of red blood cells (RBC). For this, at least 20 fields containing between 200 and 300 erythrocytes were examined. Infection was considered sub-patent when no parasites were observed in 50 fields. Smears were normally taken from day 3 to 60 post-infection.

2.7. Presentation of parasitaemic data

The geometric mean of the parasitaemia (mean Log_{10} of the number of pRBC in 10^5 RBC) of a group of mice was plotted against time (expressed in days) and the resultant graph expressed the course of infection. Vertical bars showing S.D. are included when differences in the courses of parasitaemia among different experimental groups are not self-evident.

From the data collected and their graphical presentation, five 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; (v) survival to infection in the case of 'susceptible' mice.

In all experiments, the minimum number of mice in each experimental group was 5. 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 predicted theoretical minimal level calculated using the binomial distribution (Gilks, 1988). Groups of at least 5 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 Microsoft excel version 6.0 on a Viglen Genie Professional 45x25 PC and Cricket graph III 1.5 on an Apple Macintosh Performa 630 microcomputer. Nonparametric statistical analysis was performed on parasitaemia data using the Microsoft excel program on a PC.

2.8. Haematology

2.8.1. Haematocrit

Heparinised capillaries were used to collect blood from the tip of the tail as described above. Samples were centrifuged for 5 min at 12,000 g in a micro-haematocrit centrifuge (Hawksley) and the proportion of the column of packed red blood cells (haematocrit) was determined using a haematocrit reader (Hawksley).

2.8.2. Reticulocytes

Peripheral blood obtained from the tail was used to determine the proportion of immature red blood cells or reticulocytaemia. For this purpose a drop of blood was smeared onto 1% brillial cresyl blue pre-treated glass slides. They were incubated for 30 minutes in a humidified chamber at RT and, after drying, stained with Giemsa's stain as for determination of parasitaemia. Remnant nuclear material present in immature erythrocytes is observed as dark blue spots distributed within the cell. The proportion of reticulocytes and mature red blood cells was determined by observation under x1000 and presented as a percentage.

2.9. Collection of serum

Large volumes of sera were collected after exsanguination by cardiac puncture immediately after sacrificing the mice. Blood was allowed to clot and this was loosened from the edges of the container and left to contract for 1 hour at 37 _C (or overnight at RT). The overlying serum was collected by pipetting and contaminating RBC were removed by centrifugation (300 g for 5 min). The cleared serum was then aliquoted and stored frozen at -20 _C until required.

For collection of smaller volumes of serum, mice were warmed in a warm box at 32_C for 10 min and up to 100 µl of blood were withdrawn from the tail. The blood was processed as for larger volumes.

For the collection of immune serum from infected mice to study the levels of specific anti-malarial antibodies or levels of nitric oxide (as nitrate) during the course of infection, serum was collected from individual mice in different experimental groups at 2-3 days intervals from days 0 to 60 post-infection. Within each group, every mouse was bled on an equal number of occasions to ensure that anaemia did not result from repeated bleeding of an individual mouse.

2.10. Determination of anti-malarial antibody titres

The slide IFAT procedure of Van Meirvenne *et al.* (1975) modified by McLean *et al.* (1982) was used to establish total anti-parasite antibody present in serum of infected mice. The technique is based on the indirect fluorescent antibody method for malaria parasites as described by Voller (1964) and O'Neill & Johnson (1970).

2.11. Preparation of malarial antigen slides

Trophozoite/schizont stage parasites were collected from infected mice and used as the source of antigen. Donor mice with a rising parasitaemia of 5-15% were bled into 0.1 ml of heparin up to a final concentration of 10 IU./ml. pRBCs were washed three times in 20 ml PBS (pH 7.2) by centrifugation (250 g for 5 min) and resuspended. The final pellet of cells was resuspended to at about a half of 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 tissue in batches of five, packed with silica gel and stored at -20 _C until required (Manawadu & Voller, 1978).

2.11.1. Assay

To avoid condensation, slides stored at -20 _C were brought up to RT in a desiccator for 1-2 hours before use. They were then fixed in absolute acetone (Rhone-Poulenc Ltd.) for 5 min and air dried. Reaction zones were marked on the antigen slides using an H series tex pen (Deacon Laboratories). The slides were washed into successive coplin jars of PBS, drained and rehydrated in a third jar of PBS (pH 7.2) for 15 min. From this point slides were never allowed to dry as this would impair fluorescence. The area around each reaction zone was dried and 20 µl of a serial 1:2 dilution of test and control sera were applied to reaction zones. Each slide had PBS (to act as a control for non-specific fluorescence), a negative serum and a hyperimmune serum as controls. The slides were incubated for 15 min at RT in a humidified chamber, then washed and rehydrated as above. They were recovered from PBS and the edges dried. Then, each slide was applied with 1 ml of FITC-conjugated sheep anti-mouse IgG (Scottish Antibody Production Unit (SAPU)) diluted 1:200 in PBS containing Evans Blue (1:10,000 w/v) (Merck) and incubated for 15 min. The Evans Blue acts as counterstain (El Nahel & Bray, 1963). Finally, the slides were washed to remove excess conjugate and rehydrated in fresh PBS before mounting under a long coverslip with a 1:1 solution of non-fluorescent PBS-glycerol (Merck).

2.11.2. Examination of slides

A Leitz Ortholux microscope was used to observe fluorescence. The microscope was linked to an Epson PX4 computer through a Leitz MPV Compact 2 microscope photometer. The overhead u.v. source used was a Wotan HBO-50 mercury lamp with 2 x kP490 exciting filters and a TK 510 dichronic beam-splitting mirror and a k515 suppression filter.

The slides were examined at x 500 using a water immersion objective. The titre (endpoint) of the serum was considered to be the last dilution of serum at which

specific fluorescence was observed. The control zones of hyper-immune and normal serum, as well as that of PBS only, were examined for comparison.

2.12. Determination of nitric oxide levels

Nitrate serum levels were determined as a measure of nitric oxide (NO) production, nitrogen oxidation of L-arginine results in the release of the highly reactive intermediate NO, which has a half-life of milliseconds before being further oxidisation to nitrite (NO_2^-) and nitrate (NO_3^-). Both NO_2^- and NO_3^- are more stable forms and can be detected in serum as a result of NO production (reviewed by Green *et al.*, 1990). Nitrate serum levels were determined using the conversion test described by Moncada *et al.* (1991). The degree of conversion was assessed by the Greiss reaction (Migliorini *et al.*, 1991) or by chemiluminescence (Palmer *et al.*, 1987; Dowes *et al.*, 1976). 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 solution of $NaNO_2$ and $NaNO_3$ respectively in distilled water. 30 μl of the standards and the samples were plated out in duplicate and then 30 μl of conversion buffer (Appendix) containing the enzyme nitrate reductase and the cofactors NADPH and FAD added. The plate was incubated at 37 _C for two hours, after which time they assayed for NO_2^- or frozen at -20 _C for later evaluation.

The Greiss reaction for nitrite analysis was carried out in 96-well flat bottomed plates. 50μl of samples and standards were plated in duplicate and 50μl of the Greiss reagent (Appendix) added to each well. After 10 minutes of incubation at room temperature, the plate was read in an ELISA reader (Titertek Multiskan MCC/340) at 540 nm. Nitrite levels were read from the standard curve.

In some cases, a chemiluminescence method was used for the determination of NO_2. Briefly, 20μl of sample were injected, using a Hamilton syringe, into a reaction vessel containing 75 ml 1% 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 chemiluminiscent product measured with a photomultiplier (Model 2107 Chemiluminescent Nitric Oxide analyser, Dasibi Environmental Corporation), and quantified by reference to 1 min infusions of authentic NO into the reflux vessel. Nitrite levels were read from the standard curve.

2.13. Preparation of spleen cell suspensions

Suspensions of spleen cells suitable for *in vitro* proliferation were prepared as follows. Mice were sacrificed and the spleens dissected out aseptically. Each spleen was placed in a 9cm Petri disc (Sterilin) containing incomplete RPMI 1640 medium. The spleens were cut into pieces and dissociated by pushing through a sterile stainless steel sieve (mesh size 0.025 mm^2) using the inside of a plunger of a syringe, and collected into the medium. The cells were disaggregated by aspiration through a Pasteur pipette, and the supernatant collected after tissue debris and clumps of cells were sedimented. The spleen cells were washed twice by centrifugation at 250g for 5 min, and the medium was changed after each wash. The final pellet was resuspended in 1 ml of 10% FCS RPMI 1640 medium.

Contaminating erythrocytes were removed by lysis. For this 1 ml of spleen cell suspension was incubated with 9 ml 0.83% Tris-ammonium chloride (pH 7.4) (Appendix) for 5 min at RT (Boyle, 1968). The suspension was washed twice with 10% FCS RPMI 1640 medium at 250 g for 5 min. The pellet was resuspended in 1 ml of 10% FCS RPMI 1640 medium and appropriate dilutions were made to determine cell viability (see below).

2.14. Determination of cell viability

The viability of spleen cell preparations was measured by the trypan blue dye exclusion test (Naysmith & James, 1968; recommended by Jerne *et al.*, 1974). A dilution 1:10 or 1:100 of spleen cells in PBS (pH 7.2) was prepared and a further 1:1 dilution was made with a solution of 0.2% w/v trypan blue (Gurr, BDH Ltd.) in PBS (pH 7.2). The suspension was mixed thoroughly and incubated at RT for 2-3 min before being examined by phase contrast under immersion oil (x1000) on a light microscope. Dead cells were unable to exclude the vital dye and stained blue, whereas viable cells remained clear. At least 100 cells were observed and the proportion of live to dead cells was expressed as a percentage viability, and adjustments to total cell numbers made accordingly.

2.15. Proliferative assays

Spleen cell suspensions were prepared as described above. The cells were adjusted to a final concentration of $1x10^6$/ml in 10% FCS RPMI 1640 medium. Aliquots of 200 µl in triplicate were incubated in 96 well flat-bottomed microtitre plates (Nunc) with a crude *P. chabaudi* antigen (200 µg/ml), a normal RBC antigen (200 µg/ml), ConA (5 µg/ml) or RPMI 1640 medium. In some cases, a fresh infected red blood cell antigen was used at a final concentration of 2 x 10^6/ml (see 2.18), and the control cultures were incubated with a non-infected RBC fresh antigen or RPMI 1640 medium. Cells were incubated at 37 _C in humidified 5% CO_2 atmosphere for 72 hours. At the end of which each well was pulsed with 1µCi (37kBq) of [^{3}H-methyl] thymidine (Amersham International) diluted in 10µl of 10% FCS RPMI 1640 medium. After a further 18 hours incubation, the wells were harvested with a semi-automatic cell harvester (Titertek, Flow) onto glass fibre filter paper (FG/A, Whatman), washed twice with distilled water and air dried. Each filter disc was transferred to plastic beta vials (LKB) and 2 ml non-aqueous scintillation fluid

(Optiscint 'safe', LKB) added using an automated dispenser (Jencons (Scientific) Ltd.). All the insert tubes were lidded, labelled and placed inside outer plastic scintillation vials ready for counting. The beta activity present in each sample was detected during 1 min using a scintillation counter (LKB Wallac 1219 Rackbeta) and quantified on a programmed computer (Viglen Genie Professional 45x25).

For individual wells, total counts per minute were measured, from which the arithmetic mean for triplicate wells could be calculated. Cells were considered to have given a positive proliferative response if their counts were ³ 2 S.D. than the values of the appropriates negative controls.

2.16. Preparation of parasitised and normal red blood cells lysates

For use in stimulation of spleen cells, soluble crude *P. chabaudi chabaudi* AS strain antigens were prepared from whole blood cells enriched for mature trophozoite/schizont-infected RBCs. Since sequestration of mature forms occurs *in vivo*, a modification of the method described by McDonald & Sherman (1980) was used obtain a mature forms of the parasite *in vitro* .

Mice to be used as the source of antigen were kept under reversed light (12 hr light between 19:00-07:00 hr) conditions for a minimum of 10 days before infection, therefore schizogony which under normal light condition would occur at around 01:00 hr, occurred between 1100-1300 hr (Jarra & Brown, 1985).

When mice reached peak parasitaemia (around 40%) they were sacrificed and bled by cardiac puncture into an heparinised syringe (0.1 ml of 10 IU./ml PBS). RBCs containing mainly late ring stages were washed twice in 5% FCS RPMI 1640 medium at 200 g for 5 min. The pellet of RBC was resuspended to a 10% haematocrit in the same medium and incubated using the candle jar method (Trager & Jensen, 1976). For this 1.5 ml of the RBCs suspension were dispensed in 35 mm Petri dishes (Cel-Cult, Sterilin), then placed together with a candle in a humidified glass dessicator. The candle lit and the lid put on with the stopcock open. When the

candle flame started to extinguished, the stopcock was closed. This provided a gas phase of approximately 3% CO_2 and 15-17% O_2. The candle jar was placed in a 37_C incubator until the parasites had reached the schizont stage; this was monitored every 30 min by examination of Giemsa's stained thin blood smears.

After harvesting, the cells were washed in 5% FCS RPMI 1640 medium at 200 g for 5 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 leukocytes (Beutler *et al.*, 1976) and the filtrate subsequently washed in 5% FCS RPMI 1640 medium at 200 g for 5 min. The pellet containing the malaria parasites was restored to its original volume in PBS (pH 7.2) and then freeze-thawed five times. Each cycle of freeze-thawing entailed snap freezing the preparation by plunging into liquid N_2, then immediately defrosting the suspension in a 37 _C waterbath (Gallencamp). The disrupted pRBC suspension was centrifuged at 1500 g for 10 min and the supernatant fluid collected. This was termed the pRBC lysate antigen and was stored in 20 µl aliquots at -20 _C until required.

Non-infected blood was used for the preparation of a RBC antigen. For this the same procedure was followed as for the preparation of the pRBC lysate antigen excluding the steps of culturing the pRBC. The antigen obtained from non- infected RBC was called nRBC lysate antigen. This antigen was used as a control for testing antigen specificity (Dodge *et al.*, 1963).

2.17. Determination of total protein concentration

A quantitative estimation of total protein present in the RBC lysate antigens was determined using the method described by Smith *et al.* (1985). The assay can be used to measure protein concentration in the range 150-1500 µg/ml, where the colour response is relative linear, allowing quick, accurate determinations. For this known protein concentrations ranging between 150-1500 µg/ml were prepared by diluting 2 mg/ml stock bovine serum albumin (BSA) standard (Pierce Chemical Co.) in

deionised water. 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 and the absorbance at 595 nm was read versus deionised water using a spectophotometer (Pye Unicam PU 8600). The net absorbance of each standard or unknown protein sample was obtained by substracting the absorbance of deionised water from each sample. A standard curve was constructed from which the protein concentration for each unknown sample was determined.

2.18. Preparation of a fresh antigen of *P. chabaudi*

A fresh antigen of *P. chabaudi* was used in some of the lymphoproliferative assays. This was prepared from infected blood of one mouse which was obtained into heparinised PBS (10 IU/ml) by cardiac puncture. Parasitaemia was determined (see 2.6) and the blood was washed twice in RPMI 1640 medium. Then, the pRBCs were resuspended in medium to obtain a concentration of 2×10^6 or 2×10^5 pRBCs/ml (for a final concentration in the well of 10^6 or 10^5 pRBCs/ml). A control antigen was prepared following identical procedure using blood of a naive mice. The concentration of RBCs of the control antigen was defined by the total number of cell at which the pRBC antigen was prepared (pRBC plus non-infected RBC).

2.19. *Salmonella typhimurium* mutants

The *S. typhimurium* BRD509 mutant is an $aroA^-$, $aroD^-$ strain derived from *S. typhimurium* SL1344 (Strugnell *et al.*, 1992). The plasmids NB/IFNγ, NB/TNFα, NB/TGFβ or NB/IL-4 were transformed into the mutant at the Department of Immunology of the University of Glasgow using previous published procedures (Carrier *et al.*, 1992). A *S. typhimurium* mutant not carrying cytokines genes, was used as a bacteria control.

2.20. Bacteria culture

Bacteria were received and routinely cultured in Millers-modified Luria Broth (L-broth) (Gibco BRL, Life Technologies) consisting of 1% SELECT Peptone 140, 0.5% Yeast Extract and 1% sodium chloride. A Millers-modified Luria agar (L-agar) (Gibco BRL, Life Technologies) containing 1% SELECT Peptone 140, 0.5% Yeast Extract , 1% sodium chloride and 1.2% Agar was also used routinely to replicate the bacteria. Both media were sterilised before use. Bacterial strains containing plasmids were cultured in the presence of ampicillin (100 mg/ml)(Sigma) to select for transformed bacteria. The mutant used as control was cultured without adding ampicillin. A single colony of *S. typhimurium* from an L-agar plate was grown in 1 l of L-broth overnight at 37 _C without shaking. The optical density of the suspension was measured at 600 nm using a spectophotometer (Pye Unicam PU 8600) and the bacteria centrifuged at 600g for 20 min at 4_C in a Mistral 3000i (Fisons) centrifuge. The pellet was resuspended in sterile PBS (pH 7.2) to obtain a bacterial concentration of approximately 1 x 10^{10}/ml. The volume, in ml, of PBS in which the pellet should be resuspended was equivalent to five times the optical density reading. The number of bacteria in the suspension was checked by culturing dilutions of the suspension on L-agar Plates.

Mice received tube 0.2 ml of the bacteria suspension by gavage . Immediately prior to the *Salmonella* inoculation the mice were given 0.1 ml 5% sodium bicarbonate orally in order to neutralise the gastric pH.

2.21 Bacterial growth and plasmid stability *in vivo*

Two of the mutants were tested for their growth and stability *in vivo*. Groups of 18 mice each were set up, each group received orally *S. typhimurium* BRD509/NB/IFNγ or *S. typhimurium* BRD509/NB/TNFα. Mice were inoculated as described above.

Colonisation of the bacteria in NIH mice was monitored by performing viable bacteria counts from homogenates of spleen, liver and mesenteric lymph nodes as previously described (O'Callaghan *et al* 1988). Three mice were sacrificed each time point, at 3 days and 1, 2, 3, 4 and 5 weeks after inoculation. Spleen, livers and mesenteric lymph nodes were obtained under sterile conditions, placed in 10 ml of sterile PBS (pH 7.2) and homogenised by passing through a sterile stainless steel sieve (mesh size 0.025 mm^2) using the inside of a plunger of a syringe to push the cells through. Ten fold dilutions of the homogenate of each individual organ were plated in a volume of 100 µl onto L-agar plates both with and without 100 µl/ml ampicillin. Samples were incubated overnight at 37_C. The number of colonies which grew in the absence of ampicillin was used to calculate *Salmonella* colonisation of organs and the number growing with ampicillin was used to calculate plasmid stability.

2.22 IFN-gamma ELISA

To quantify IFNγ in spleen cell supernatants a capture ELISA was performed. Spleen cells were processed and suspensions prepared as described above (see 2.16). Aliquots of 1 ml in duplicate were plated in 24 well flat-bottomed tissue culture plates (Nunc) with a crude *P. chabaudi* antigen (200 µg/ml), a normal RBC antigen (200 µg/ml), and ConA (5 µg/ml). Control cultures were unstimulated. Cells were incubated at 37 _C in humidified 5% CO_2 and 24, 48 or 72 hours later supernatants were collected and stored at -70 _C until they were assayed for IFNγ production.

Flat-bottomed 96-well plates (Immulon IV) were coated with 50 µl per well of a 0.5 µg/ml dilution of the purified rat anti-mouse IFNγ (Pharmingen) capture antibody in PBS . Plates were incubated at 4 _C overnight. The wells were blocked with 150 µl of 10% FCS in PBS and incubated for 45 min at 37 _C. The plates were then washed three times with PBS/0.05% Polyoxyethylenesorbitan monolaurate (Tween 20) (Sigma) for three minutes each time. The IFNγ recombinant standards were prepared in 10% FCS RPMI and the samples to be assayed were defrosted at 37 _C. 50 µl of

standards and samples were plated in each well and incubated at RT for 2 hr. The plates were washed as above and 50 µl of rat anti-mouse IFNγ biotinylated antibody (Pharmingen) in 1% BSA/PBS-Tween 20 added and incubated for 1 hour at room temperature. After a washing step, 75 µl of streptavidin peroxidase in 1% BSA/PBS-Tween 20 was added to each well and incubated for 1 hour at room temperature. The plates were washed and 100 µl of substrate (TMB peroxidase)was added per well. Plates were read after 15 minutes in an ELISA reader (Titertek Multiskan MCC/340) at a wavelength of 630 nm. Units of IFNγ were calculated from the standard curve constructed from the recombinant cytokine standard.

2.23. Reverse transcription- Polymerase chain reaction (RT-PCR)

2.23.1. RNA extraction

A semiquantitative RT-PCR technique (Osborne, personal communication) was used to measure production of IFNγ mRNA in livers and spleens of NIH mice inoculated with *S. typhimurium*/IFNγ 8 days before *P. chabaudi* infection. Control groups were included.

Organs were extracted aseptically and kept at -70° C. Livers from uninfected mice were used fresh as controls.

Samples were defrosted when required and total RNA was isolated using GIBCO TRIzol™ reagent. Presence of RNA was confirmed by running of the samples on a MOPS gel. For this a 50ml mini-gel was prepared (see appendix), and 1-5µl RNA sample were added to 1µl 500ng/ml EtBr, 1µl dye and 5µl formamide. The solution was incubated at 65° C for 10 min, loaded and run in a MOPS buffer in fumehood. Quality of RNA was checked by U.V.

2.23.2. DNAase Treatment

RNA samples were DNAase treated using a GIBCO DNAase I Amplification Grade™ kit. Samples of 1μg of RNA, 1μl DNAase buffer and 1 μl DNAase enzymes were mixed and incubated at room temperature for 15 min. Then 1 μl 20mM EDTA and 6 μl DEPC treated water were added and the solution was incubated at 65 C for min.

2.23.3. Reverse Transcription

Reverse transcription was carried out by adding 2-5 μg of RNA (DNAase-treated) to 2 μl random hexamers and 0.5 μl RNAsin (Promega). This solution was incubated at 65°C for 10 min, pulsed and left in ice for 5 min. Then 4 μl of RT Buffer (Promega), 2 μl 10mM dNTPs (Perkin Elmer) and 1 μl RT enzyme (Promega) were added and the resultant solution was incubated at 37°C for 60 min.

2.23.4. Polymerase Chain Reaction (PCR)

For PCR, an Amplitaq DNA polymerase kit (Perkin Elmer) was used. 50 μl reaction mixture were prepared by mixing 4 μl DNA, 5 μl 10x Buffer, 37.5 μl sterile ddH_2O, 1 μl dNTPs (10 mM), 1 μl (20 uM) of each primer and 0.5 μl amplitaq enzyme. The solution was incubated at 94°C for 45 sec, 60°C for 45 seconds, 72°C for 2 min, for 30 cycles. Finally, they were incubated at 72°C for 7 min. Quality of the product was checked on a 1.2% agarose gel.

2.23.5. Primers

Primers of IFNγ and β-actin (control) were obtained from CRUACHEM and were prepared as follows (Roberts, personal communication):

- 3′β-actin

CTC TTT gAT gTC ACG CAC gAT

- 5′β-actin

  gTg ggC CgC TCT Agg CAC CAA

- 3′IFNγ

  CgA CTC CTT TTC CgC TTC CTg Ag

- 5′IFNγ

  TgA Acg CTA CAC ACT gCA TCT Tgg

2.24. Cytochemical identification of macrophages

An assay for the detection *in situ* of macrophages in liver and spleen samples was standardised based on a technique previously described by Dockrell *et al.*(1978). Macrophages posses a high non-specific esterase activity. Using a-naphthyl acetate as substrate, macrophages show strong cytoplasmic staining. In addition tests for acid phosphatase, an important marker of lysosomes, give strong positive reactions with macrophages.

Livers and spleens obtained at different time-points from mice inoculated with *S. typhimurium*/IFNγ and from controls, were analysed for the presence of non-specific esterase and acid phosphatase. They were fixed in formol sucrose at 4°C for 24 hr and rinsed for further 24 hr using cacodylate-sucrose. They were frozen at -20°C and kept until required for cryostat sectioning.

Before placing the sections, the slides were immersed in a solution of gelatine (5 gm) and chromium potassium sulphate (0.5 gm) (SIGMA) in 1 lt dH_2O. Slides were dipped at room temperature, drained and allowed to dry. Cryostat sections of the tissues were cut at a thickness of 10 μm and mounted on glass slides before staining for non-specific sterase or acid phosphatase.

2.24.1. Demonstration of non-specific sterase

A stock solution containing α-naphthyl acetate 1 gm, acetone 50 ml and dH_2O 50 ml was prepared and named solution A. A phosphate buffer 0.1 M pH 7.3 was prepared by mixing 23 ml sodium dihydrogen orthophosphate anhydrous 0.2 M, 77 ml disodium hydrogen orthophophate anhydrous (0.2M), and 100ml dH2O. This buffer was named solution B.

A fresh working substrate was prepared with 2 ml solution A, 15 ml solution B, 15 ml dH_2O and 20 gm Fast Red TR salt (Sigma). This substrate was filtered before use.

Mounted samples were incubated in the substrate for 30-40 min at room temperature. Then, they were rinse in tap water and counterstain with 1% methyl green (Sigma) for 1 min. Finally they were rinse in tap water and mounted in glycerine gelatine (Sigma) before observation. Enzymatic activity was seen as reddish granules.

2.24.2. Standard coupling Azo-dye technique for demonstration of acid phosphatase

A mixture of 10 mg sodium-naphthyl phosphate in 20 ml veronal acetate buffer 0.1 M was prepared. To this, 1.5 gm polyvinyl pirrolidine (Sigma) were added and allowed to dissolve at 37°C, then 20 mg fast Red Garnet GBC salt (Sigma) were added. The solution was shaked and filtered.

Dry sections were incubated in the stain at room temperature for 30 min, wash in tap water and counterstain with 1% methyl green for 1 min. Samples were washed in tap water and mounted in glycerin-gelatine (SIGMA). Sites of acid phosphatase activity are seen as reddish brown areas.

CHAPTER THREE

EFFECT OF INTERFERON-GAMMA ON THE COURSE OF *Plasmodium chabaudi* INFECTION

3.1. Introduction

IFNγ is a potent inducter of maturation of mononuclear phagocytes into macrophages, and increases the synthesis of the enzymes that mediate the respiratory burst (reviewed by Stevens, 1995). Other effects of IFNγ include enhancement of cellular and humoral immune responses through increased expression of MHC I and II; increased T- and B-cell differentiation; neutrophil and natural killer cells activation and enhancement of TNFα transcription and synthesis (reviewed by Stevens, 1995).

Evidence from several experimental murine models leads to the conclusion that IFNγ is important in the acquisition of immunity against malaria. The cytokine has been demonstrated to have an important activity both *in vivo* and *in vitro* against pre-erythrocytic stages of *Plasmodium* (Ferreira, 1986; Mellouk *et al.*, 1991; Maheshwari *et al.*, 1990).

The use of neutralising anti-IFNγ antibodies enabled identification of IFNγ as responsible for killing blood forms of *P. yoelii* through activation of macrophages (Ockenhouse & Shear, 1984). In *P. chabaudi* infection, both plasma levels and production of IFNγ *in vitro* by spleen cells, increases and peaks two to three days before the peak of parasitaemia (Slade & Langhorne, 1989; Stevenson *et al.*, 1990c), indicating a role for IFNγ in the control of the primary parasitaemia. Recent findings confirmed that IFNγ, in combination with TNFα, is responsible for the induction of one or more factors, other than NO, which are involved in parasite killing (Jacobs *et al.*, 1996a).

Protective mechanisms associated with IFNγ during primary parasitaemia are not completely antibody-independent. Treatment with anti-IFNγ antibodies resulted in suppression of production of protective antiplasmodial IgG2a immunoglobulin isotype in mice infected with *P. berghei* (Waki *et al.*, 1995).

Research on the effects of IFNγ injection on the course of a malaria infection and its role in protection against the disease has been limited. Exogenous IFNγ does not

consistently provide complete protection, which could be partially explained by the fact that IFNγ is a short lived cytokine (Sedegah *et al.*, 1994).

The phenomenon of sequestration observed in *P. chabaudi* might allow close contact of the parasite with effector cells and their products in organs such as liver and spleen. Recently, Faure *et al.* (1995) have concluded that schizonts are responsible for a significant homing or multiplication of lymphomyeloid cells in the extravascular compartment of the liver. This may imply accumulation of effector molecules locally in the liver which come in close contact with mature forms of the parasite while sequestration occurs. *S. typhimurium* invades deep organs and multiplies within them, and therefore IFNγ delivered by a mutant is expected to accumulate in organs such as the liver, and contribute to better control of a malaria infection. This constitutes an important advantage over experiments where the cytokine is administered by injection as inoculation into the bloodstream does not guarantee its location in those areas where sequestration occurs.

The experiments presented in this chapter investigated the effect of the oral inoculation of a *S. typhimurium* mutant, carrying the murine IFNγ gene, on the course of a *P. chabaudi* infection in 'resistant' NIH mice. The bacteria were given at different times before malaria infection to achieve IFNγ production at different stages of primary parasitaemia. Other immune parameters such as NO, and antibody production, and *in vitro* proliferative responses and cytokines production, were also studied in the mice. Additionally, the course of *P. chabaudi* infection was followed in susceptible' A/J mice which were inoculated with the *S. typhimurium* mutant before the malaria infection.

3.2. Results

3.2.1. Colonisation and stability of the *S. typhimurium* mutant in organs of NIH mice

Eighteen age-matched female NIH naive mice were used in this experiment. A stock of the *S. typhimurium* BRD509/NB/IFNγ (*S. typhimurium*/IFNγ) mutant was replicated in L-broth with Ampicillin (100μg/ml) and cultured overnight. The concentration of the bacteria was adjusted with PBS (pH 7.2) to approximately 1 x 10^{10}/ml. Mice were administered 0.1 ml 5% sodium bicarbonate by gavage tube followed by 2 x 10^9 bacteria in 0.2 ml of PBS.

The ability of the bacteria to invade organs of NIH mice and the *in vivo* stability of the plasmid were evaluated by culturing homogenates of liver, spleen and mesenteric lymph nodes in L-agar. The plasmid was selected by adding ampicillin to the medium as the IFNγ gene was always expressed with an ampicillin resistance gene . Thus, lack of expression of the cytokine due to reversion of the mutation was associated with susceptibility to the antibiotic.

Samples from three mice were obtained each time at different time points after inoculation. *S. typhimurium*/IFNγ grew well *in vivo*. Viable organisms were first isolated from mesenteric lymph nodes at day 3 p.i.. The bacteria persisted until day 28 p.i. (Figure 4.a.). Liver homogenates were positive for the bacteria expressing the construct at days 7 and 14 p.i.(Figure 4.b.). Isolation of *S. typhimurium*/IFNγ from spleen was made at day 14 p.i (Figure 4.c.).

The IFNγ gene was shown to be stable *in vivo* as is evident from the high number of bacteria growing in medium with Ampicillin (Figure 4. a., b. & c.).

3.2.2. Effect of *S. typhimurium* delivered IFNγ on the course of a malaria infection in NIH mice.

In order to study the *in vivo* effect of IFNγ on *P. chabaudi*, a series of experiments were carried out in which NIH mice inoculated with *S. typhimurium*/IFNγ, were infected with the parasite at different times after administration of the bacteria

In a first experiment, 12 mice were administered orally the IFNγ mutant 7 days before infection with 10^5 pRBCs with *P. chabaudi*. Two control groups were set up each

consisting of six mice, the mice of one group were given the *S. typhimurium* control and 7 days were later infected with *P. chabaudi* at the same time as the experimental group and the second control group was also infected with the parasite.

Mice in the experimental group had a primary patent parasitaemia from day 3 p.i. to 16 p.i.. A peak of parasitaemia was observed in the experimental group at day 8 p.i. (mean 43.45% $^{+}$/- 7.81) which was not different from controls. Similarly, no differences were observed in the resolution of this primary parasitaemia with all groups reaching subpatency at day 17 p.i. A recrudescent parasitaemia was observed in the experimental mice from day 29 p.i. to 44 p.i., while in the control groups this occurred earlier (from day 23 p.i. to 36 p.i.). The mean peak, at day 35 p.i., of this recrudescent parasitaemia was 0.013% ± 0.019, which was lower and delayed when compared with the recrudescent peak parasitaemia of both control groups, occurring at day 31 p.i.. No differences were observed in the course of the parasitaemia between the two control groups (Figure 5).

In *P. chabaudi* infection, a role for IFNγ, has been suggested in the control of the primary parasitaemia as levels of the cytokine increase to reach a peak two to three days before the peak of parasitaemia (see above) (Slade & Langhorne, 1989; reviewed by Stevenson *et al.*, 1990b). This finding suggests that the time of IFNγ production during the malaria infection might be crucial in mediation of a protective effect. To assess this, three groups of six mice each were given the *S. typhimurium*/IFNγ 10 or 3 days before or on the same day as the malaria infection. Three groups of six mice each were given the *S. typhimurium* control construct at the same time points as the experimental groups. Finally a control group for the malaria infection alone was included in the experiment. All mice were infected i.v. with 1 x 10^6 pRBC.

Mice inoculated with the *S. typhimurium*/ IFNγ 10 days before the malaria infection had a primary patent parasitaemia from day 3 p.i. to 14 p.i. with a peak (mean 43.33 % ± 7.67) at day 6 p.i.. This course of infection was not different from that observed in the two control groups (Figure 6.a., Table 1). However two of the mice (20%) of

the malaria control group died at day 8 p.i. due to intense parasitaemia. A recrudescent parasitaemia was observed in half of the mice from the experimental group between day 32 p.i. and 50 p.i. no recrudescences were observed in the remaining mice of this group during the period of study (60 days). The mice given the *S. typhimurium* control bacteria had no recrudescences, while in the *P. chabaudi* control group a recrudescent parasitaemia was observed between days 35 p.i and 45 p.i. The group of mice inoculated with the *S. typhimurium*/IFNγ construct 3 days before the malaria infection had a primary parasitaemia which reached a peak (mean 50.5% ± 10.14) at day 6 p.i. and resolved at day 14 p.i. (Figure 6.b., Table 1). This primary parasitaemia resolved earlier in the experimental group than in the two control groups, at day 12 p.i. an apparent was observed between the parasitaemias. A recrudescent parasitaemia was observed in half of the mice of the experimental group between days 31 p.i. and 51 p.i.. In the *S. typhimurium* group no recrudescences were observed. In the malaria control group, however, a recrudescence was present in all surviving mice from day 35 p.i. to 45 p.i.. When the *S. typhimurium*/IFNγ construct was administered on the same day of the *P. chabaudi* infection, a primary patent parasitaemia was observed from day 3 p.i. to 12 p.i. with a peak parasitaemia (42.65% ± 7.76) at day 6 p.i. (Figure 6.c., Table 1). Although, no differences were observed in the peak parasitaemia between the groups, in the *S. typhimurium* control group one mouse died at day 6 p.i. due to severe parasitaemia. Moreover, resolution of this primary parasitaemia occurred 2 days earlier in the *S. typhimurium*/ IFNγ group compared with the two control groups, differences between them in the parasitaemia were observed at day 12 p.i.. One mouse in the experimental group had a recrudescent parasitaemia from day 32 p.i. to 37 p.i. In the *S. typhimurium* control group a recrudescent parasitaemia was observed in 2 (of 5) mice between days 33 p.i. and 38 p.i.. In the malaria control group this was observed in all mice between days 35 p.i. and 45 p.i. No differences were seen in the course of the recrudescent parasitaemia, when present, between the groups.

In all the following experiments the *S. typhimurium* constructs were inoculated 8 days before the infection with 1 x 10^5 pRBC since in a first experiment an obvious difference in the course of infection was seen in mice from the experimental group. These had a delay in the onset of the primary parasitaemia which was evident as a one day delay to reach a 2% parasitaemia when compared with control mice. In addition, the peak parasitaemia at day 8 p.i. in the IFNγ recipient mice was lower (mean 13.44% ± 5.86, n=8) than control mice (28.59% ± 6.83, n=10) which occurred at day 7 p.i.

In a different experiment, three groups of at least 13 mice each were studied as follows: (i) inoculated with *S. typhimurium*/IFNγ and infected 8 days later with 1 x 10^5 pRBC, (ii) inoculated with *S. typhimurium* control and infected 8 days later with 1 x 10^5 pRBC, and (iii) infected with 1 x 10^5 pRBC only. In the mice inoculated with the cytokine construct a primary parasitaemia was observed from day 3 p.i. to day 12 p.i., while in the two control groups this patent parasitaemia persisted for 2 more days (Figure 7, Table 2). Moreover, the peak of this parasitaemia was higher in the two control groups, mean 19.2% (± 7.99, n=14) for the *S. typhimurium* and mean 17.60% (± 9.73, n=14) for the *P. chabaudi*, than in the experimental group (mean 9.68% ± 5.01, n=13). The mice in this experiment were followed for 16 days and therefore differences in the recrudescent parasitaemia could not be studied. However in two additional experiments which were followed for 60 days (see 3.2.2), no differences in the course of the recrudescent parasitaemia were detected.

Finally, an experiment where the *S. typhimurium*/IFNγ and TNFα (see Chapter 4) constructs were tested concurrently was performed. In this, 4 groups of six mice each were infected with 1 x 10^5 pRBC 8 days after they were given the constructs as follows: (i) *S. typhimurium*/IFNγ, (ii) *S. typhimurium*/TNFα (results are presented in chapter 4), (iii) *S. typhimurium*/TNFα and IFNγ, and (iv) *S. typhimurium* control. A fifth group was only infected with *P. chabaudi* and was the malaria control group. In the *S. typhimurium*/ IFNγ a primary parasitaemia was observed from day 3 p.i. to 11 p.i., with a mean peak parasitaemia, on day 8 p.i., of 26.59% (± 9.75) (Figure 8.a.,

Table 3). The mice which were recipients of the IFNγ and TNFα constructs had a primary parasitaemia which also resolved at day 11 p.i. and with a mean peak at day 8 p.i. of 27.53% (± 12.92) (Figure 8.b., Table 3). No differences were observed during the peak primary parasitaemia among these two groups and the control mice. Mice given the *S. typhimurium* control bacteria, had a mean peak of infection on day 9 p.i. of 31.33% (± 7.28) (Table 3), while control mice infected with *P. chabaudi* only, had a peak parasitaemia at day 8 p.i. (mean 25.18% ± 7.76). All mice given the IFNγ or IFNγ-TNFα constructs resolved this primary parasitaemia one day earlier than the control groups. One recrudescence was observed in all groups between days 23 p.i. and 32 p.i.. No differences were observed in the course of this recrudescent parasitaemia between the experimental and the control groups. (Values of peak parasitaemias of the experiments performed in NIH mice are presented in Table4)

3.2.3. Effect of *S. typhimurium* delivered IFNγ on the course of a malaria infection in A/J mice.

While studying the effect of *S. typhimurium*/TNFα on the course of a malaria infection in 'susceptible' A/J in comparison with that in 'resistant' NIH mice, it was noted that there were differences in the A/J mice after administration of the construct compared with control mice, while the course of infection remained unmodified in the NIH mice, confirming previous observations which suggest that the role of this cytokine in the control of malaria was dependent on the genetic background of the mice (Stevenson & Ghadirian, 1989). It was then decided to carry out two experiments studying the effect of *S. typhimurium*/IFNγ on the course of *P. chabaudi* infection in A/J.

In the first of these experiments, a group of six mice were administered the *S. typhimurium*/IFNγ construct three days before infection with 1 x 10^5 pRBC. The mice had a primary patent parasitaemia from day 3 p.i., with a peak at day 7 (mean 37.04% ±10.29) and which became subpatent at day 22 p.i.. They survived the

infection and no recrudescences were observed over a period of 60 days after infection. No differences were observed in the course of parasitaemia of the experimental group when compared with controls, in which the mean peak parasitaemia was 30.17% (±4.9) in the *S. typhimurium* control group, and 45.16% (±7.31) in the malaria control group. All mice from control groups became subpatent at day 16 p.i. and survived the infection without recrudescences in the parasitaemia (Figure 9, Table 5).

A second experiment evaluating the effect of administration of the IFNγ construct 8 days before the malaria infection with 10^5 pRBC, resulted in similar finding as the previous experiments. The course of a malaria infection was studied in 3 groups of six A/J mice: one experimental group was given *S. typhimurium*/IFNγ, a control group received the *S. typhimurium*/control bacteria, and a second control group was only infected with *P. chabaudi*. The experimental mice showed a patent parasitaemia from day 3 p.i. to day 18 p.i. with a mean peak of infection at day 8 p.i. of 34.65% (±5.87). Mice inoculated with the control mutant had a patent parasitaemia with a mean of 28.4% (±8.43) at peak (day 8 p.i.), while in the malaria control group this was 35.22% (±7.12) at day 8 p.i.. Parasitaemia became subpatent at day 19 p.i. in both control groups. No differences were observed in the course of parasitaemia between the experimental mice and the control groups.

3.2.3. Immune responses in *S. typhimurium* recipient NIH mice

3.2.3.1. Nitric oxide production

Serum samples collected during three different experiments were assayed for NO production. For this, mice were inoculated with *S. typhimurium*/IFNγ and 8 days later were given 10^5 pRBC. At least three samples from different mice in experimental and control groups were obtained daily during the primary patent parasitaemias. NO

levels before the malaria infection and after the inoculation of the *S. typhimurium* mutants were measured in one experiment.

In the experimental group the serum samples no rise of serum NO was detected during the primary parasitaemia (Figure. 10.a.). However, control groups infected with *S. typhimurium* control and *P. chabaudi*, or *P. chabaudi* only, showed a sharp rise of NO levels around peak parasitaemia (Figure. 10.b. & c.). Neither the *S. typhimurium*/IFNγ nor the *S. typhimurium* control bacteria were able to induce production of NO to detectable levels in serum before the malaria infection.

3.2.3.2. Proliferative responses

NIH mice were inoculated with *S. typhimurium*/IFNγ and 8 days later were given 10^5 pRBC. Four groups of mice were set up (i) *S. typhimurium*/IFNγ-*P. chabaudi*; (ii) *S. typhimurium*/control-*P. chabaudi;* (iii) *P. chabaudi* (control), and (iv) Naive mice. . Spleens of two mice from each group were collected as described in Chapter 2. Cells were cultured with antigen of fresh *P. chabaudi* pRBCs (at concentrations of 10^5/ml and 10^6/ml), fresh RBC (see 2.18), RPMI medium (negative control), or ConA. Cultures were performed from 2 days before *P. chabaudi* infection (6 after *S. typhimurium* inoculum) to day 10 after the malaria infection. Cells were cultured for 72h and pulsed for the last 18h with [^{3}H-methyl] thymidine (see 2.15).

High incorporation of the isotope was observed in all groups when cultured in the presence of ConA but not when a *P. chabaudi* antigen was added. In assays performed before day 8 p.i., both control groups had a significantly higher proliferative response when stimulated with Con A than the experimental group (Figure 11). All groups exhibited increased proliferative responses in response to ConA at day 6 p.i.. This was followed by a significant suppression at around peak parasitaemia (Figure 11).

3.2.3.3. IFNγ production

Samples from liver and spleen were obtained to study differences in IFNγ mRNA production between mice inoculated with *S. typhimurium*/IFNγ and controls. Organs from mice given the mutants (*S. typhimurium*/IFNγ and *S. typhimurium* control) and infected 8 days later with *P. chabaudi*, and from mice infected only with malaria, were aseptically obtained and kept frozen at -70_ C for six months.

RT-PCR was performed as described in 2.23. Fresh tissue samples were included as controls.

RT-PCR was always successful when fresh samples were used. However RNA was no detectable in any of the frozen samples from experimental mice. It was concluded that samples should be kept at -196_ C for optimal preservation of mRNA.

3.2.3.4. Macrophage migration

To study changes in the pattern of macrophage migration between mice inoculated with *S. typhimurium*/IFNγ and controls, liver and spleen samples were processed and staining for non-specific esterase and acid phosphatase was carried out (see 2.24). Organs from mice administered the mutants (*S. typhimurium*/IFNγ and *S. typhimurium* control) and infected 8 days later with *P. chabaudi*, and from mice infected only with malaria, were aseptically obtained fixed and kept frozen at -20_ C for one year.

Staining for acid phosphatase and non-specific esterase were successfully standardised in fresh spleen samples from malaria infected and non infected mice. However, the techniques failed when applied to liver samples. One run made using frozen tissues showed that structures were not well preserved due to dehydration occurred during the time they were kept frozen. Therefore, it was concluded that in order to obtain optimal results, fresh samples of spleen are best. The problems

encountered with standardisation of the technique using liver samples remain to be addressed.

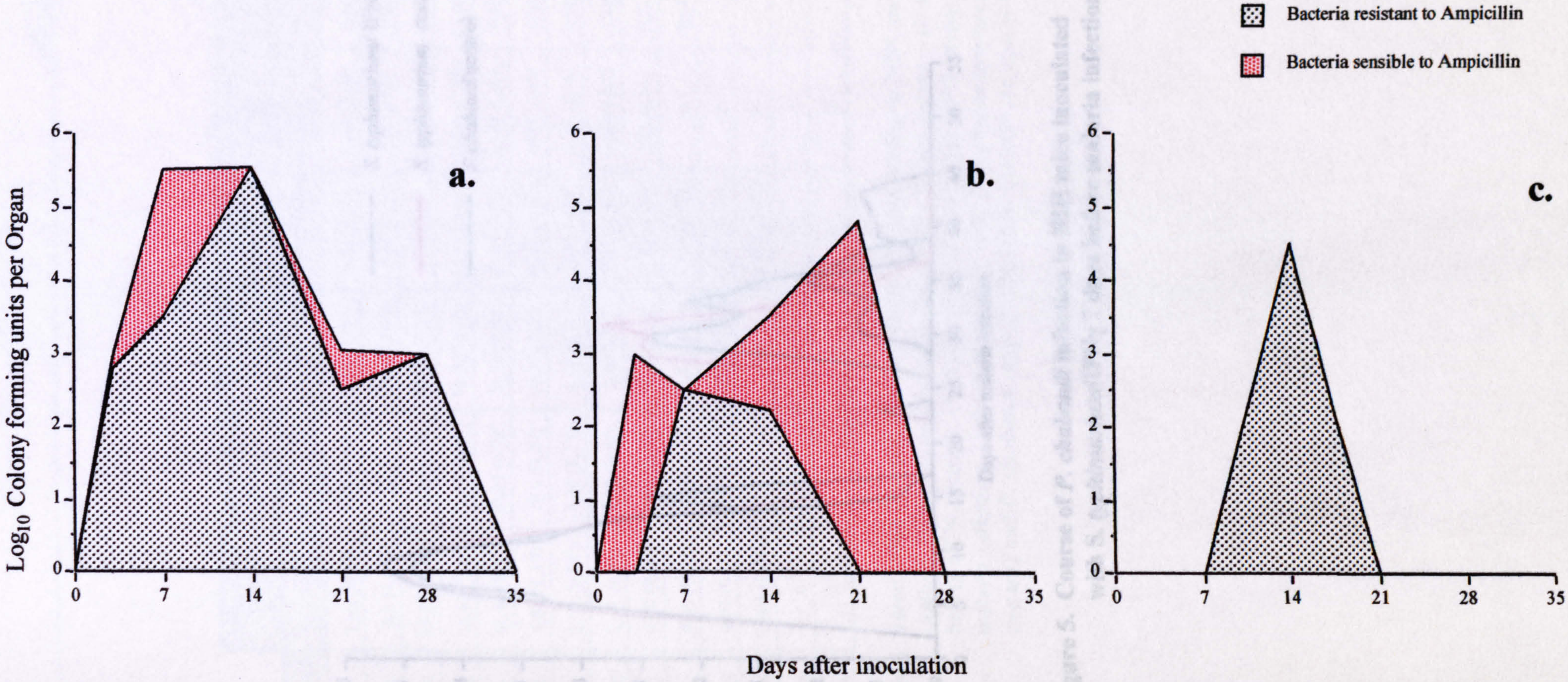


Figure 4. Colonisation and stability of *S. typhimurium*/IFNγ mutant in mesenteric lymph nodes (a), liver (b), and spleen (c) of NIH mice. Bacteria resistant ro ampicillin express the IFNγ construct. Each point represents the average of three mice.

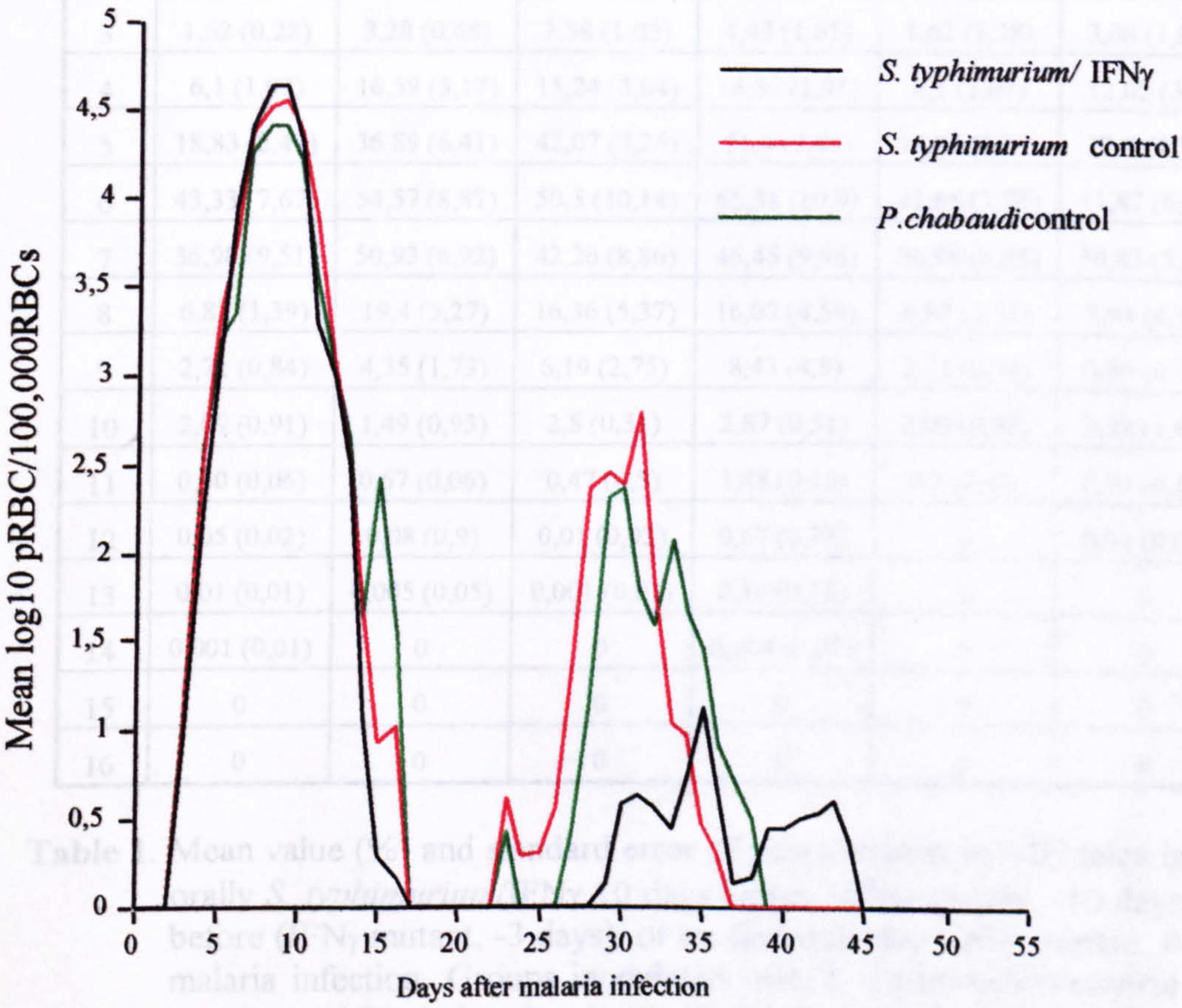


Figure 5. Course of *P. chabaudi* infection in NIH mice inoculated with S. *typhimurium*/IFNγ 7 days before malaria infection.

	IFNγ mutant -10 days n=6	Control mutant -10 days n=6	IFNγ mutant -3 days n=6	Control mutant -3 days n=6	IFNγ mutant 0 days n=6	Control mutant 0 days n=5	*P. chabaudi* control n=4
Day p.i.	**% mean parasitaemia (S.D.)**						
3	1,62 (0,28)	3,28 (0,68)	3,58 (1,02)	4,43 (1,65)	1,62 (1,28)	3,08 (1,02)	6,29 (1,01)
4	6,1 (1,07)	16,59 (3,17)	15,24 (3,04)	16,36 (2,95)	6,1 (1,07)	12,02 (3,7)	27,10 (5,73)
5	18,83 (2,41)	36,89 (6,41)	42,07 (7,25)	51,4 (9,94)	18,83 (3,23)	22,8 (4,21)	61,51 (10,3)
6	43,33 (7,67)	54,57 (8,87)	50,5 (10,14)	65,31 (10,9)	42,65 (7,76)	41,87 (6,96)	77,6 (12,32)
7	36,98 (9,51)	50,93 (6,92)	42,26 (8,86)	46,45 (9,98)	36,98 (8,05)	30,83 (5,67)	38,72 (5,78)
8	6,87 (1,39)	19,4 (5,27)	16,36 (5,37)	16,03 (4,59)	6,87 (2,35)	7,94 (4,18)	12,24 (3,27)
9	2,71 (0,84)	4,35 (1,73)	6,19 (2,75)	8,43 (4,9)	2,71 (0,74)	0,86 (0,75)	4,2 (2,03)
10	2,09 (0,91)	1,49 (0,93)	2,8 (0,31)	2,87 (0,51)	2,09 (0,93)	3,38 (1,62)	2,89 (0,81)
11	0,30 (0,06)	0,67 (0,06)	0,47 (0,5)	1,48 (0,16)	0,3 (0,43)	0,14 (0,16)	1,64 (0,75)
12	0,05 (0,02)	0,08 (0,9)	0,01 (0,02)	0,67 (0,72)	0	0,01 (0,02)	0,89 (0,86)
13	0,01 (0,01)	0,005 (0,05)	0,001 (0,01)	0,14 (0,15)	0	0	0,23 (0,22)
14	0,001 (0,01)	0	0	0,004 (0,07)	0	0	0,008 (0,01)
15	0	0	0	0	0	0	0,003 (0,01)
16	0	0	0	0	0	0	0

Table 1. Mean value (%) and standard error of parasitaemias in NIH mice inoculated orally *S. typhimurium*/IFNγ 10 days before (IFNγ mutant, -10 days), 3 days before (IFNγ mutant, -3 days), or on the same day (IFNγ mutant, 0 days) of malaria infection. Groups inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

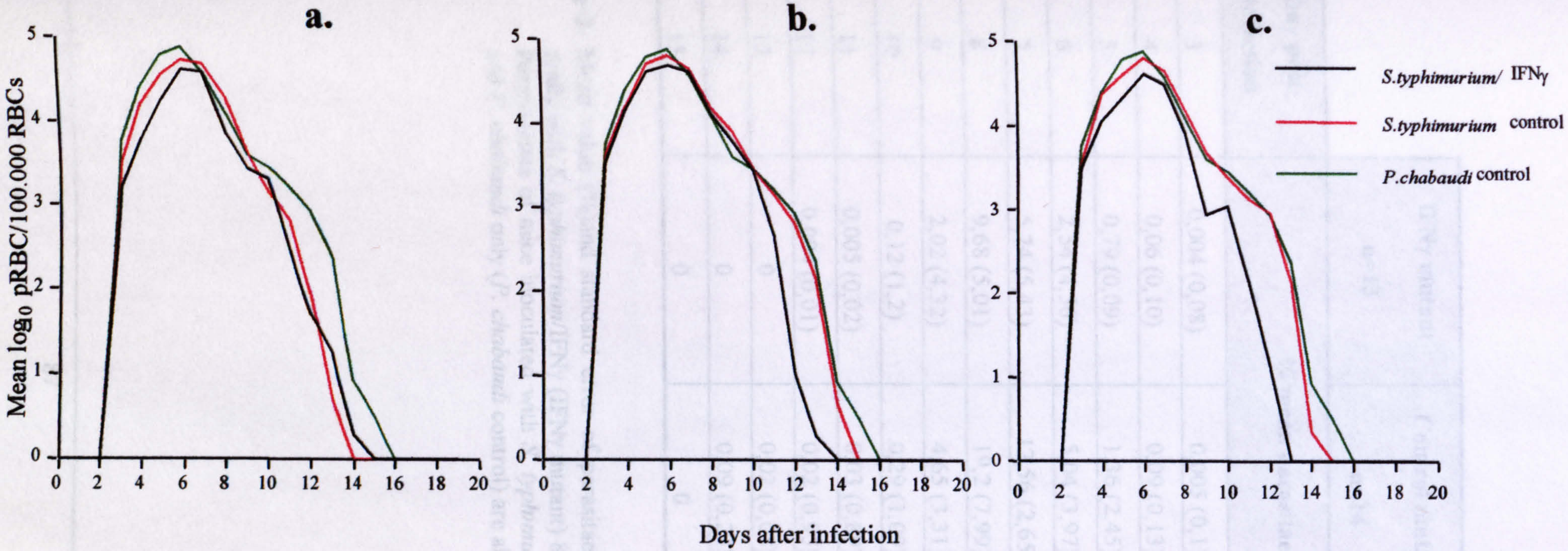


Figure 6. Course of *P. chabaudi* infection in NIH mice inoculated with *S. typhimurium* mutants 10 days before (a), 3 days before (b), or on the same day (c) of the malaria infection. In each experiment one group was inoculated with *S. typhimurium*/IFNγ. Two control groups were included: *S. typhimurium* control and *P. chabaudi* control. Each group consisted of 6 mice.

	IFNγ mutant n=13	Control mutant n=14	*P. chabaudi* control n=14
Day post-infection	**% mean parasitaemia (S.D.)**		
3	0,004 (0,08)	0,005 (0,1)	0,006 (0,13)
4	0,06 (0,10)	0,09 (0,13)	0,21 (0,79)
5	0,79 (0,09)	1,36 (2,45)	1,37 (1,05)
6	2,54 (4,56)	5,04 (3,97)	5,02 (2,34)
7	5,74 (5,43)	12,56 (2,65)	12,76 (2,09)
8	9,68 (5,01)	19,2 (7,99)	17,6 (9,73)
9	2,02 (4,32)	4,65 (3,31)	5,21 (2,41)
10	0,12 (1,2)	0,29 (1,09)	0,349 (0,95)
11	0,005 (0,02)	0,03 (0,89)	0,07 (0,12)
12	0,004 (0,01)	0,02 (0,95)	0,03 (0,51)
13	0	0,02 (0,08)	0,008 (0,064)
14	0	0,09 (0,23)	0,003 (0,032)
15	0	0	0

Table 2. Mean value (%)and standard error of parasitaemias in NIH mice inoculated orally with *S. typhimurium*/IFNγ (IFNγ mutant) 8 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

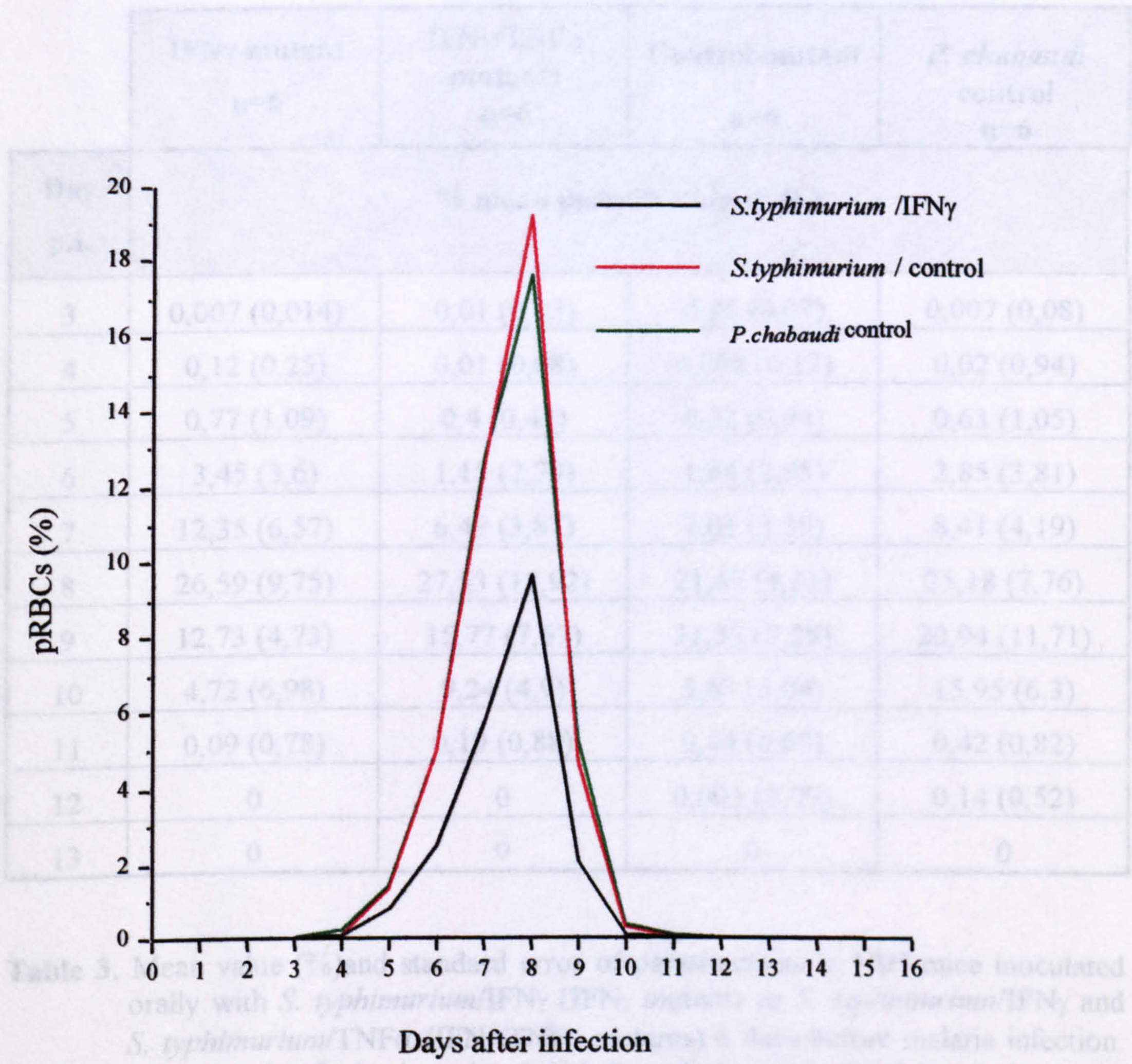


Figure 7. Course of *P. chabaudi* infection in NIH mice inoculated with S. *typhimurium*/IFNγ 8 days before malaria infection.

	IFNγ mutant n=6	IFNγ/TNFα mutants n=6	Control mutant n=6	*P. chabaudi* control n=6
Day p.i.	**% mean parasitaemia (S.D.)**			
3	0,007 (0,014)	0,01 (0,03)	0,01 (0,07)	0,007 (0,08)
4	0,12 (0,25)	0,01 (0,08)	0,098 (0,12)	0,02 (0,94)
5	0,77 (1,09)	0,4 (0,47)	0,52 (0,94)	0,63 (1,05)
6	3,45 (3,6)	1,15 (2,79)	1,84 (2,95)	2,85 (3,81)
7	12,35 (6,57)	6,49 (3,87)	7,01 (3,59)	8,41 (4,19)
8	26,59 (9,75)	27,53 (12,92)	21,67 (9,31)	25,18 (7,76)
9	12,73 (4,73)	15,77 (7,67)	31,33 (7,28)	20,94 (11,71)
10	4,72 (6,98)	9,24 (4,9)	3,63 (3,04)	15,95 (6,3)
11	0,09 (0,78)	0,19 (0,88)	0,44 (0,69)	0,42 (0,82)
12	0	0	0,005 (0,09)	0,14 (0,52)
13	0	0	0	0

Table 3. Mean value (%)and standard error of parasitaemias in NIH mice inoculated orally with *S. typhimurium*/IFNγ (IFNγ mutant) or *S. typhimurium*/IFNγ and *S. typhimurium*/TNFα (IFNγ/TNFα mutants) 8 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

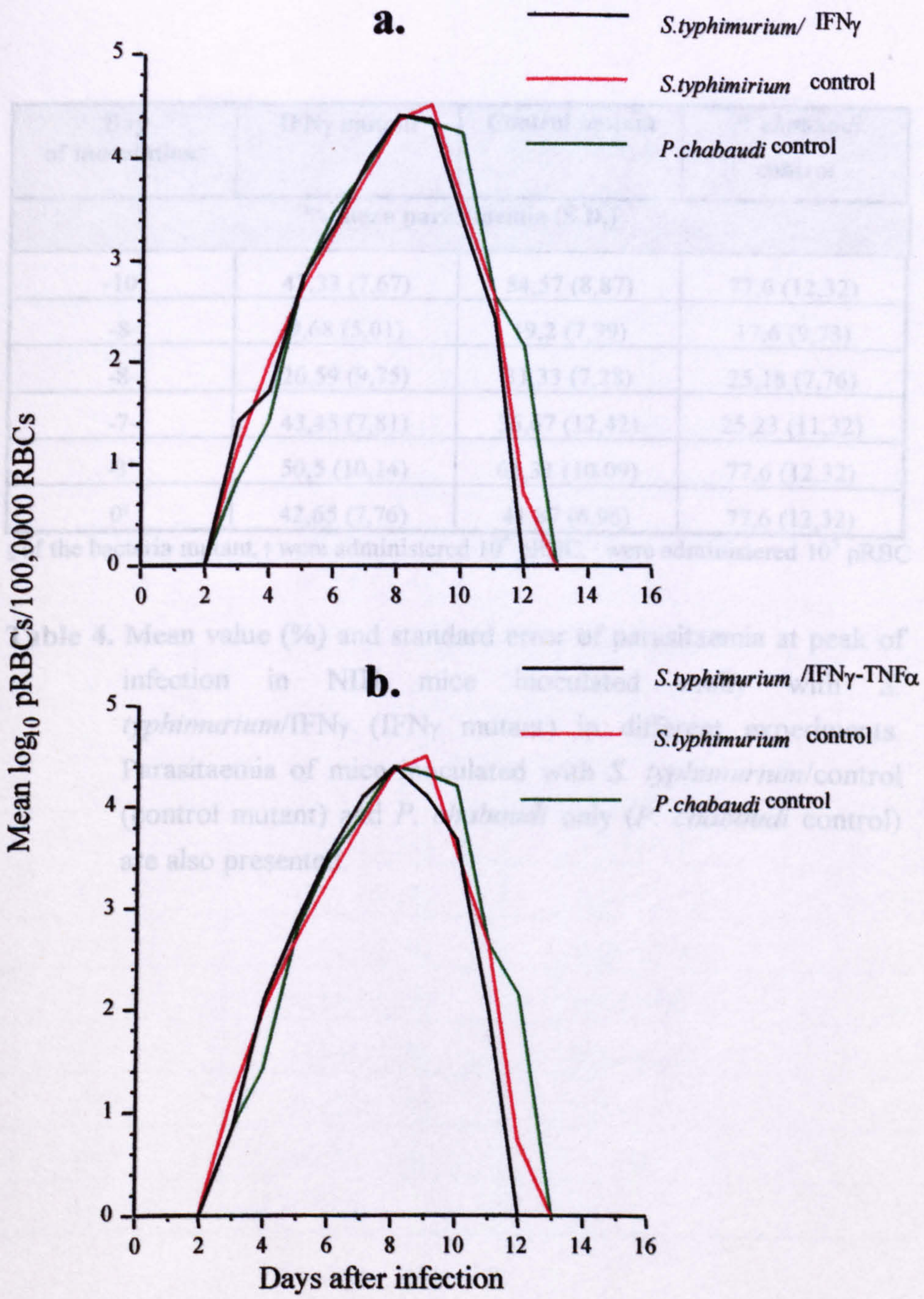


Figure 8. Course of *P. chabaudi* infection in NIH mice inoculated with S.*typhimurium* mutants 8 days before malaria infection. Two groups were inoculated with *S. typhimurium*/IFNγ (a) or *S.typhimurium*/IFNγ and *S.typhimurium*/TNFα (b). Two control groups were included, one received *S. typhimurium* control and the other *P. chabaudi* only. Each point represents the average parasitaemia of six mice.

Day of inoculation§	IFNγ mutant	Control mutant	*P. chabaudi* control
% mean parasitaemia (S.D.)			
-10†	43,33 (7,67)	54,57 (8,87)	77,6 (12,32)
-8-	9,68 (5,01)	19,2 (7,99)	17,6 (9,73)
-8-	26,59 (9,75)	31,33 (7,28)	25,18 (7,76)
-7-	43,45 (7,81)	36,67 (12,42)	25,23 (11,32)
-3†	50,5 (10,14)	65,31 (10,09)	77,6 (12,32)
0†	42,65 (7,76)	41,87 (6,96)	77,6 (12,32)

§ of the bacteria mutant, † were administered 10^6 pRBC, - were administered 10^5 pRBC

Table 4. Mean value (%) and standard error of parasitaemia at peak of infection in NIH mice inoculated orally with *S. typhimurium*/IFNγ (IFNγ mutant) in different experiments. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

	IFNγ mutant n=6	Control mutant n=6	*P. chabaudi* control n=6
Day post-infection	**% mean parasitaemia (S.D.)**		
3	0,02 (0,01)	0,02 (0,02)	0,14 (0,04)
4	0,91 (0,56)	0,42 (0,082)	0,99 (0,43)
5	5,04 (1,69)	2,88 (1,16)	3,68 (3,11)
6	16,59 (5,47)	9,2 (1,6)	23,4 (5,46)
7	37,04 (10,29)	30,08 (4,28)	45,16 (7,31)
8	37,02 (7,99)	30,17 (4,9)	42,88 (11,03)
9	18,27 (7,02)	28,54 (10,77)	22,11 (9,17)
10	3,74 (2,35)	10,74 (7,56)	2,36 (1,33)
11	0,41 (0,24)	1,17 (0,47)	0,25 (0,15)
12	0,21 (0,16)	0,21 (0,11)	0,14 (0,06)
13	0,22 (0,09)	0,06 (0,06)	0,11 (0,05)
14	0,16 (0,15)	0,04 (0,03)	0,28 (0,23)
15	0,14 (0,09)	0,13 (0,14)	0,15 (0,15)
16	0,12 (0,09)	0,03 (0,03)	0,3 (0,31)
17	0,05 (0,06)	0	0
18	0	0	0

Table 5. Mean value (%) and standard error of parasitaemias in A/J mice inoculated orally with *S. typhimurium*/IFNγ (IFNγ mutant) 3 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

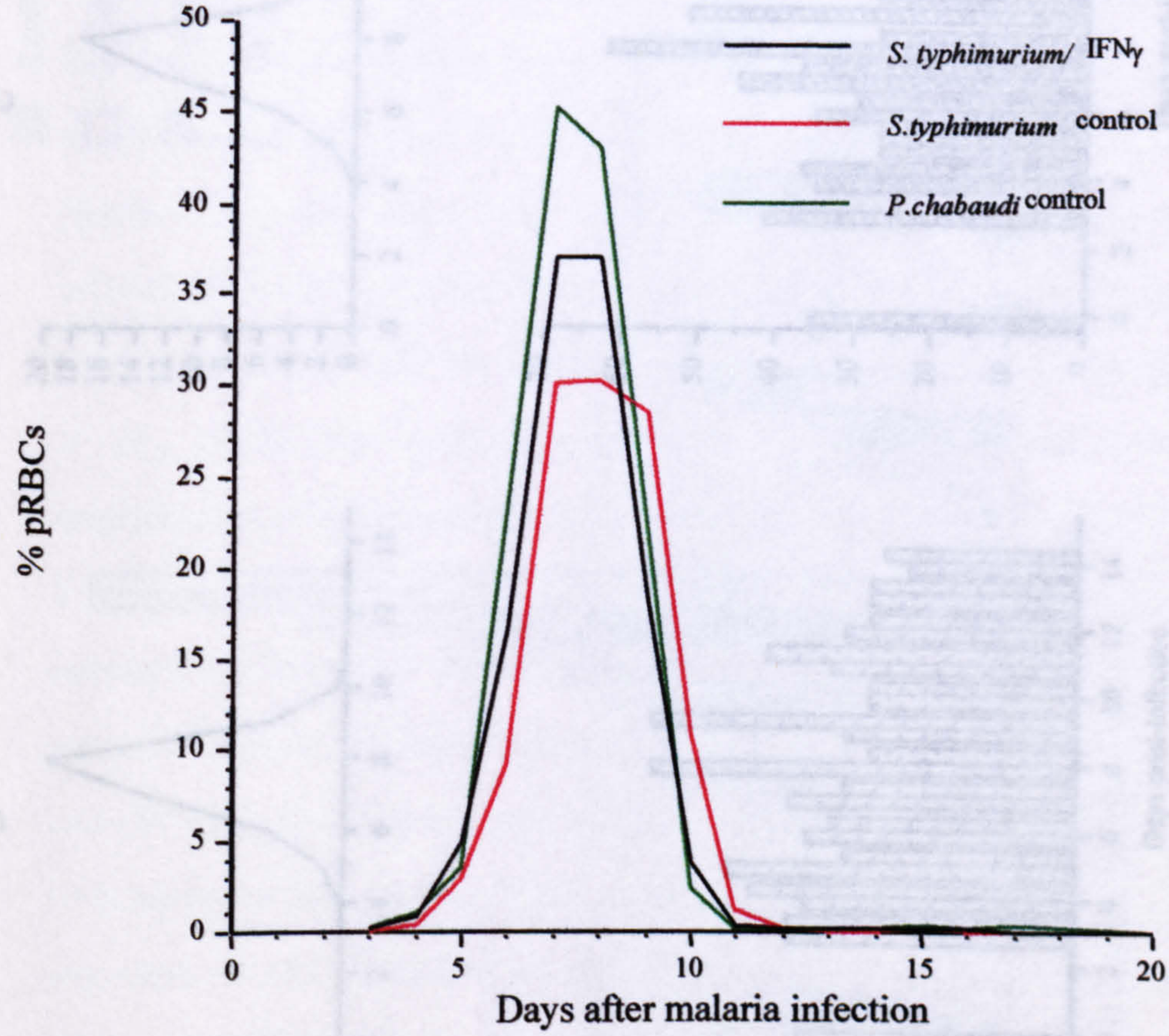


Figure 9. Course of *P. chabaudi* infection in A/J mice inoculated with S. *typhimurium*/IFNγ 3 days before malaria infection.

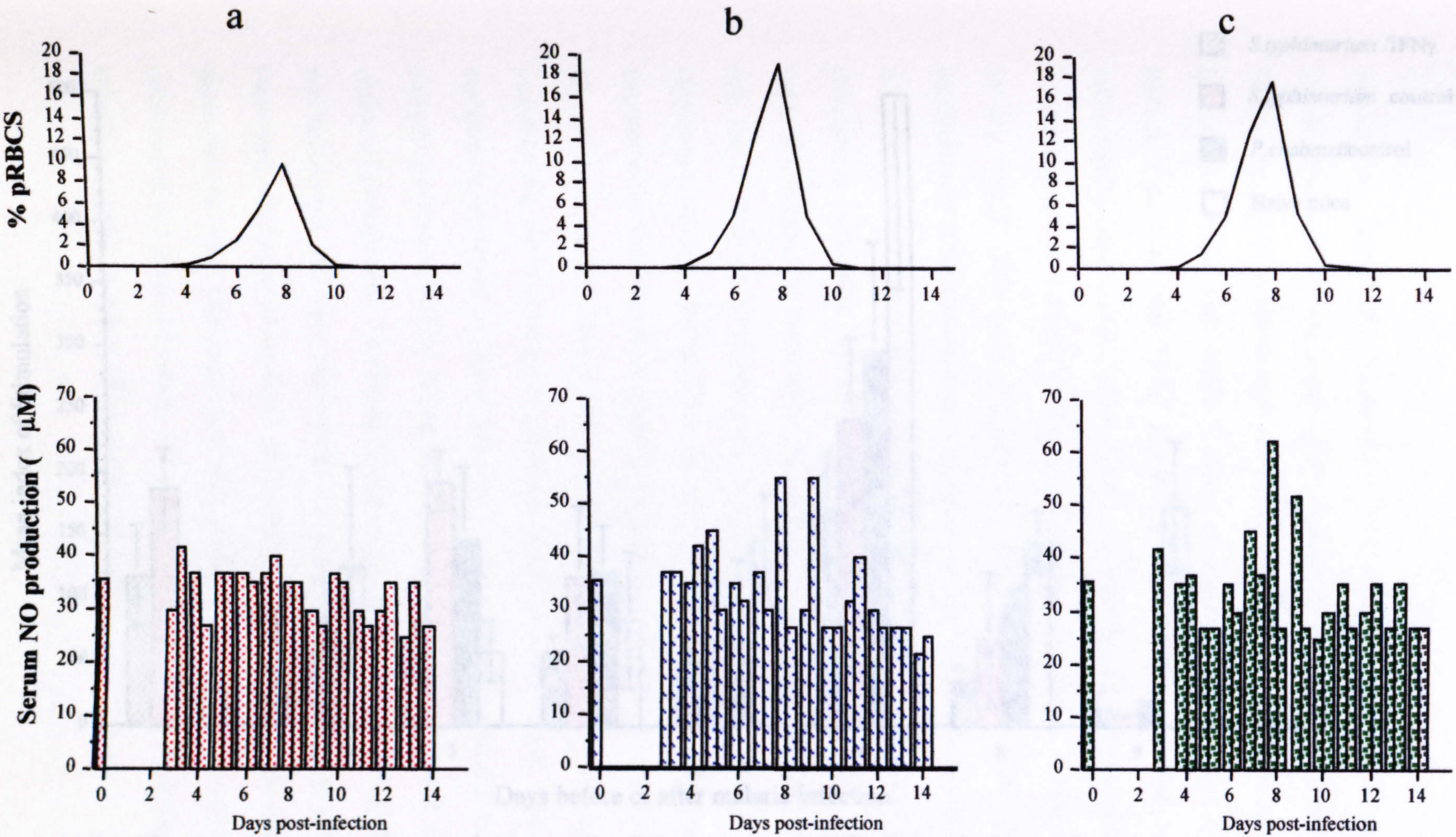


Figure 10.Parasitaemia and serum nitric oxide levels of mice inoculated with *S. typhimurium* mutants 8 days before *P. chabaudi* challenge. One group was inoculated with *S. typhimurium*/IFNγ (a). One control group was given *S. typhimurium* control (b) and the other *P. chabaudi* only (c). Each bar represents one individual.

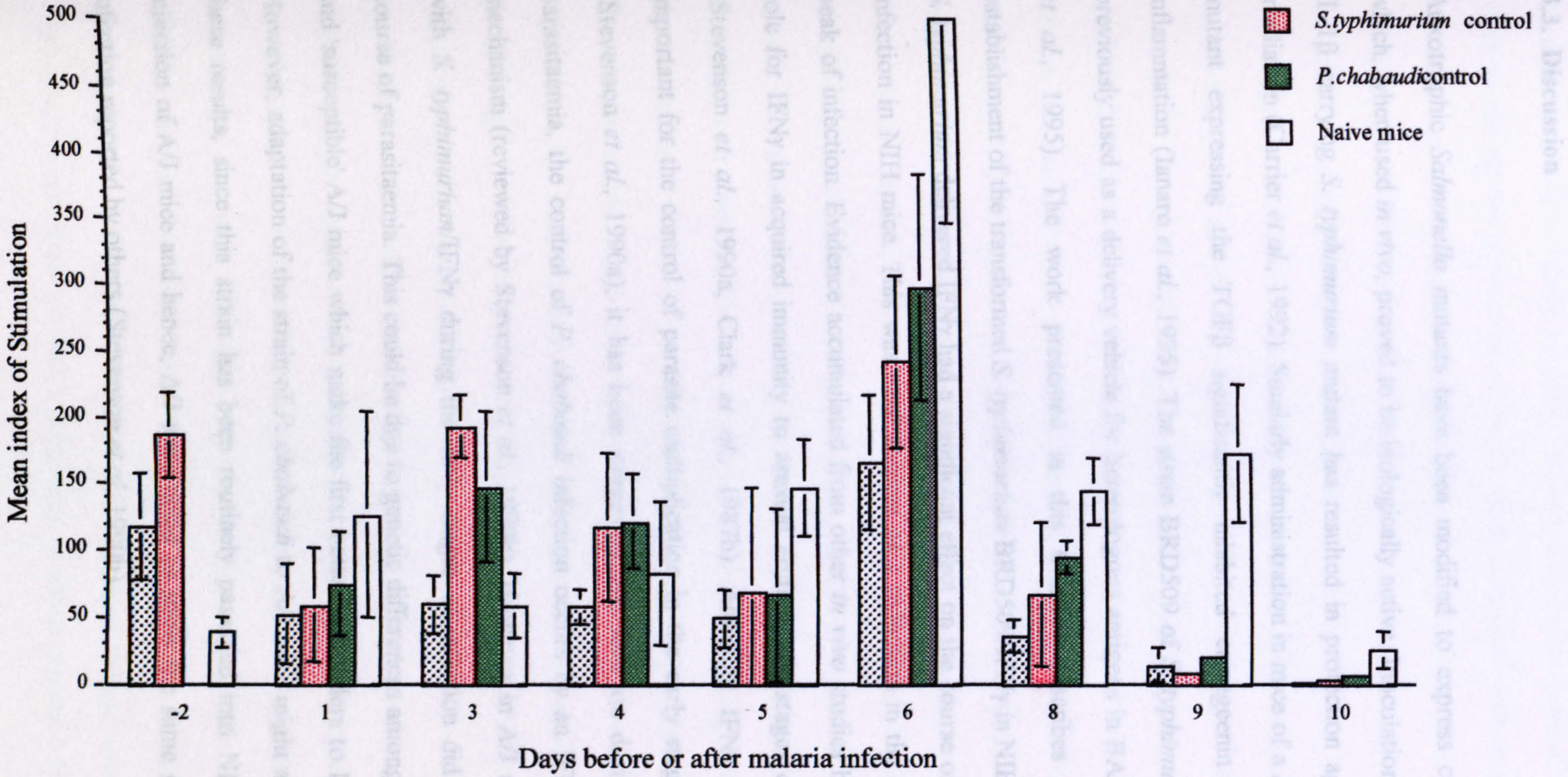


Figure 11.Proliferative response to Con A of spleen cells of *S. typhimurium*/IFNγ 8 days before malaria infection. One cpntrol group was given *S. typhimurium* control 8 days before malaria infection and the other group was infected with *P. chabaudi* only. Each point represents the average of two mice.

3.3. Discussion

Auxotrophic *Salmonella* mutants have been modified to express cytokines genes which, when used *in vivo*, proved to be biologically active. Inoculation into mice of a IL-1β carrying *S. typhimurium* mutant has resulted in protection against lethal γ-irradiation (Carrier *et al.*, 1992). Similarly administration in mice of a *S. typhimurium* mutant expressing the TGFβ significantly inhibited carrageenin induced local inflammation (Ianaro *et al.*, 1995). The strain BRD509 of *S. typhimurium* has been previously used as a delivery vehicle for heterologous antigens in BALB/c mice (Xu *et al.*, 1995). The work presented in this Chapter describes the successful establishment of the transformed *S. typhimurium* BRD509/IFNγ in NIH mice.

S. typhimurium delivered IFNγ had a significant effect on the course of a *P. chabaudi* infection in NIH mice. This was most evident as a reduction in the parasitaemia at peak of infection. Evidence accumulated from other *in vivo* studies has suggested a role for IFNγ in acquired immunity to asexual erythrocytic stages of *P. chabaudi* (Stevenson *et al.*, 1990a, Clark *et al.*, 1987b). Although IFNγ appears to be important for the control of parasite multiplication in the early stages of infection (Stevenson *et al.*, 1990a), it has been suggested that later on during the primary parasitaemia, the control of *P. chabaudi* infection occurs by an IFNγ-independent mechanism (reviewed by Stevenson *et al.*, 1990b). However in A/J mice, treatment with *S. typhimurium*/IFNγ during the early stages of infection did not affect the course of parasitaemia. This could be due to genetic differences among 'resistant' NIH and 'susceptible' A/J mice which make the first better responders to IFNγ treatment. However, adaptation of the strain of *P. chabaudi* to A/J mice might also account for these results, since this strain has been routinely passaged into NIH mice before infection of A/J mice and hence, A/J mice failed to show the same susceptibility to infection reported by others (Stevenson *et al*, 1990b).

Administration of *S. typhimurium*/IFNγ to NIH mice 8 and 3 days before, or simultaneously with malaria infection resulted in a better control of the malaria infection during the primary parasitaemia when compared with controls. A more consistent result was obtained when the mutant bacteria were administered 8 days before infection with $1x10^5$ pRBC. Characteristically, peak parasitaemia was lower and primary patent parasitaemia resolved earlier than in controls. Mice inoculated with *S. typhimurium*/IFNγ 10 days before *P. chabaudi* infection exhibited a similar pattern of infection as controls. Taken together these results suggest that the interval between the *S. typhimurium*/IFNγ administration and the malaria infection, is important and the maximum antimalarial activity of delivered IFNγ is seen when there are high levels of expression of the cytokine in early stages of infection.

The effect of IFNγ on reducing parasite multiplication in NIH mice might explain the results obtained in a single experiment when mice were inoculated with the construct 7 days before malaria infection. No difference in the course of primary patent parasitaemia was observed between the groups, but a significantly reduced and delayed recrudescent parasitaemia was observed in the experimental group when compared with controls. Since this result was recorded on a single occasion only, it can be speculated that it is a consequence of a more effective parasite killing during the subpatent stages between primary and recrudescent parasitaemias.

High IFNγ levels might result in increased NO production from macrophages. A role for NO against blood-stage *P. chabaudi* AS infection in NIH mice has been suggested by other workers (Taylor-Robinson *et al.*, 1993). In order to assess the role for NO in the enhanced control of parasitaemia in *S. typhimurium*/IFNγ recipient NIH mice, serum samples from around peak parasitaemia were assayed for *in vivo* NO production. A sharp rise of NO levels was observed in control groups at peak parasitaemia. However, NO levels in the *S. typhimurium*/IFNγ recipient mice remained at baseline levels along the course of primary parasitaemia. Taylor-Robinson *et al.*, (1996) have observed persistence of background levels of NO production in mice reinfected with *P. chabaudi*, in which increased NO levels can be

detected only at peak parasitaemia during a first infection, subsequent challenge was not associated with increased levels of the molecule unless the mice were re-infected with a large parasite dose several months after clearance. This might indicate the presence of a prolonged refractory period for NO production after the initial burst of NO during the first malaria infection. NO production in *S. typhimurium* recipient mice was studied before *P. chabaudi* infection, and such a refractory period of NO production in these mice could not be confirmed. These results suggest that, in the NIH model, NO is not exclusively responsible for the control of blood forms of *P. chabaudi* during primary parasitaemia. A plausible explanation for the persistence of background levels in the *S. typhimurium*/IFNγ treated mice, is that the low antigenic load is not enough stimulus to trigger NO production to detectable levels. Furthermore, IFNγ might be responsible for mediation of other immune mechanisms that have been shown to be effective in the control of malaria parasites including production of reactive oxygen intermediates (Shear *et al.*, 1989; reviewed by Weidanz *et al.*, 1990) and TNFα (Bate *et al.*, 1988) from activated macrophages. IFNγ is the major macrophage activating factor and therefore is the primary cytokine responsible for inducing non-specific cell-mediated mechanisms of host defence (reviewed by Farrar & Schreiber, 1993). In addition, IFNγ enhances the ability of the macrophage to participate in other immune effector functions.

The ability of monocytes/macrophages to participate in antibody dependent cellular cytotoxicity (ADCC) reactions is enhanced through increased expression of high affinity Fc, receptors particularly FcγRI (Erbe *et al.*, 1990). However, Bouharoun-Tayoun et al. (1995) suggested that increase by IFNγ of ADCC intensity was not related to induction of FcγRI, but rather was the result of IFNγ-dependent increased ability of monocytes to release TNFα, which is triggered by FcγRII cross-linking (Muñoz-Fernandez *et al.*, 1992; Polat *et al.*, 1993). Evidence for the role of specific ADCC activity *in vivo* during malaria parasitaemia, is conflicting, with some authors reporting non-specificity in murine models of *P. berghei* (Coleman *et al.*, 1975) *P. chabaudi* (McDonald & Phillips, 1978), and in *P. falciparum* in humans (Brown &

Smalley, 1980). However, enhancement of cytotoxicity against these parasites has been observed *in vitro* in the presence of immune serum (reviewed by Phillips, 1994). IFNγ has been shown to enhance the biosynthesis of a variety of complement proteins (such as C2, C4, and Factor B) by macrophages and fibroblasts (Strunk *et al.*, 1994) and regulates the expression of complement receptors on the mononuclear phagocyte plasma membrane thereby promoting humoral immunity through enhancement of complement activity (reviewed by Farrar & Schreiber, 1993). There is conflicting evidence about the importance of complement in immunity to erythrocytic stages of *Plasmodium*. Reduction of serum complement levels has been observed in acute infections, and *in vitro* studies with *P. berghei* have demonstrated a reduction of phagocytosis by rat macrophages in the absence of complement (reviewed by Phillips, 1994). On the other hand, *in vitro* immune phagocytosis of *P. falciparum* pRBC by human monocytes and neutrophils, and inhibition of multiplication of *P. knowlesi* by immune serum was independent of complement (reviewed by Phillips, 1994). Further work on the role of IFNγ delivered by a *S. typhimurium* mutant should include the study of effector mechanisms responsible for the better control of *P. chabaudi* infection in NIH mice. This work should include: (i) expression of iNOS in organs such as a liver and spleen of infected mice (this is a better indicator of increase of NO production than serum NO determination), (ii) proliferative responses of spleen cells against malaria antigen, (iii) *in vitro* cytokine production by spleen cells stimulated with malarial antigen, and (iv) evaluation, using histochemistry, of macrophage and neutrophil activation *in situ*

In conclusion, the results presented in this chapter confirm that a *S. typhimurium* mutant can be successfully used as a delivery vehicle for IFNγ in order to study its role in acquisition of immunity against *P. chabaudi* malaria and confirm the important role of IFNγ in the control of the parasite during early stages of the infection in NIH mice.

CHAPTER FOUR

EFFECT OF TUMOUR NECROSIS FACTOR-ALPHA ON THE COURSE OF *Plasmodium chabaudi* INFECTION

4.1. Introduction

In vivo and *in vitro* evidence suggest that, along with IFNγ, tumour necrosis factor (TNF) contributes in an important way to immunity to blood-stage *P. falciparum* infection in humans (Peyron *et al.*, 1990; Ockenhouse *et al.*, 1984; Kumaratilake *et al.*, 1991). Macrophages produce TNFα in response to stimulation with lipopolysaccharide as well to a variety of bacterial toxins (Hacket & Stevens, 1992; 1993). Lymphocytes produce a structurally and functionally similar protein known as TNFβ (Stevens, 1995).

TNFα production can originate from a small pool of intracellular mRNA that is maintained in an untranslated state, or after production of newly transcribed TNFα mRNA (reviewed by Stevens, 1995). Stimuli (such as LPS) trigger an acceleration of gene transcription which is followed by rapid translation of the newly synthesised mRNA and the stored mRNA. IFNγ increases TNFα synthesis at the translational level. On the other hand, cytokines such as IL-10, IL-1 and IL-6 act as potent inhibitors of TNFα synthesis by mononuclear cells, probably at the gene transcriptional level (Wang *et al.*, 1994).

In addition to its cytoxicity against tumour cells, TNFα has been shown to stimulate and prime neutrophils to increase oxygen radical production (Jupin *et al.*, 1989) and to promote their degranulation (Ferrante *et al.*, 1993). Furthermore, TNFα exerts a wide range of immunomodulatory effects on lymphocytes, macrophages, endothelial cells and haematopoietic progenitor cells (reviewed by Stevenson & Ghadirian, 1989).

TNFα has been shown to have be important in defence against *Entamoeba histolytica* (Denis & Chadee, 1989), *Candida albicans* (Ferrante, 1989) and *Staphylococcus aureus* (Ferrante *et al.*, 1993) infections. In some models of bacterial infection a dual effect of TNFα has been demonstrated. For example, in *Listeria* infection excessive production of TNFα may be associated with dramatic shock and organ failure, while inhibition of TNFα has been associated with increased quantities of the bacteria and

decreased circulating granulocytes (Van Furth *et al.*, 1994). Paradoxical findings of the role of TNFα have also been reported in *Mycobacterium tuberculosis* infection because, although there is much evidence for a protective role, there is also evidence that it plays a part in the tissue damage present during the disease (Rook *et al.*, 1990; Rook, 1988; Hernandez-Pardo & Rook, 1994). Similar observations have been made in murine infection with *Toxoplasma gondii* (reviewed by Beaman *et al.*, 1992) and human and murine malaria infections. In *P. falciparum* infection, the data collected so far suggest that very high levels of TNFα are associated with severe disease and fatal outcome while a moderate increase in TNFα production appears to be a normal and possibly beneficial host response during the infection (reviewed by Clark *et al.*, 1992; reviewed by Urquhart, 1994). In a experimental model of cerebral malaria, mice infected with *P. berghei* showed increased levels of TNFα coincident with the development of the neurological syndrome, and passive immunisation with anti-TNFα antibody protected them against cerebral malaria and death (Grau *et al.*, 1987). Further support for the involvement of TNFα in cerebral malaria comes from experiments with non- infected CBA/Ca mice, in which the clogging of cerebral blood vessel with monocytes that has been associated with cerebral malaria was reproduced after administration of high doses of recombinant TNFα (Grau *et al.*, 1989b) More recently, it has become apparent that it is not the systemic level of the cytokine which is important in cerebral malaria, but rather the increased local concentrations of TNFα resultant from sequestered monocytes within the cerebral blood vessels (reviewed by Taylor-Robinson, 1995). Clark *et al.* (1992) have suggested that one way the rise in TNFα levels during malaria infection could alter brain function is to generate NO. It is hypothesised that TNFα stimulates endothelial cells to release NO which diffuses into the brain and disrupts the regulation of glutamate-induced neural NO, resulting in altered neurotransmission and coma (reviewed by Taylor-Robinson, 1995). However, the involvement of NO in the actual processes causing neurovascular damage at advanced stages of the disease remain controversial.

While TNFα is an important mediator of the pathophysiology associated with malaria infection, it is also known to play an important role in immunity against the parasite. TNFα and lymphotoxin have been shown to enhance the killing of intraerythrocytic forms (Kumaratilake *et al.*, 1990 & 1991) and merozoites (Kumaratilake *et al.*, 1992) of *P. falciparum* by human neutrophils. Further support for the role of TNFα in the development of acquired immunity against malaria comes from recent studies in a murine model of *P. chabaudi*, in which treatment with a synthetic TNFα peptide was effective in suppressing parasitaemia (Kumaratilake *et al.*, 1995). Earlier, Stevenson and Ghadirian (1989) demonstrated that injection of recombinant TNFα (rTNFα) into *P. chabaudi* susceptible A/J mice, resulted in a significantly increased survival rate. However, treatment of C57BL/6 resistant mice with similar concentrations of rTNFα, had no effect on the course of infection (Stevenson & Ghadirian, 1989). They concluded that TNFα dependent immune mechanisms that result in better protection in A/J mice, might be under genetic regulation (Stevenson *et al.*, 1990b).

In vivo studies on the immunoregulatory properties of TNFα have been limited due to its toxicity (reviewed by Balkwill, 1994; Schiller *et al.*, 1992). Administration of the cytokine in D-galactosamine-sensitised mice causes marked toxic effects and death within 48 hours when given at 0.25 mg/kg body weight (Kumaratilake *et al.*, 1995). The use of a *S. typhimurium* to deliver TNFα might offer the advantage of low systemic concentrations (thus less TNFα toxicity) but increased production in organs invaded by the bacteria. As *P. chabaudi* undergoes sequestration, high concentration of TNFα in tissues as liver and spleen may result in increased effector mechanisms against the sequestered parasites and better clearance of the parasitaemia.

The experiments described in this chapter were designed to evalute the feasibility of using a *S. typhimurium* mutant for the delivery of murine TNFα *in vivo* and to study its role in immunity against *P. chabaudi*. For this, two strains of mice, 'resistant' NIH and 'susceptible' A/J, were inoculated with the mutant and later infected with $1x10^5$ *P. chabaudi* pRBC. Following infection with *P. chabaudi* , A/J mice have been reported to develop a fulminant parasitaemia which results in 100% mortality within 2

weeks of infection (Stevenson & Ghadirian, 1989). Resistant NIH mice develop a moderate level of peak parasitaemia which resolves spontaneously before 4 weeks and it is followed by one or two recrudescences before an apparent sterile immunity develops.

4.2. Results

4.2.1. Colonisation and stability of the *S. typhimurium* mutant in organs of NIH mice

In order to evaluate the *in vivo* penetration and stability of the *S. typhimurium* BRD509/NB/TNFα (*S. typhimurium*/TNFα) mutant, 20 age-matched female NIH naive mice were inoculated with the bacteria. For this, a stock of the mutant was cultured overnight in L-broth with ampicillin (100μg/ml). The concentration of the bacteria was adjusted with PBS to approximately 1 x 10^{10}/ml. Mice were given 0.1 ml 5% sodium bicarbonate by gavage tube followed by 0.2 ml of the bacterial suspension. Homogenates of liver , spleen and mesenteric lymph nodes were obtained at 3, 7, 14, 21, 28 and 35 days after inoculation. These were cultured in L-agar and the plasmid was selected by adding ampicillin (100μg/ml) to the medium. The difference between the numbers of bacteria susceptible and resistant to the antibiotic reflected the proportion of reversion of the TNFα gene.

Samples from three mice were obtained at each of the time points and these indicated that the *S. typhimurium*/TNFα mutant grew well *in vivo*, as evident by the isolation of viable bacteria from day 3 p.i. from mesenteric lymph nodes. The bacteria persisted until day 14 p.i.(Figure 12.a.). Isolation of the bacteria from livers and spleens was achieved from day 3 p.i until day 28 p.i. (Figure 12.b. & c.).

The TNFα mutation was shown to be conserved throughout the period of infection as isolates resistant to ampicillin were obtained at 7, 14 and 21 days post-inoculation in

samples from liver and spleen. Mesenteric lymph nodes samples showed bacterial growth at 7 and 14 days post-inoculation.

4.2.2. Effect of *S. typhimurium* delivered TNFα on the course of malaria infection in NIH mice

In the first experiment, a group of six mice was inoculated with the *S. typhimurium*/TNFα construct and on the same day they were infected with 1 x 10^5 pRBC. After three days, the mice developed a primary parasitaemia which peaked at day 7 p.i. (mean 45%, ± 5.89) and resolved after day 14 p.i.. The onset of this parasitaemia in the experimental groups was no different from that in the controls, all groups reaching a mean 2% parasitaemia at day 5 p.i.. A recrudescent parasitaemia was detected after day 33 p.i., with a peak at day 37 p.i. and which became subpatent at day 40 p.i.. No differences were observed in either primary or recrudescent parasitaemias in the mice receiving the *S. typhimurium*/TNFα construct when compared with the two control groups (Figure 13).

In a second experiment NIH and A/J mice were inoculated with *S. typhimurium*/TNFα and 5 days later were infected with 1 x 10^5 pRBC. NIH mice inoculated with the *S. typhimurium*/TNFα mutant had a primary parasitaemia from day 3 p.i. to 15 p.i., which reached a mean peak of 33.62% ± 7.83, at day 8 p.i. A recrudescent parasitaemia was observed after day 25 p.i. which peaked at day 28 p.i. (mean 0.92% ± 1.098) and became subpatent after day 31 p.i.. Both control groups exhibited a similar course of infection (Figure 14.a., Table 6). Results obtained with A/J mice are presented in 4.2.3..

A third experiment was carried out in NIH mice by inoculating them with the TNFα construct followed by infection with 1 x 10^5 pRBC 3 days later. The mice develop a primary parasitaemia from day 3 p.i. which peaked (mean 26.58% ± 9.29) at day 8 p.i. and became subpatent after day 13 p.i.. Neither the onset nor the peak of this primary parasitaemia were different from the controls. A recrudescent parasitaemia

was observed after day 25 p.i. which reached its peak (mean 0.29% ± 0.16) at day 28 p.i.. Control groups exhibited a similar recrudescent parasitaemia as the experimental group. Parasitaemia in all groups was subpatent from day 31 p.i. to 60 p.i..

4.2.2.2. Course of infection in A/J mice

In the first experiment with A/J mice a group of six was inoculated with *S. typhimurium*/TNFα and 3 days later the mice were infected i.v. with 1 x 10^5 pRBC. Control groups were inoculated with the *S. typhimurium* control three days before malaria infection and a control group, infected only with *P. chabaudi*, was included. The mice developed a patent parasitaemia from day 4 p.i. which peaked at day 8 p.i.(mean 6.94%, ± 7.06) and resolved at day 23 p.i. Mice inoculated with the *S. typhimurium* control had a similar course of parasitaemia. This became patent from day 3 p.i. and peaked at day 8 p.i (mean 17.25%, ± 13.31) and resolved at day 22 p.i.. Control mice which were not administered *S. typhimurium,* exhibited a primary parasitaemia from day 3 p.i., had a peak parasitaemia at day 8 p.i. (mean 28.3%, ±12.7) and resolved the infection after day 24 p.i.. No recrudescences were observed in any of the groups and all mice survive the infection (Figure 15, Table 8).

In a second experiment (performed simultaneously in NIH mice, results are presented in 4.2.2.), six mice were inoculated with *S. typhimurium*/TNFα and 5 days later were infected with 1 x 10^5 pRBC. The mice developed a primary parasitaemia from day 3 p.i. which peaked at day 9 p.i. (mean 24.36%, ±12.74) and resolved after day 24 p.i. Control mice inoculated with the *S. typhimurium* had a similar course of parasitaemia, with a peak at days 9 p.i (mean 30.45%, ±6.28) and resolved after day 22 p.i.. Control mice infected only with *P. chabaudi* had a peak parasitaemia at day 9 p.i. (mean 37.51%, ±11.43) and resolved the infection after day 22 p.i.. No recrudescences were observed in any of the groups (Figure 14.b., Table 7).

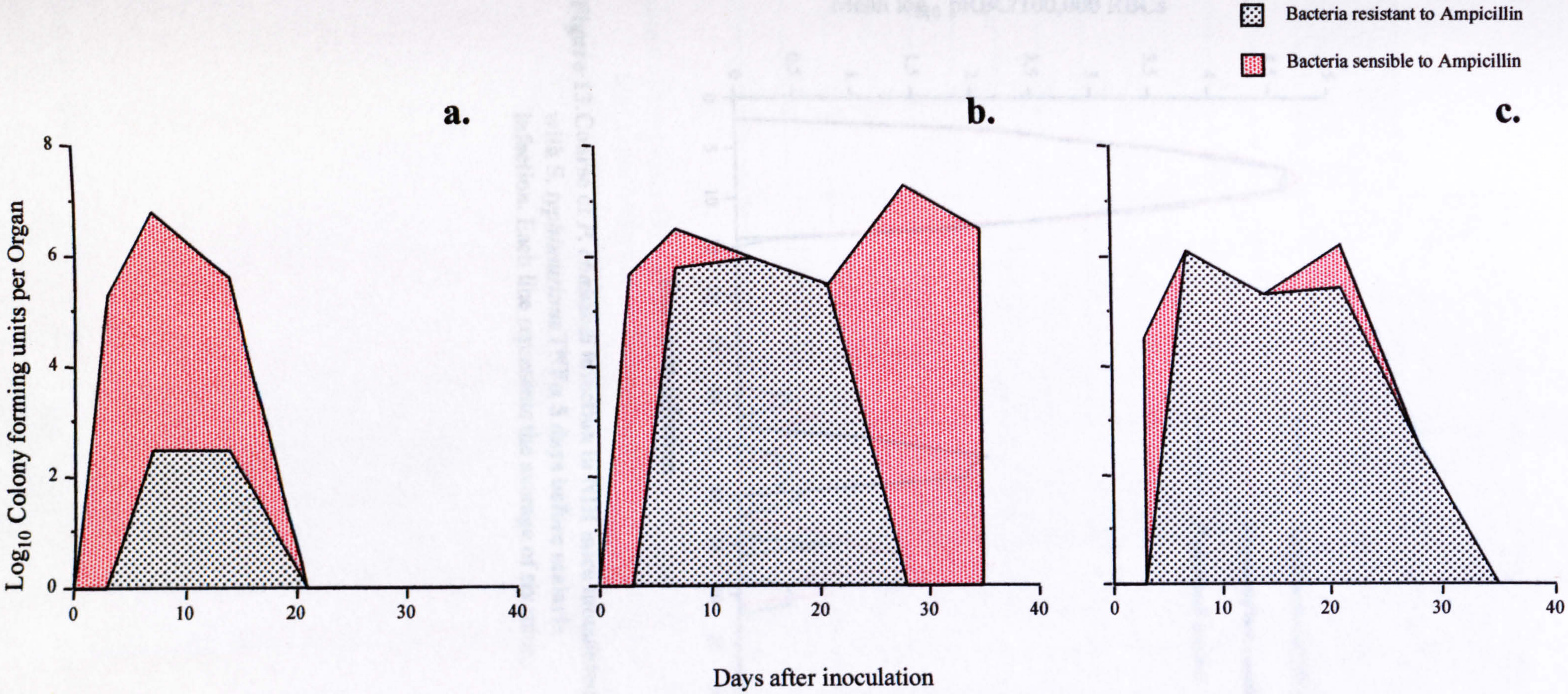


Figure 12.Colonisation and stability of *S. typhimurium*/TNFα mutant in mesenteric lymph nodes (a), liver (b), and spleen (c) of NIH mice. Bacteria resistant ro ampicillin express the TNFα construct. Each point represents the average of three mice.

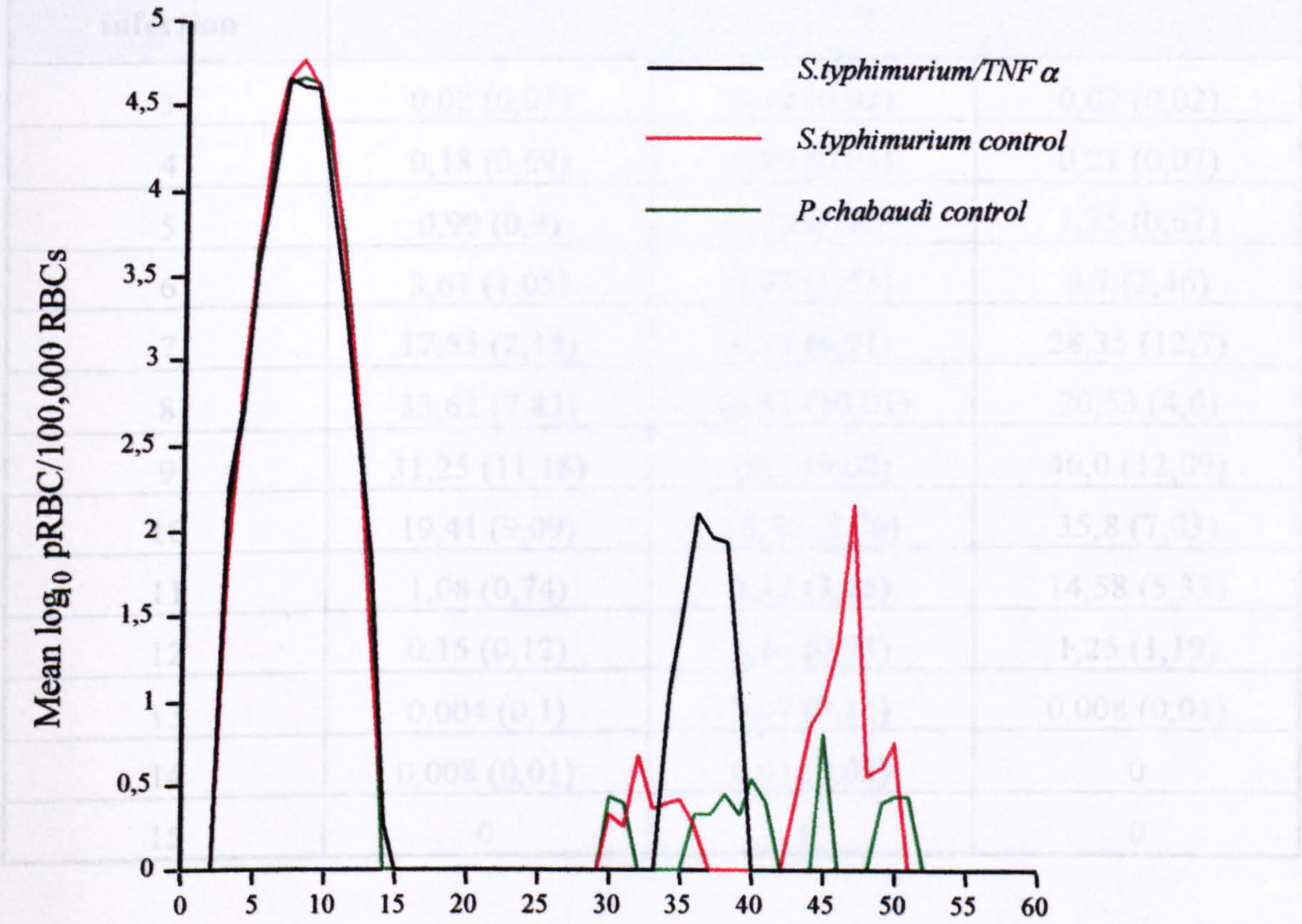


Figure 13.Course of *P. chabaudi* infection in NIH mice inoculated with S. *typhimurium*/TNFα 5 days before malaria infection. Each line represents the average of six mice.

	TNFα mutant n=6	Control mutant n=6	*P. chabaudi* control n=6
Day post-infection	**% mean parasitaemia (S.D.)**		
3	0,02 (0,01)	0,12 (0,02)	0,02 (0,02)
4	0,18 (0,69)	0,06 (0,04)	0,21 (0,07)
5	0,99 (0,4)	0, 32 (0,22)	1,75 (0,67)
6	3,61 (1,05)	1,83 (1,53)	8,7 (2,46)
7	17,55 (2,15)	6,94 (6,91)	28,35 (12,7)
8	33,62 (7,83)	16,81 (10,01)	20,53 (4,0)
9	31,25 (11,18)	20,5 (9,02)	46,0 (12,09)
10	19,41 (9,09)	16,26 (8,76)	35,8 (7,03)
11	1,08 (0,74)	5,12 (3,16)	14,58 (5,33)
12	0,15 (0,12)	1,16 (0,94)	1,25 (1,19)
13	0,004 (0,1)	0,07 (0,11)	0,008 (0,01)
14	0,008 (0,01)	0,01 (0,03)	0
15	0	0	0

Table 6. Mean value (%) and standard error of parasitaemias in NIH mice inoculated orally with *S. typhimurium*/TNFα (TNFα mutant) 5 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

	TNFα mutant n=6	Control mutant n=6	*P. chabaudi* control n=6
Day p.i.	**% mean parasitaemia (S.D.)**		
3	0,01 (0,02)	0	0,02 (0,01)
4	0,08 (0,07)	0,05 (0,03)	0,55 (0,73)
5	0,29 (0,21)	0,23 (0,06)	0,43 (0,3)
6	2,29 (1,95)	1,28 (0,42)	2,54 (1,32)
7	9,1 (9,51)	6,94 (2,83)	12,41 (5,72)
8	24,25 (15,37)	18,04 (4,75)	29,16 (8,37)
9	24,36 (12,74)	30,45 (6,28)	37,51 (11,43)
10	15,65 (3,31)	20,77 (11,26)	21,29 (7,31)
11	6,77 (6,16)	3,16 (1,8)	2,98 (3,75)
12	5,22 (6,72)	2,07 (1,54)	0,75 (0,86)
13	1,04 (1,55)	0,18 (0,17)	0,02 (0,02)
14	0,91 (1,07)	0,17 (0,18)	0,004 (0,01)
15	0,47 (0,73)	0,13 (0,11)	0,04 (0,06)
16	0,51 (0,73)	0,23 (0,21)	0,04 (0,05)
17	0,72 (1,17)	0,27 (0,22)	0,1 (0,09)
18	0,65 (1,17)	0,15 (0,12)	0,34 (0,27)
19	0,74 (1,08)	0,10 (0,07)	0,42 (0,44)
20	0,35 (0,58)	0,10 (0,11)	0,25 (0,58)
21	0,13 (0,09)	0,02 (0,03)	0,11 (0,09)
22	0,03 (0,05)	0,008 (0,02)	0,02 (0,05)
23	0,02 (0,04)	0	0
24	0,005 (0,01)	0	0
25	0	0	0

Table 7. Mean value (%) and standard error of parasitaemias in A/J mice inoculated orally *S. typhimurium*/TNFα (TNFα mutant) 5 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

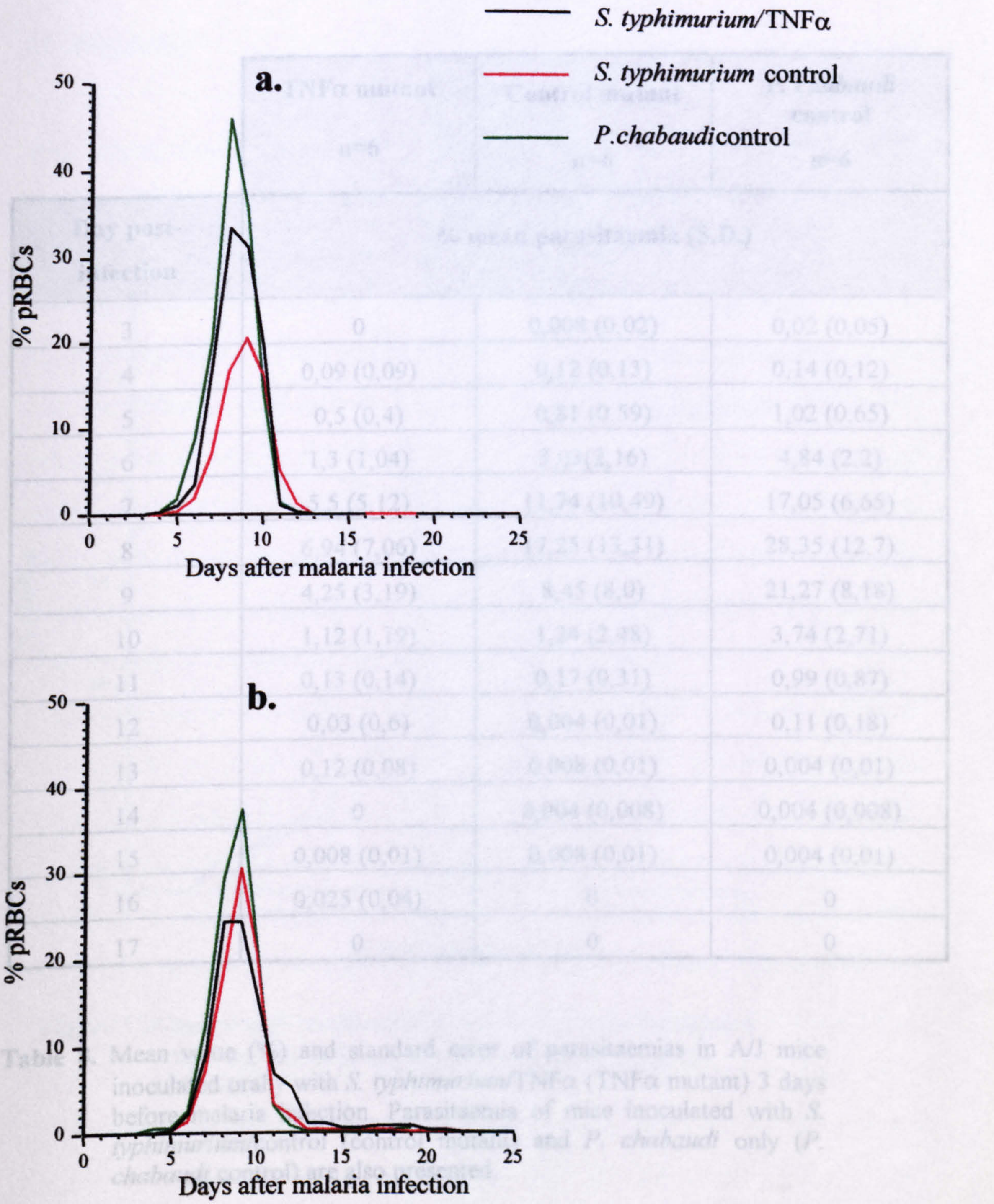


Figure 14.Course of *P. chabaudi* infection in NIH (a) and A/J (b) mice inoculated with S. *typhimurium*/TNFα 5 days before malaria infection. Each point represents the average of six mice.

	TNFα mutant n=6	Control mutant n=6	*P. chabaudi* control n=6
Day post-infection	**% mean parasitaemia (S.D.)**		
3	0	0,008 (0,02)	0,02 (0,05)
4	0,09 (0,09)	0,12 (0,13)	0,14 (0,12)
5	0,5 (0,4)	0,81 (0,59)	1,02 (0,65)
6	1,3 (1,04)	3,03(2,16)	4,84 (2,2)
7	5,5 (5,12)	11,74 (10,49)	17,05 (6,65)
8	6,94 (7,06)	17,25 (13,31)	28,35 (12,7)
9	4,25 (3,19)	8,45 (8,0)	21,27 (8,18)
10	1,12 (1,19)	1,24 (2,48)	3,74 (2,71)
11	0,13 (0,14)	0,17 (0,31)	0,99 (0,87)
12	0,03 (0,6)	0,004 (0,01)	0,11 (0,18)
13	0,12 (0,08)	0,008 (0,01)	0,004 (0,01)
14	0	0,004 (0,008)	0,004 (0,008)
15	0,008 (0,01)	0,008 (0,01)	0,004 (0,01)
16	0,025 (0,04)	0	0
17	0	0	0

Table 8. Mean value (%) and standard error of parasitaemias in A/J mice inoculated orally with *S. typhimurium*/TNFα (TNFα mutant) 3 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

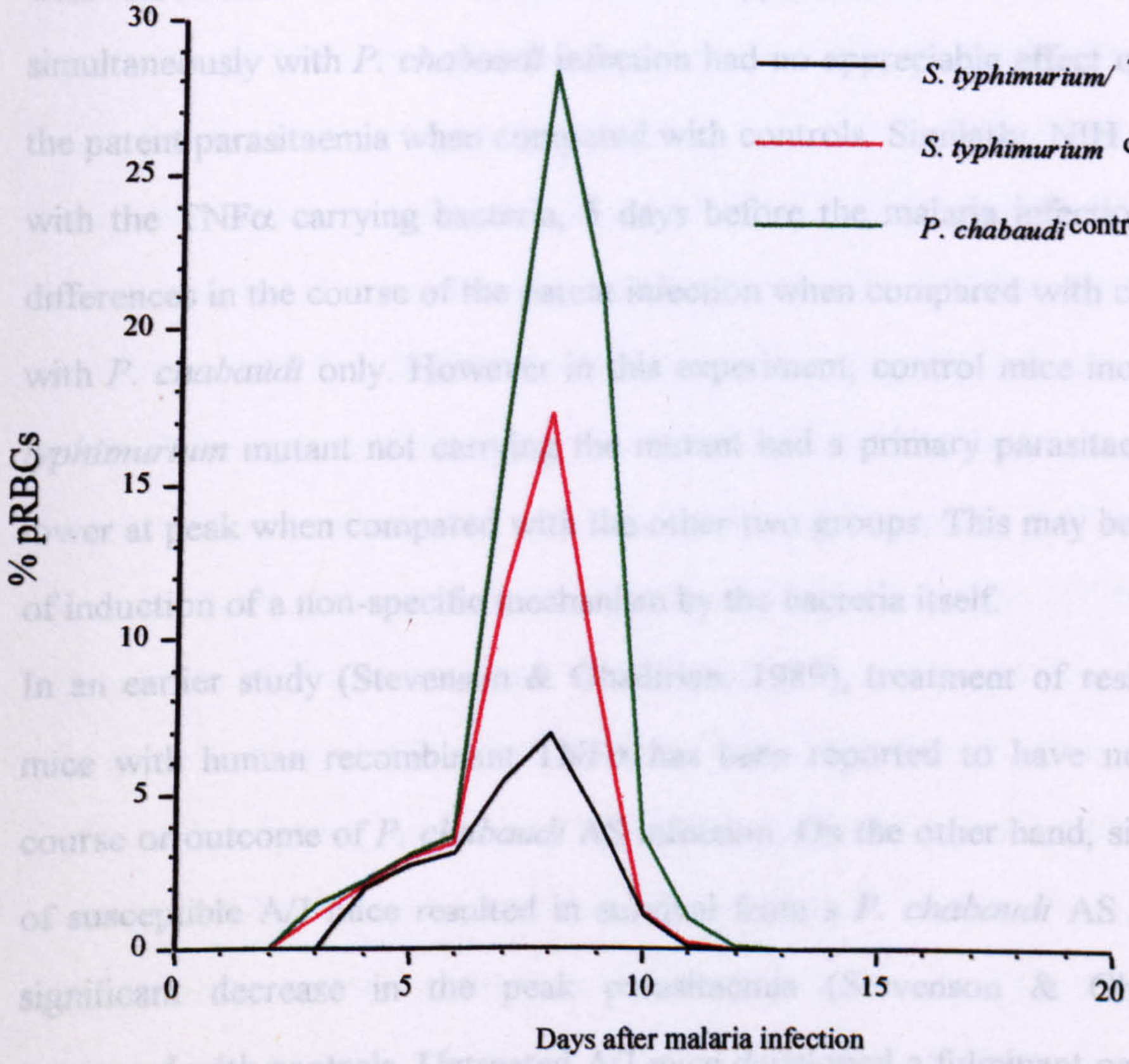


Figure 15.Course of *P. chabaudi* infection in A/J mice inoculated with S. *typhimurium*/TNFα 3 days before malaria infection.

4.3. Discussion

This chapter describes the successful establishment of a *S. typhimurium* mutant carrying the murine TNFα gene in liver, spleen and mesenteric lymph nodes of NIH. Oral inoculation of these mice with *S. typhimurium*/TNFα 7 days before or simultaneously with *P. chabaudi* infection had no appreciable effect on the course of the patent parasitaemia when compared with controls. Similarly, NIH mice inoculated with the TNFα carrying bacteria, 5 days before the malaria infection, exhibited no differences in the course of the patent infection when compared with controls infected with *P. chabaudi* only. However in this experiment, control mice inoculated with *S. typhimurium* mutant not carrying the mutant had a primary parasitaemia which was lower at peak when compared with the other two groups. This may be a consequence of induction of a non-specific mechanism by the bacteria itself.

In an earlier study (Stevenson & Ghadirian, 1989), treatment of resistant C57BL/6 mice with human recombinant TNFα has been reported to have no effect on the course or outcome of *P. chabaudi* AS infection. On the other hand, similar treatment of susceptible A/J mice resulted in survival from a *P. chabaudi* AS infection and a significant decrease in the peak parasitaemia (Stevenson & Ghadirian, 1989) compared with controls. Untreated A/J mice developed a fulminant parasitaemia with 100% mortality and a mean survival time of 10 days. However, the experiments reported in this chapter failed to confirm the very marked susceptibility of A/J mice to *P. chabaudi* AS infection. The mice survived after development of an acute parasitaemia which was controlled before 30 days p.i.. It can be speculated that differences in diet, population of A/J mice and/or parasite strain and its adaptation to the inbred strain of mice, are responsible for the different course of malaria infection observed in A/J mice in this study. Nevertheless, an important difference observed in the course of infection between the A/J and NIH mice was the absence of recrudescences after a primary patent parasitaemia was resolved in A/J mice when compared with NIH. NIH mice had a primary peak of patent parasitaemia. In

addition, the course of the parasitaemia remain unaltered in NIH after treatment (at any time before infection) with the *S. typhimurium*/TNFα mutant, while A/J mice inoculated with the construct 5 or 3 days before malaria infection had a significantly lower peak parasitaemia when compared with controls, supporting a previous suggestion that the genetic background might be a determinant of the relative importance of TNFα in acquired immunity against *P. chabaudi* infection (Stevenson & Ghadirian, 1989).

Elimination of the asexual blood stage of *P. chabaudi* by resistant C57BL/6 mice has been demonstrated to involve the sequential activation of $CD4^+$ TH1 cells followed by activation of $CD4^+$ TH2 cells. In contrast, in susceptible A/J mice, induction of a strong TH2 response early during the malaria infection has been suggested to lead to the severe course of infection and lethal outcome (Stevenson & Tam, 1993). It has been confirmed that, early during malaria infection, resistant C57BL/6 mice have higher levels of TNFα messenger RNA (mRNA) in spleen and liver than susceptible mice (Jacobs *et al.,* 1996b). Furthermore, increased levels of TNFα in serum and of TNFα mRNA in liver later during infection, were found in susceptible A/J mice. This suggests that TNFα has a protective role during a *P. chabaudi* infection when it is produced in the spleen and the liver early during infection and has a deleterious effect when it is found high only in the liver. Jacobs *et al.* (1996b) suggested that the paradoxical role of protection (or resistance) versus pathology (or susceptibility) of TNFα in malaria infection may be dependent on the amount of TNFα released and the timing and site of its expression. The way in which orally delivered TNFα exerts an effect on resistance to *P. chabaudi* is not known. *S. typhimurium* mutants establish in deep organs of the host where, after a limited cycles of replication, the cytokine is released after bacterial death. Therefore oral inoculation of *S. typhimurium*/TNFα mutant might result in increased levels of the cytokine in organs such as liver and spleen early in the infection and might be responsible for a better control of malaria infection. Although TNFα has no direct parasiticidal effect *in vitro* (Taverne *et al.,* 1987), its production in the spleen leads to production of NO which has been shown

to protect the host against oxygen radical-mediated tissue damage during blood-stage malaria (Jacobs *et al.*, 1995; Jacobs *et al.*, 1996a). However, it has been found that expression of TNFα in the liver of resistant C57BL/6 mice does not increase NO production and therefore other mechanisms may be responsible for the enhanced control of infection in these strains of mice (Jacobs *et al.*, 1996a). TNFα in the liver may induce the production of acute-phase reactants (Urquhart, 1994), which have been found to increase during acute *P. falciparum* infection (Graninger *et al.*, 1994)

In conclusion, *S. typhimurium* delivered TNFα had an effect on the course of *P. chabaudi* infection in A/J mice which is comparable with the results obtained by injection of recombinant TNFα, indicating that this murine model is suitable for the study of the effects of TNFα on a malaria infection.

CHAPTER FIVE

EFFECT OF TRANSFORMING GROWTH FACTOR-BETA ON THE COURSE OF *Plasmodium chabaudi* INFECTION

5.1. Introduction

Transforming growth factor-β (TGFβ) belongs to the transforming growth factor superfamily. Members of this family are a group of structurally related proteins which are among the most versatile carriers of growth and differentiation signals (reviewed by Wahl *et al.*, 1994). They participate in setting up the basic body plan during embryogenesis in mammals, frogs and flies by controlling formation of the neural tube, limbs, cartilage, bone and sexual organs; suppress epithelial cell growth; promote wound repair and influence immune and endocrine functions. This family include among others the TGFβs, activins, inhibins, bone morphogenetic proteins, müllerian inhibiting substance (Shull & Doetschman, 1994). TGFβ is a homodimeric protein of 25kD which is secreted locally from platelet stores during early stages of inflammation and then is generated by inflammatory cells themselves (reviewed by Wahl, 1994). Also macrophages and lymphocytes produce TGFβ as part of a cytokine network (reviewed by Wahl, 1994). Initially, the cytokine mediates the egress of undifferentiated leukocytes and, subsequently, promotes tissue repair and resolution of inflammation (Wahl, 1992; McCartney-Francis & Wahl, 1994). TGFb has the capability of inducing human peripheral blood monocytes to express the type III receptor for the FC portion of IgG (FcγRIII/CD16) (Welch *et al.*, 1990), promote their chemotaxis (Wahl *et al.*, 1987), and increase their synthesis of IL-1 and TNFα (Wahl, 1992). In addition, the cytokine down-regulates activation and the production of free oxygen radicals (Tsunawaki *et al.*, 1988) and NO (Ding *et al.*, 1990; Nelson *et al.*, 1991; James, 1995) by macrophages. This inhibition of NO has also been observed in mice and rat hepatocytes, although it was not demonstrated in humans (Nussler *et al.*, 1995). However, TGFβ can promote immuno-suppression as recent evidence suggests that systemic TGFβ targets endothelial cells, where it inhibits E-selectin expression to block adhesion and targeting of leukocytes to the site of infection (Gamble *et al.*, 1993). As part of this immuno-suppressive role, TGFβ inhibits IL-1 dependent thymocyte proliferation, (Kehrl *et al.*, 1986a) IL-2-dependent

T-cell proliferation (Kehrl *et al.*, 1986a; Wahl *et al.*, 1988), B-cell proliferation and immunoglobulin secretion (Kehrl *et al.*, 1986b), and IFNα enhancement of NK cell activity (Rook *et al.*, 1986).

In malaria, the precise role of TGFβ in immunity and pathology has still to be elucidated and research on the role of the cytokine is very limited. V gamma 9^+ T cells of malaria non-exposed donors produce TGFβ, along with IFNγ, TNFα, TNFβ and IL-8, when exposed to *P. falciparum* schizont antigens (Goodier *et al.*, 1995)

Mice susceptible to malaria have been found to have reduced expression of TGFβ, while resistance to cerebral malaria was associated with high expression of TGFβ (de Kossodo & Grau, 1993). More recently, humans infected with *P. falciparum* have been found to have decreased levels of TGFβ in their serum which correlated inversely with serum TNFα levels. These patients showed background TGFβ production after treatment with antimalarials (Wenisch *et al.*, 1995). TGFβ delivered by a *S. typhimurium* mutant administered orally has been shown to reduce inflammation following administration of carrageenin in the footpad of mice (Ianaro *et al.*, 1995). This was accompanied by suppression of an ongoing TH1-like response, characterised by the production of IL-2 and IFNγ, while IL-10 production was enhanced . The results of the inoculation of the same mutant and the effect of the cytokine on the course of *P. chabaudi* infection, are presented in this chapter. The bacteria were inoculated into two strains of mice: A/J and NIH, which were selected on the basis of the different effects observed in the two inbred strains after inoculation with the *S. typhimurium*/TNFα mutant (see Chapter 4).

5.2. Results

5.2.2. Effect of *S. typhimurium* delivered TGFβ on the course of a malaria infection in NIH mice

A group of 6 mice was inoculated with 2 x 10^9*S. typhimurium*/TGFβ bacteria and 4 days later were infected with 1 x 10^5 pRBC. Two control groups were set up: one was inoculated with the *S. typhimurium* mutant control and the parasite, and the other was only infected with the parasite. Mice from the experimental group had a primary patent parasitaemia from day 3 p.i. to day 17 p.i.. The parasitaemia had a mean peak, at day 8 p.i., of 33.56% (± 4.51) in the experimental group and was not different from controls. All groups showed a recrudescent parasitaemia between days 20 p.i. and 30 p.i., with no significant differences among them (Figure 16).

In a second experiment, six mice were inoculated with the TGFβ mutant and 7 days later were infected with 1 x 10^5 pRBC,. They developed a primary patent parasitaemia from day 5 p.i. to day 15 p.i., with a peak of on day 8 of 25.79% ±4.88). This peak parasitaemia was not different from controls. A recrudescent parasitaemia was observed in the experimental mice from day 28 p.i. to 34 p.i, with a mean peak of parasitaemia at day 31 p.i. of (0.18% ±0.93). A recrudescent parasitaemia was also observed in control groups between days 29 p.i. and 36 p.i.. No significant differences were observed in the course of infection in the experimental mice when compared with controls (Figure 17)

5.2.3. Effect of *S. typhimurium* delivered TGFβ on the course of a malaria infection in A/J mice

One experiment evaluating the effects of *S. typhimurium*/TGFβ was performed in A/J mice. In this, a group of six mice were inoculated orally with the TGFβ construct and 3 days later they were infected with 1 x 10^5 pRBC. Two control groups were set up:

one was given the *S. typhimurium* control bacteria and 3 days later was infected with *P. chabaudi*, another was only infected with malaria.

The experimental mice showed a patent parasitaemia from day 3 p.i. to 18 p.i., with a mean peak parasitaemia of 33.77% ±12.2), at day 7 p.i.. Control mice inoculated with the *S. typhimurium* mutant had a similar onset of infection, with a mean peak of parasitaemia of 30.17% ±4.9 at day 8 p.i.. In the malaria control group mean peak parasitaemia was 42.88% ±11.03. The *S. typhimurium* inoculated mice showed a better control of the infection in comparison with the malaria control group. The parasitaemia of the two control groups became subpatent by day 18 p.i. (Figure 18, Table 9).

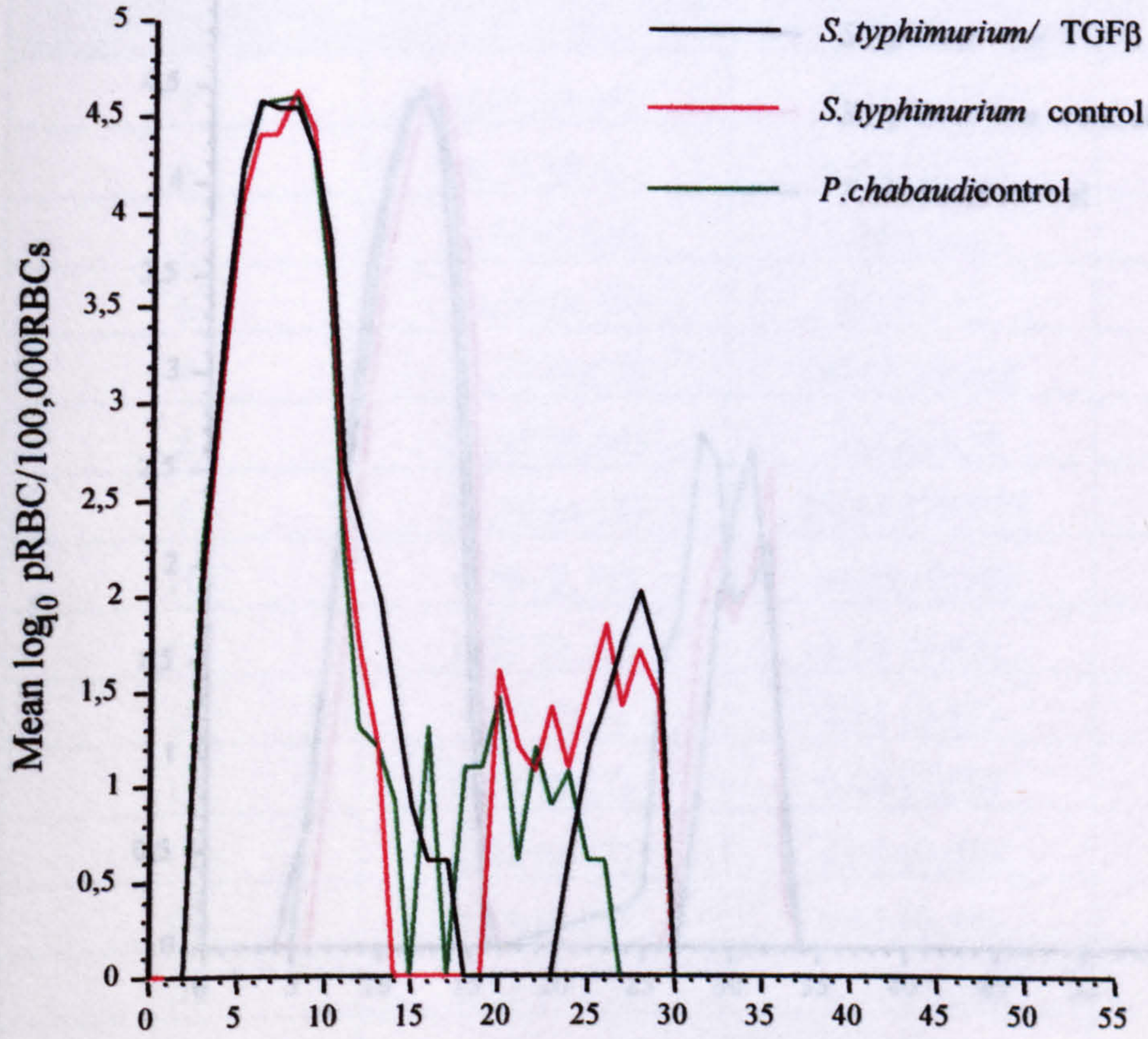


Figure 16.Course of *P. chabaudi* infection in NIH mice inoculated with S. *typhimurium*/TGFβ 4 days before malaria infection.

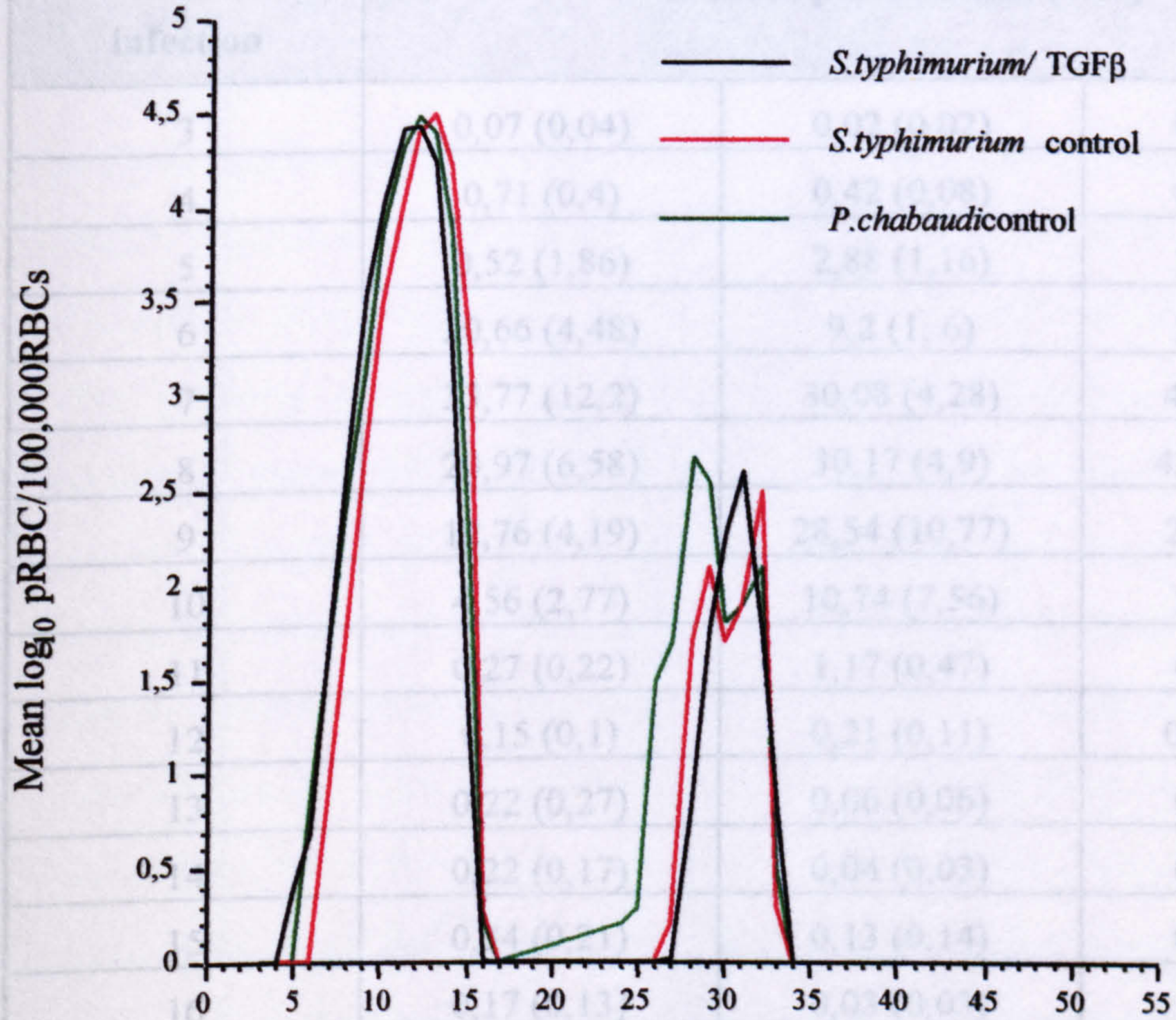


Figure 17.Course of *P. chabaudi* infection in NIH mice inoculated with S. *typhimurium*/TGFβ 7 days before malaria infection.

	TGFβ mutant n=6	Control mutant n=6	*P. chabaudi* control n=6
Day post-infection	**% mean parasitaemia (S.D.)**		
3	0,07 (0,04)	0,02 (0,02)	0,14 (0,04)
4	0,71 (0,4)	0,42 (0,08)	0,99 (0,43)
5	0,52 (1,86)	2,88 (1,16)	3,68 (3,11)
6	20,66 (4,48)	9,2 (1, 6)	23,4 (5,46)
7	33,77 (12,2)	30,08 (4,28)	45,16 (7,31)
8	20,97 (6,58)	30,17 (4,9)	42,88 (11,03)
9	12,76 (4,19)	28,54 (10,77)	22,11 (9,17)
10	4,56 (2,77)	10,74 (7,56)	2,36 (1,33)
11	0,27 (0,22)	1,17 (0,47)	0,14 (0,11)
12	0,15 (0,1)	0,21 (0,11)	0,14 (0,064)
13	0,22 (0,27)	0,06 (0,06)	0,11 (0,05)
14	0,22 (0,17)	0,04 (0,03)	0,28 (0,23)
15	0,34 (0,21)	0,13 (0,14)	0,15 (0,15)
16	0,17 (0,13)	0,03 (0,03)	0,3 (0,31)
17	0,17 (0,24)	0,01 (0,13)	0
18	0,002 (0,006)	0	0
19	0	0	0

Table 9. Mean value (%) and standard error of parasitaemias in A/J mice inoculated orally with *S. typhimurium*/TGFβ (TGFβ mutant) 3 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

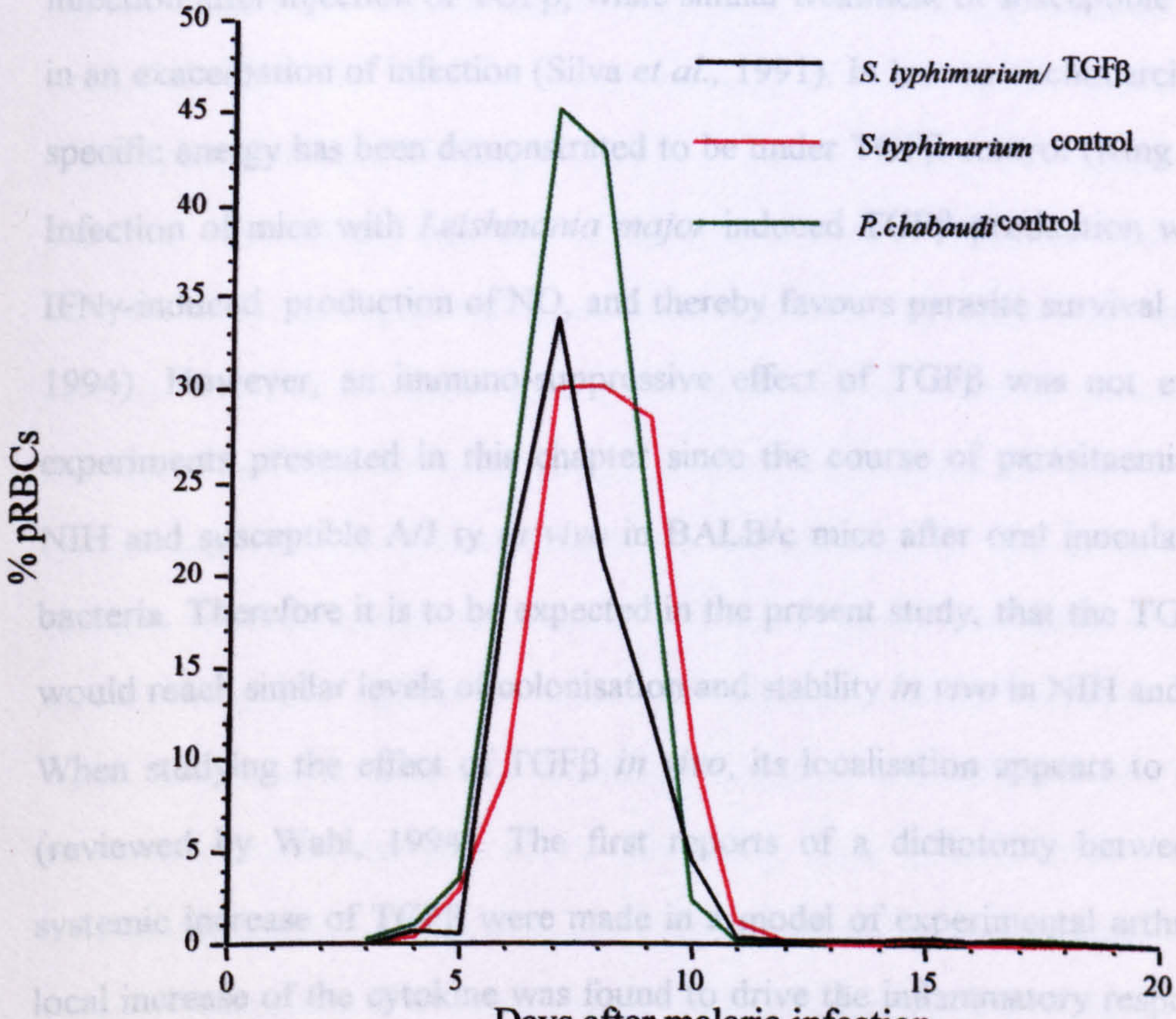


Figure 18.Course of *P. chabaudi* infection in A/J mice inoculated with S. *typhimurium*/TGFβ 3 days before malaria infection.

5.3. Discussion

TGFβ has shown to suppress immune responses in a number of different parasitic infections. Resistant mice infected with *Trypanosoma cruzi* became susceptible to infection after injection of TGFβ, while similar treatment of susceptible mice resulted in an exacerbation of infection (Silva *et al.*, 1991). In human onchocerciasis, parasite-specific anergy has been demonstrated to be under TGFβ control (King *et al.*, 1993). Infection of mice with *Leishmania major* induced TGFβ production which blocked IFNγ-induced production of NO, and thereby favours parasite survival (Green *et al.*, 1994). However, an immuno-suppressive effect of TGFβ was not evident in the experiments presented in this chapter since the course of parasitaemia in resistant NIH and susceptible A/J ty *in vivo* in BALB/c mice after oral inoculation with the bacteria. Therefore it is to be expected in the present study, that the TGFβ construct would reach similar levels of colonisation and stability *in vivo* in NIH and A/J mice .

When studying the effect of TGFβ *in vivo*, its localisation appears to be important (reviewed by Wahl, 1994). The first reports of a dichotomy between local and systemic increase of TGFβ were made in a model of experimental arthritis in which local increase of the cytokine was found to drive the inflammatory response whereas a high systemic concentration was inhibitory (Allen *et al.*, 1990; Wahl *et al.*, 1993). The mechanisms involved in the local anti-inflammatory effect of TGFβ include leukocyte recruitment to the site of inflammation with increased adhesion and fibroblast accumulation (reviewed by Wahl., 1994). On the other hand, systemic TGFβ inhibits leukocyte recruitment to the site of infection. Thus, the route of injection and the resultant local or systemic levels of TGFβ, could be crucial when studying its role on malaria infection.

As an immunomodulatory agent, TGFβ has been found to inhibit IL-2-dependent T cell proliferation (Kehrl *et al.*, 1986 a & b, Wahl *et al.*, 1988), IL-1-dependent murine thymocyte proliferation (Kehrl *et al.*, 1986 a), IFNγ enhancement of NK cell activity (Rook *et al.*, 1986), and B-cell proliferation and immunoglobulin secretion (Kehrl *et*

al., 1986 b). TGFβ has been reported to inhibit reactive oxygen and nitrogen intermediates production by macrophages (Tsunawaki *et al.*, 1988; Vodovotz *et al.*, 1993).

Ianaro et al. (1995) have demonstrated that oral inoculation of mice with *S. typhimurium*/TGFβ did not increase systemic levels of the cytokine. If the same occurred in the *S. typhimurium*/TGFβ-*P. chabaudi* model, then an immunomodulatory effect might have been exerted locally in organs such as spleen and liver. Accordingly with the model of inflammation discussed above, a localised increase of TGFβ might result in a better control of the parasite since the sequestered parasite would be in contact with activated macrophages and its products. However, the fact that important macrophage effector mechanisms for parasite killing (i.e. oxygen and nitrogen intermediates) can be inhibited by the cytokine, may make changes in parasitaemia not obvious. To better assess the effect of TGFβ in the immune response against *P. chabaudi* infection, further experiments exploring variables such as systemic and localised (at least in liver and spleen) TGFβ and NO production should be performed. Moreover, since pRBCs adhere to E-selectin (Ockenhouse *et al.*, 1992) and TGFβ inhibit its expression by endothelial cells, it would be interesting to evaluate changes in sequestration of *P. chabaudi*.

Finally, it is evident from experiments performed with other constructs (See 3.2 & 4.2), that timing between the construct inoculation and parasite infection is crucial in order to observe a protective response. Ianaro *et al.*, (1995), demonstrated that an ongoing TH1-like response could be suppressed if the bacteria were inoculated 6 days before induction of oedema. In the experiments presented here, NIH mice were given the bacteria 4 and 7 days before malaria infection, while in A/J it was done 3 days before *P. chabaudi* infection and no changes in the course of parasitaemia were observed. Further experiments with changes in the timing between the *S. typhimurium*/TGFβ construct inoculation and the *P. chabaudi* infection should be performed before any effect of the cytokine on malaria infection is ruled out.

CHAPTER SIX

EFFECT OF INTERLEUKIN-4 ON THE COURSE OF *Plasmodium chabaudi* INFECTION

6.1. Introduction

Interleukin 4 (IL-4) was originally characterised in 1985 (Oliver *et al.*), by its ability to stimulate differentiation of resting B lymphocytes . This cytokine is a growth factor for activated T lymphocytes, thymocytes, NK cells (Spits *et al.*, 1987 ; Defrance *et al.*, 1987; Mosmann *et al.*, 1986) and mast cells (Mosmann *et al.*, 1986; Lee *et al.*, 1988) Furthermore, it induces maturation of monocytes on which it can exert an stimulatory effect by increasing MHC II expression and antigen presenting ability (Zlotnik *et al.*, 1987). On the other hand, IL-4 appears to inhibit some parameters of monocyte activation including superoxide production (Abramson & Gallin, 1990) and inhibition of release of prostaglandin E2 , IL-1β (Hart *et al.*, 1989), IL-6 (Yanagawa *et al.*, 1991), IL-8 (Standiford *et al.*, 1990), and TNFα (Essner *et al.*, 1989).

TH0 $CD4^+$ cells produce mainly IL-2 in response to primary stimulation. Persistent stimulation results in differentiation into TH1 cells, which mainly produce IFNγ, and TH2 cells, which mainly produce IL-4. TH2 cells secrete other cytokines including IL-5, IL-6, IL-9, IL-10 and IL-13 (reviewed by Kopf *et al.*, 1995). Mast cells and eosinophils can also secrete these TH2 cytokines.

Parasitic infections tend to result in a polarised TH1 or TH2 response (reviewed by Sher & Coffman, 1992). In general, protozoa such as *L. major* (Wang *et al.*, 1994) and *T. gondii* (Gazzinelli *et al.*, 1994) trigger a dominant TH1 response. In murine malaria, acquisition of immunity against *P. vinckei* after two drug treated infections has been associated with a TH1 response. While mice infected with *P. chabaudi* exhibited characteristics of a TH1 response early during infection, a TH2 response became predominant after two weeks of infection (reviewed by von der Weid & Langhorne, 1993b; Taylor-Robinson *et al.*, 1993).

In order to study the role of IL-4 on a *P. chabaudi* AS strain infection further, susceptible A/J mice and resistant NIH mice were inoculated with a *S. typhimurium* carrying the murine IL-4 gene and the course of the malaria infection was followed.

6.2. Results

6.2.2. Effect of *S. typhimurium* delivered IL-4 on the course of a malaria infection in NIH mice

In the first experiment with the *S. typhimurium*/IL-4 construct, the bacteria were given to a group of 6 NIH mice and 4 days later they were infected with 1 x 10^5 pRBC. Control groups were set up as follows: (i) 6 mice were given the *S. typhimurium* control bacteria and 4 days later they were infected with *P. chabaudi*, and (ii) 6 mice were only infected with *P. chabaudi*. All mice had a primary parasitaemia from day 3 p.i. to day 16 p.i. which peaked at day 8 p.i.. In the experimental group the mean peak parasitaemia was (34.42% ±4.35), which was not different from the mean peak parasitaemia of controls (41.9% in the *S. typhimurium* control group, and 38.4% in the malaria control group) and became subpatent at day 16 p.i.. All groups had a recrudescent parasitaemia between days 20 p.i. and 32 p.i., with no significant differences between them (Figure 19).

In a second experiment, six mice were mice given the *S. typhimurium*/IL-4 mutant 7 days before injection with 1 x 10^5 pRBC. These mice showed a primary patent parasitaemia from day 3 p.i. to day 13 p.i., with a mean peak parasitaemia, at day 8, of 23.45% ±6.78. This peak parasitaemia was not different from controls. A recrudescent parasitaemia was observed in the experimental mice from day 27 p.i. to 37 p.i, with a mean peak of parasitaemia at day 32 p.i. of 0.94% (±0.92). The control groups had a recrudescent parasitaemia between days 29 p.i. and 36 p.i.. No differences were observed in the course of infection in the experimental mice when compared with controls.

6.2.3. Effect of *S. typhimurium* delivered IL-4 on the course of a malaria infection in A/J mice

A single experiment was carried out to evaluate the effects of *S. typhimurium*/IL-4 in the course of *P. chabaudi* infection in 'susceptible' A/J mice. In this, six mice were inoculated orally with the *S. typhimurium*/IL-4 construct and 3 days later they were infected with 1 x 10^5 pRBC. Two control groups were set up: one was given the *S. typhimurium* control bacteria and 3 days later was infected with *P. chabaudi*, while another was only infected with malaria.

The experimental mice had a patent parasitaemia from day 3 p.i. to 17 p.i., with a mean peak parasitaemia of 35.33% (± 7.52), at day 7 p.i.. Control mice inoculated with the *S. typhimurium* mutant had a similar onset of infection, with a mean peak of parasitaemia of 30.17% (± 4.9) at day 8 p.i..The malaria control groups had a mean peak parasitaemia of 42.88 ±11.03). Between the two control groups there were differences, the *S. typhimurium* inoculated mice showing a better control of the infection in comparison with the malaria control group. The parasitaemia of the two control groups became subpatent by day 18 p.i. (Figure 20, Table 10). No recrudescences were observed in any of the groups and all mice survived the infection

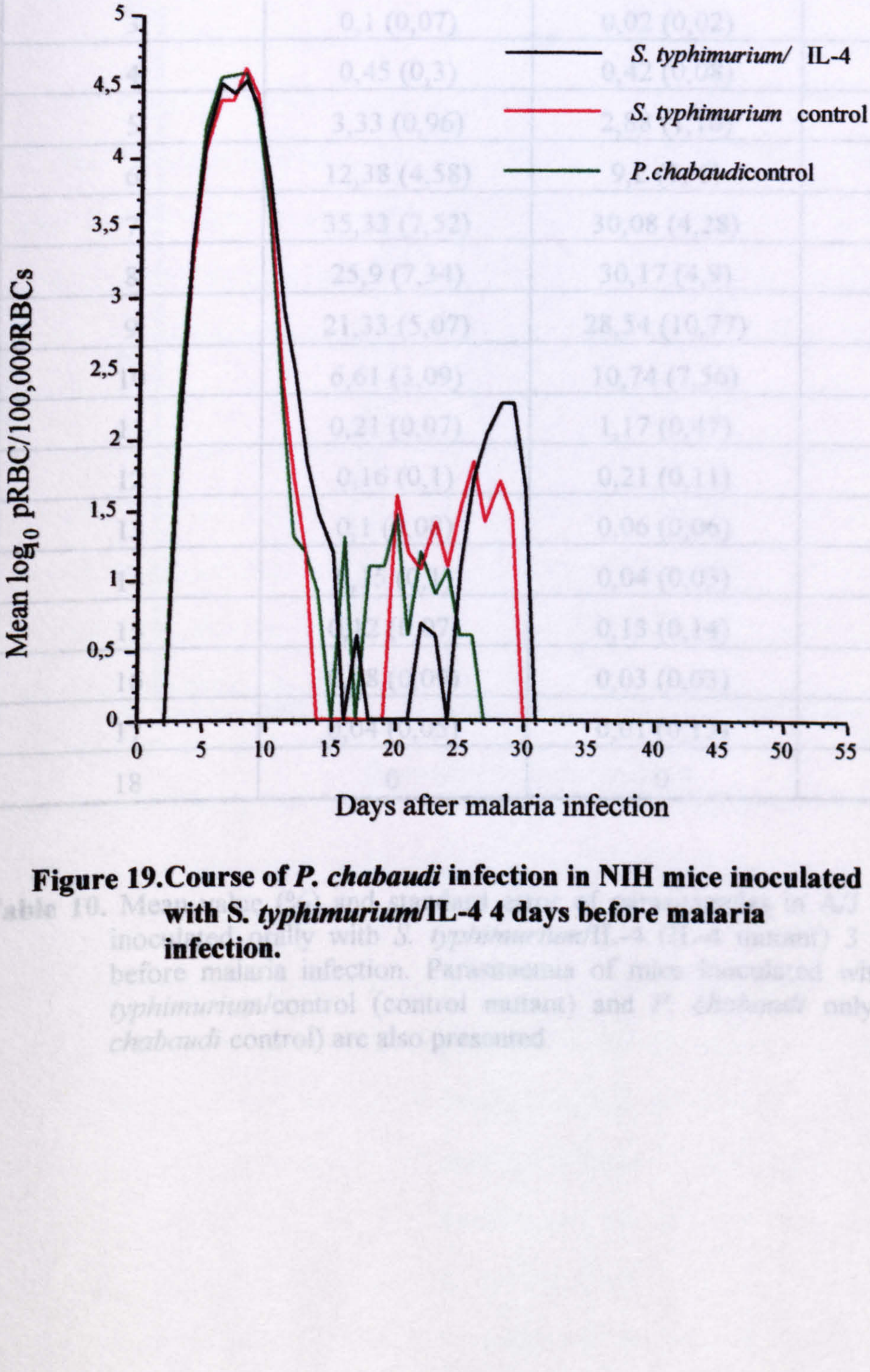


Figure 19.Course of *P. chabaudi* infection in NIH mice inoculated with S. *typhimurium*/IL-4 4 days before malaria infection.

	IL-4 mutant n=6	**Control mutant n=6**	***P. chabaudi* control n=6**
Day post-infection	**% mean parasitaemia (S.D.)**		
3	0,1 (0,07)	0,02 (0,02)	0,14 (0,04)
4	0,45 (0,3)	0,42 (0,08)	0,99 (0,43)
5	3,33 (0,96)	2,88 (1,16)	3,68 (3,11)
6	12,38 (4,58)	9,2 (1, 6)	23,4 (5,46)
7	35,33 (7,52)	30,08 (4,28)	45,16 (7,31)
8	25,9 (7,34)	30,17 (4,9)	42,88 (11,03)
9	21,33 (5,07)	28,54 (10,77)	22,11 (9,17)
10	6,61 (3,09)	10,74 (7,56)	2,36 (1,33)
11	0,21 (0,07)	1,17 (0,47)	0,14 (0,11)
12	0,16 (0,1)	0,21 (0,11)	0,14 (0,064)
13	0,1 (0,08)	0,06 (0,06)	0,11 (0,05)
14	0,15 (0,1)	0,04 (0,03)	0,28 (0,23)
15	0,12 (0,07)	0,13 (0,14)	0,15 (0,15)
16	0,08 (0,09)	0,03 (0,03)	0,3 (0,31)
17	0,04 (0,05)	0,01 (0,13)	0
18	0	0	0

Table 10. Mean value (%) and standard error of parasitaemias in A/J mice inoculated orally with *S. typhimurium*/IL-4 (IL-4 mutant) 3 days before malaria infection. Parasitaemia of mice inoculated with *S. typhimurium*/control (control mutant) and *P. chabaudi* only (*P. chabaudi* control) are also presented.

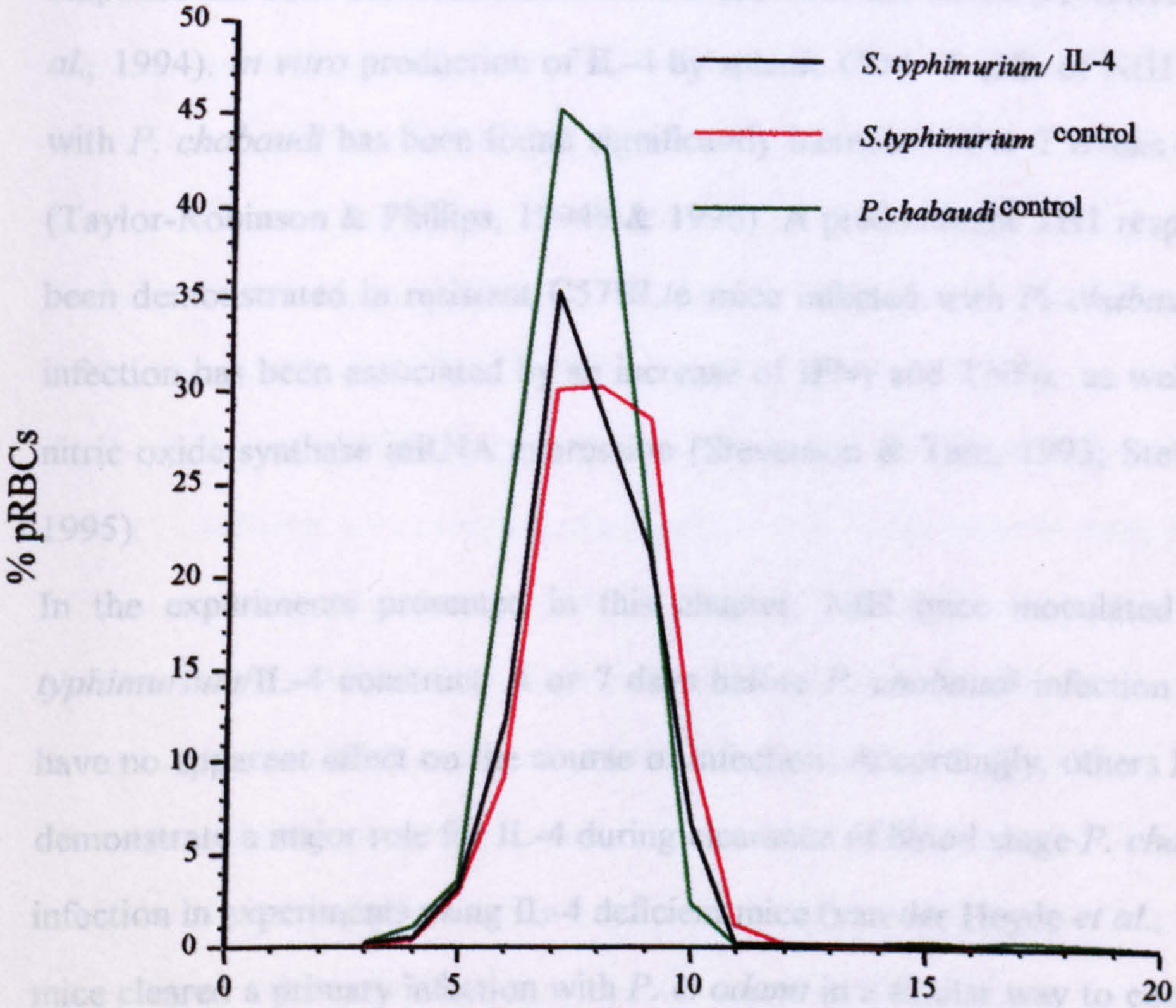


Figure 20. Course of *P. chabaudi* infection in A/J mice inoculated with S. *typhimurium*/IL-4 4 days before malaria infection.

6.3. Discussion

The important role of TH1 $CD4^+$ T cells in the control of an acute malaria infection with *P. chabaudi* has been confirmed in NIH mice. Moreover, a TH2 type immune response has been demonstrated to follow this TH1 activation (reviewed by Phillips *et al.*, 1994). *In vitro* production of IL-4 by splenic $CD4^+$ T cells of NIH mice infected with *P. chabaudi* has been found significantly increased after 2 weeks post-infection (Taylor-Robinson & Phillips, 1994b & 1996). A predominant TH1 response has also been demonstrated in resistant C57BL/6 mice infected with *P. chabaudi* and early infection has been associated by an increase of IFNγ and TNFα as well as inducible nitric oxide synthase mRNA expression (Stevenson & Tam, 1993; Stevenson *et al.*, 1995).

In the experiments presented in this chapter, NIH mice inoculated with the *S. typhimurium*/IL-4 construct 4 or 7 days before *P. chabaudi* infection showed it to have no apparent effect on the course of infection. Accordingly, others have failed to demonstrate a major role for IL-4 during clearance of blood stage *P. chabaudi adami* infection in experiments using IL-4 deficient mice (van der Heyde *et al.*, 1997). These mice cleared a primary infection with *P. c. adami* in a similar way to controls and did not develop a persistent malaria indicating that IL-4 is not required to activate the antibody mediated immunity that ultimately sterilises infection.

In contrast to the findings when studying resistant C57BL/6 mice, susceptible A/J mice that develop a higher peak parasitaemia and succumb to infection, showed high production, *in vivo* and *in vitro*, of IL-5 (a TH2 cell marker) early in the *P. chabaudi* infection (Stevenson & Tam, 1993), suggesting that an early TH2 response may lead to a severe and lethal course of malaria. However, when treated with anti-IL-4 monoclonal antibodies, peak parasitaemia was not affected when compared with untreated controls (Jacobs *et al.*, 1996a). This later finding is in agreement with the results obtained when A/J mice were inoculated with the IL-4 construct and infected with *P. chabaudi* three days later. These and the control mice, which were given *S.*

typhimurium control had a similar course of parasitaemia, and both of them were significantly lower when compared with the malaria only controls. The better control of the malaria infection in the mice which were given any of the bacteria, could be secondary to mediation of non-specific mechanisms by the bacteria itself. A period of non-specific immunity occurs as the initial replication of the attenuated organisms reaches a plateau, during which resistance to infection with *Listeria monocytogenes*, as well as to virulent *Salmonella*, has been reported (Hormaeche *et al.*, 1991). This non-specific protection is mediated primarily by activated macrophages and NK cells (Schafer & Eisenstein, 1992), and can be inhibited by anti-IFNγ monoclonal antibodies (Motile & Make, 1993).

Taylor-Robinson & Phillips (1993) have demonstrated that transfer of TH2 cell lines from *P. chabaudi* AS reinfected mice were able to protect naive mice but transfer of B cells was also required to be fully protective in immunocompromised hosts. In line with this, mice depleted from birth of B cells by treatment with anti-μ antibodies can control but not clear *P. chabaudi* AS infections (Meding & Langhorne, 1991; von der Weid & Langhorne, 1993a). Recently, a lack of clearance of *P. chabaudi* AS infection has been also confirmed in gene-targeted mice lacking B cells (von der Weid *et al.*, 1996). These findings suggest the presence of B cells may be required for the effective clearance of the parasite at the later stages of infection. IL-4 might have some role in the control of *P. chabaudi* AS infection, not only through activation of B cells to produce specific antibodies, but also by inhibiting TH1 differentiation which contributes to the TH1-TH2 $CD4^+$ cells switch observed in resistant mice.

In the experiments presented in this Chapter, *S. typhimurium* delivered IL-4 had no appreciable effect on the course of infection with *P. chabaudi* in NIH and A/J mice. Furthermore, although preliminary, the results obtained from the experiments with the A/J mice suggest that the establishment of a TH2 cells pattern early during *P. chabaudi* infection does not affect the outcome of the infection. Further experiments using the construct at different time points of the malaria infection, in both resistant and susceptible mice, should be carried out in order to better assess the role of IL-4

on *P. chabaudi* infection. These experiments should also evaluate TH1 and TH2 cytokines production, proliferative responses against malarial antigen and humoral responses.

CHAPTER SEVEN

ROLE OF NITRIC OXIDE IN THE CONTROL OF A *Plasmodium chabaudi* INFECTION

7.1. Introduction

Nitric Oxide (or nitrogen monoxide) (NO), is a gaseous free radical that exhibits multiple biological properties. The molecule was first shown in 1987 to be produced by living cells and was confirmed to be identical to the endothelium-derived relaxing factor (Ignarro *et al.*, 1987; Palmer *et al.*, 1987).

Nitric oxide is generated in conjunction with L-citrulline from the guanidino nitrogen of L-arginine, this process being catalysed by the enzyme NO synthase (NOS) (Figure 21). Several isoforms of NOS have been isolated (reviewed by Schmidt *et al.*, 1992). The NOS which is always present in cells, is a constitutive (cNOS) low-output form which results in production of low amounts of NO for short periods (reviewed by James, 1995). cNOS activity is dependent on intracellular concentrations of calcium and the enzyme is activated after the binding of a calcium-calmodulin complex (Bredt & Snyder, 1990; Lowenstein *et al.*, 1992). At least two isoforms of cNOS have been described, one restricted to neurones and the other to endothelial cells (reviewed by James, 1995).

The calcium-independent NOS can be induced in a number of cells including macrophages, neutrophils, hepatocytes, muscle cells and endothelial cells. Enhanced transcription of the gene for these inducible NOS (iNOS) is observed in response to stimulation with LPS and cytokines such as IFNγ and TNFα (Drapier *et al.*, 1988; Ding *et al.*, 1988). The enzyme is down-regulated by other cytokines including IL-4, IL-5, IL-8 and IL-10, and by some growth factors and glucocorticoids (reviewed by Cox & Liew, 1992). Production of iNOS results in a much higher concentration of NO (reviewed by Clark & Rockett, 1996).

NO has been shown to be important in hypertension associated with kidney failure and pregnancy, hypotension of septic shock, control of sphincters in the gastrointestinal and genito-urinary tracts. It has also contributed to explain the mechanisms involved in immunosuppression, insulin production, growth factor action, platelet adhesion and aggregation, childhood-onset diabetes, and killing of bacteria

and protozoa by host defences (reviewed by Clark & Rockett, 1996). Indeed, microbicidal activity of the molecule has been demonstrated in *Schistosoma mansoni*, *L. major* , *T. gondii*, *T cruzi* and *E. histolytica*, among other (reviewed by James, 1995). In blood stage malaria (Taylor-Robinson *et al.*, 1993; Jacobs *et al.*, 1996a) increased levels of NO have been associated with acute infection but the precise mechanisms of parasite killing mediated by the molecule remain unclear.

High levels of nitrate, an oxidised form of NO, have been observed in *P. chabaudi* infected mice. These developed a primary parasitaemia and a sharp peak of nitrate production which was associated with the peak of infection (Taylor-Robinson *et al.*, 1993). Others have demonstrated increased levels of nitrate in plasma of individuals infected with *P. falciparum* or *P. vivax* (Cot *et al.*, 1994; Nüssler *et al.*, 1994). Furthermore, it has been suggested that NO may have a protective effect in patients with cerebral malaria as affected children with relatively higher levels of nitrate in plasma had a shorter duration of coma (Cot *et al.*, 1994).

To assess further the role of NO in a model of *P. chabaudi* infection, mice lacking iNOS were infected and the course of parasitaemia followed. Other immunological parameters assessing humoral and cellular responses were studied.

Mice homozygous for the iNOS mutation have previously been shown to be susceptible to *L. major* infection in contrast to wild type and heterozygous mice (Wei *et al.*, 1995). The experiments presented in this chapter were performed in homozygous mice lacking iNOS and heterozygous mice were used as controls.

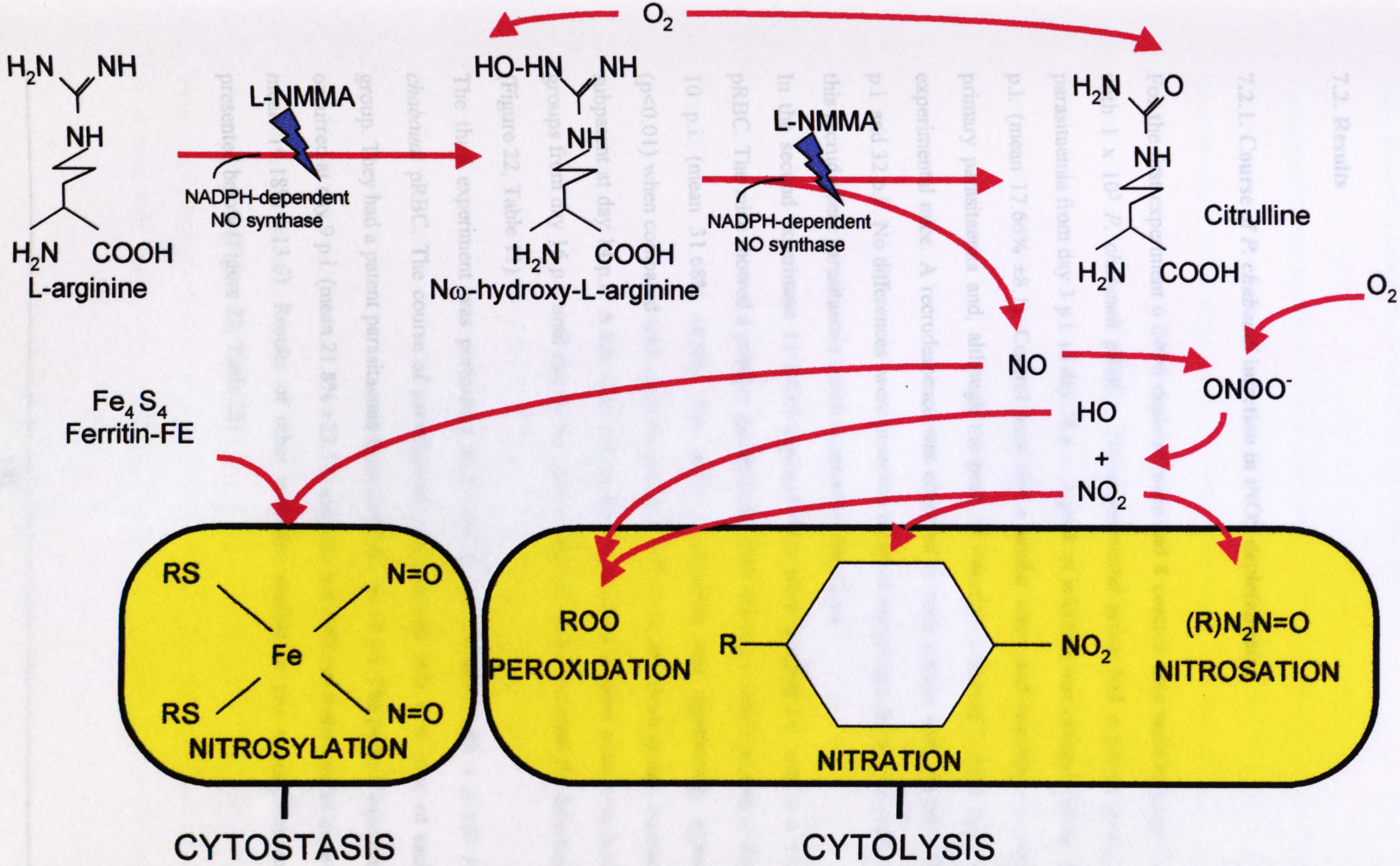


Figure 21.Nitric oxide biosynthesis from L-arginine. Some of the potential reactions of NO and its interrelated forms with parasite antigens

7.2. Results

7.2.1. Course of *P. chabaudi* infection in iNOS depleted mice

For the first experiment 6 iNOS depleted mice and 8 control mice were infected i.v. with 1 x 10^5 *P. chabaudi* pRBC. The experimental group had a patent primary parasitaemia from day 3 p.i. to day 18 p.i.. A peak of infection was observed at day 8 p.i. (mean 17.66% ±8.1). Control mice had a similar onset and resolution in their primary parasitaemia and, although the peak of infection occurred 2 days later in experimental mice. A recrudescence was observed in both groups between days 28 p.i. and 32 p.i.. No differences were observed between the groups during the peak of this recrudescent parasitaemia which occurred at day 30 p.i..

In the second experiment 11 iNOS depleted mice were infected i.v. with 1 x 10^5 pRBC. The mice showed a primary parasitaemia from day 3 p.i. which peaked at day 10 p.i. (mean 31.68% ±8.98). This peak parasitaemia was significantly higher ($p<0.01$) when compared with controls (mean 17.85% ±12.24). Both groups became subpatent at day 14 p.i.. A low level parasitaemia was noted in some mice from both groups from day 16 p.i. until day 30 p.i., after which all mice controlled the infection (Figure 22, Table 11).

The third experiment was performed in 27 iNOS mice infected with 1 x 10^5 *P. chabaudi* pRBC. The course of parasitaemia was followed only in 6 mice of each group. They had a patent parasitaemia from day 3 p.i. to 14 p.i. The peak of infection occurred at day 9 p.i. (mean 21.8% ±23.11) and was not different from control mice (mean 19.18% ±13.6). Results of other variables studied in this experiment are presented below (Figure 23, Table 12).

7.2.2. Sequestration of *P. chabaudi* at peak parasitaemia

Possible differences in sequestration of the parasite in deep organs, such as liver and spleen, were evaluated in the third experiment. For this 27 iNOS depleted mice were infected with 1 x 10^5 pRBC, and a control group of 27 mice was included. The parasitaemia of 6 malaria infected mice was followed every six hours between days 8 p.i. and 10 p.i.. The peak of infection in these mice occurred at day 9 p.i..

At day 8 p.i. a maximum of sequestration (mean parasitaemia 10.03% ±6.76) was observed at 00:00 hr in the experimental mice, which was not different from controls (mean parasitaemia 9.61% ±8.74) (Figure 24). Six hours later (06:00 hr) a mean peak parasitaemia of 19.18 % (±13.6) was observed in the experimental group, while in the control group it was 21.81% (±23.11). The proportion of parasites in circulation dropped gradually in both groups during day 9 p.i. reaching the lowest point (mean 6.1% ±5.48 in experimental mice, mean 8.92% ±7.52 in control mice) at 00.00 hr. No differences were observed in the pattern of sequestration among the two groups (Figure 24).

7.2.3. Haematology

Comparisons of the haematological parameters such as anaemia and reticulocytaemia between the iNOS depleted mice and controls infected with malaria, were made in the mice from the second experiment.

In order to assess the degree of anaemia, samples of blood were taken from the tail and the haematocrit measured. Two mice were bled from each group before malaria infection and on days 6, 11, 19, 23, 28, 33 and 41 p.i.

The basal haematocrit of iNOS depleted mice was not different from that of controls. Peak parasitaemia occurred at day 10 p.i. and was higher in the iNOS depleted mice . In addition, their haematocrit was slightly lower when compared with controls. This

pattern persisted until day 23 p.i., after which both groups had recovered from anaemia and their haematocrit returned to pre-infection levels (Figure 25).

Thin smears were made from two mice at days 0, 6, 11, 15 and 19 p.i., and they were stained to evaluate the proportion of reticulocytes (see 2.8.2.). Both groups showed an increase of reticulocytaemia simultaneously with peak infection. This peak of reticulocytes in circulation was higher in iNOS depleted mice (Figure 25.).

7.2.4. Antibody production

In the second experiment using iNOS depleted mice (See 7.2.1.) humoral responses were studied by assessing total specific IgG production during *P. chabaudi* infection. Samples of serum were taken from the same two mice before malaria infection and 6, 11, 15, 19 days p.i..

The mice had a peak of infection at day 10 p.i. and this was higher in the iNOS depleted mice (see 7.2.1., second experiment). IgG was detected from day 11 in all groups with no significant differences among them. Maximum IgG titres were reached at day 15 p.i. in iNOS mice and these were not different from those observed in control mice.

7.2.5. Serum levels of nitric oxide

Serum nitrate levels were evaluated as a measure of NO levels. Nitrogen oxidation of L-arginine results in the release of the highly reactive intermediate NO, which has a half-life of milliseconds before udergoing further oxidisation to nitrite (NO_2^-) and nitrate (NO_3^-). Both NO_2^- and NO_3^- are more stable forms and can be detected in serum as a result of NO production (reviewed by Green *et al.*, 1990). Samples from at least two mice were taken before malaria infection and from day 5 to 12 p.i.

iNOS depleted mice showed background levels of NO during the period of evaluation. On the other hand control mice showed an increase of NO levels at day 9 and 10 p.i. (Figure 26). These findings were confirmed in two separate experiments.

7.2.6. Proliferative response of spleen cells

Spleen cells from three *P. chabaudi* infected iNOS depleted mice were taken at different time points during infection. They were incubated with fresh *P. chabaudi* antigen (see 2.18) or ConA and two controls were included: non-infected RBC and medium. After 72 hours of incubation, the cells were pulsed for 18 hours, then harvested and incorporation of [^{3}H-methyl] thymidine was measured.

In all the assays performed, spleen cells failed to proliferate with the fresh *P. chabaudi* antigen. However, proliferation was observed in response to ConA (Figure 27). Spleen cells from iNOS depleted mice obtained at day 0 of malaria infection proliferate in response to ConA to a similar degree as controls. However, six days after infection, cells from the experimental group had a higher mean rate of proliferation than cells from control mice. A marked reduction of proliferation in response to ConA was observed at day 10 p.i. in coincidence with peak parasitaemia in both groups. By day 14 p.i. spleen cells from both experimental and control mice recovered the ability to proliferate when stimulated with ConA and no differences were observed in the proliferative rate between the cells from groups. Similarly, at day 18 p.i. cells from experimental and control mice showed similar rates of proliferation in response to Con A (Figure 27).

7.2.7. IL-4 and IFNγ production by spleen cells

Supernatants of ConA stimulated spleen cells from two malaria infected mice from each, were collected 24, 48 and 72 hours after incubation.

At day 6 p.i., IFNγ production by cells from iNOS depleted mice was higher (381 μg/ml) in comparison with the two controls (70.8 μg/ml and 113 μg/ml). In both groups, cells taken from mice after 10 days of infection, did not show production of the cytokine. However, at day 14 p.i. cells from experimental mice produced higher levels of IFNγ (582 μg/ml & 476 μg/ml) than cells from control mice (143 μg/ml & 71 μg/ml). By day 18 p.i. IFNγ production was similar in both groups (Figure 28.a.).

IL-4 was detected at day 18 p.i. in both groups in supernatants collected 24 hours after incubation (Figure 28.b.). The level of IL-4 production in the iNOS depleted mice and in the controls was similar (between 13 and 17 μg/ml).

	iNOS deficient n=11	**Controls n=12**
Day post-infection	**% mean parasitaemia (S.D.)**	
3	0,02 (0,02)	0,01 (0,03)
4	0,06 (0,5)	0,18 (0,39)
5	0,43 (0,19)	0,42 (0,49)
6	1,21 (0,42)	1,65 (1,72)
7	6,14 (2,84)	3,77 (2,58)
8	18,16 (6,12)	9,79 (3,92)
9	30,15 (10,44)	15,82 (5,8)
10	31,68 (8,98)	17,85 (12,24)
11	11,86 (6,87)	3,37 (2,85)
12	1,32 (1,51)	0,24 (0,19)
13	0,15 (0,18)	0,1 (0,2)
14	0,002 (0,007)	0,06 (0,08)
15	0	0,02 (0,07)
16	0	0

Table 11. Mean value (%) and standard error of parasitaemias in iNOS depleted mice and control mice.

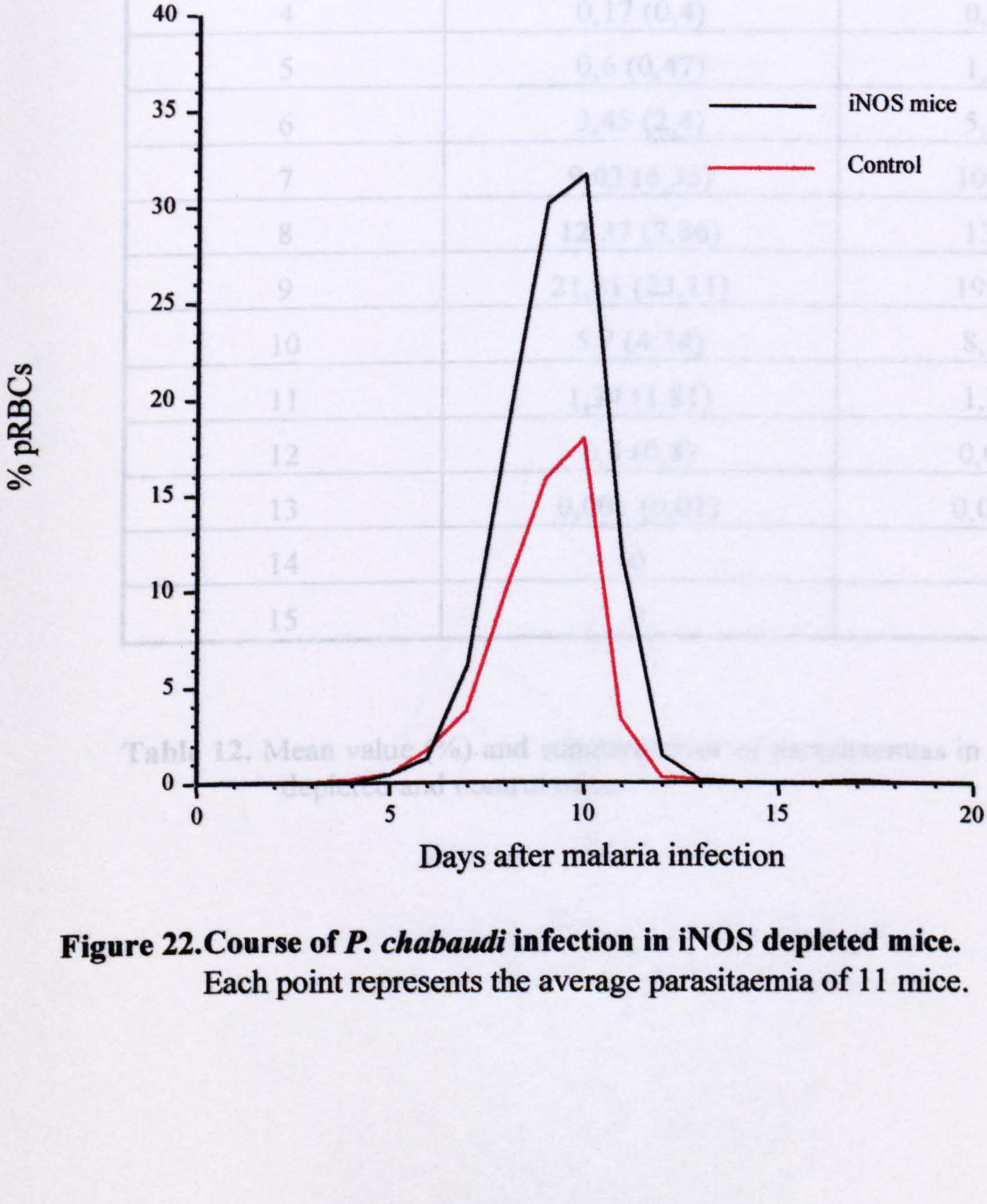


Figure 22.Course of *P. chabaudi* infection in iNOS depleted mice.
Each point represents the average parasitaemia of 11 mice.

	iNOS deficient n=6	Controls n=6
Day post-infection	**% mean parasitaemia (S.D.)**	
3	0,04 (0,03)	0,12 (0,23)
4	0,17 (0,4)	0,35 (0,19)
5	0,6 (0,47)	1,39 (0,97)
6	3,45 (2,4)	5,49 (3,37)
7	9,03 (6,35)	10,26 (5,73)
8	12,37 (7,86)	13,6 (9,36)
9	21,81 (23,11)	19,18 (13,6)
10	5,7 (4,34)	8,77 (6,97)
11	1,34 (1,81)	1,37 (1,69)
12	0,4 (0,8)	0,02 (0,075
13	0,001 (0,01)	0,001 (0,01)
14	0	0
15	0	0

Table 12. Mean value (%) and standard error of parasitaemias in iNOS depleted and control mice.

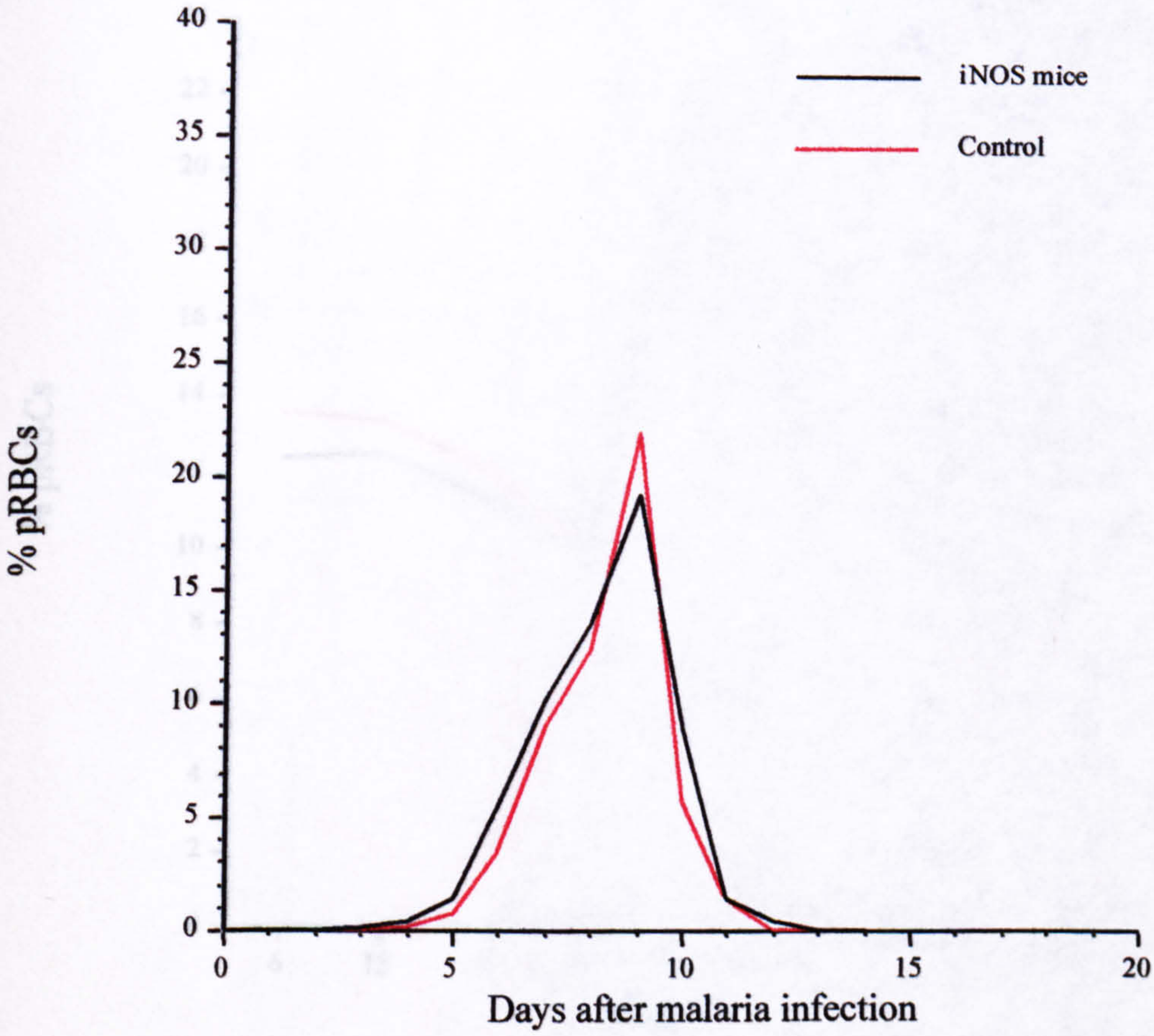


Figure 23.Course of *P. chabaudi* infection in iNOS depleted mice.
Each point represents the average parasitaemia of 6 mice.

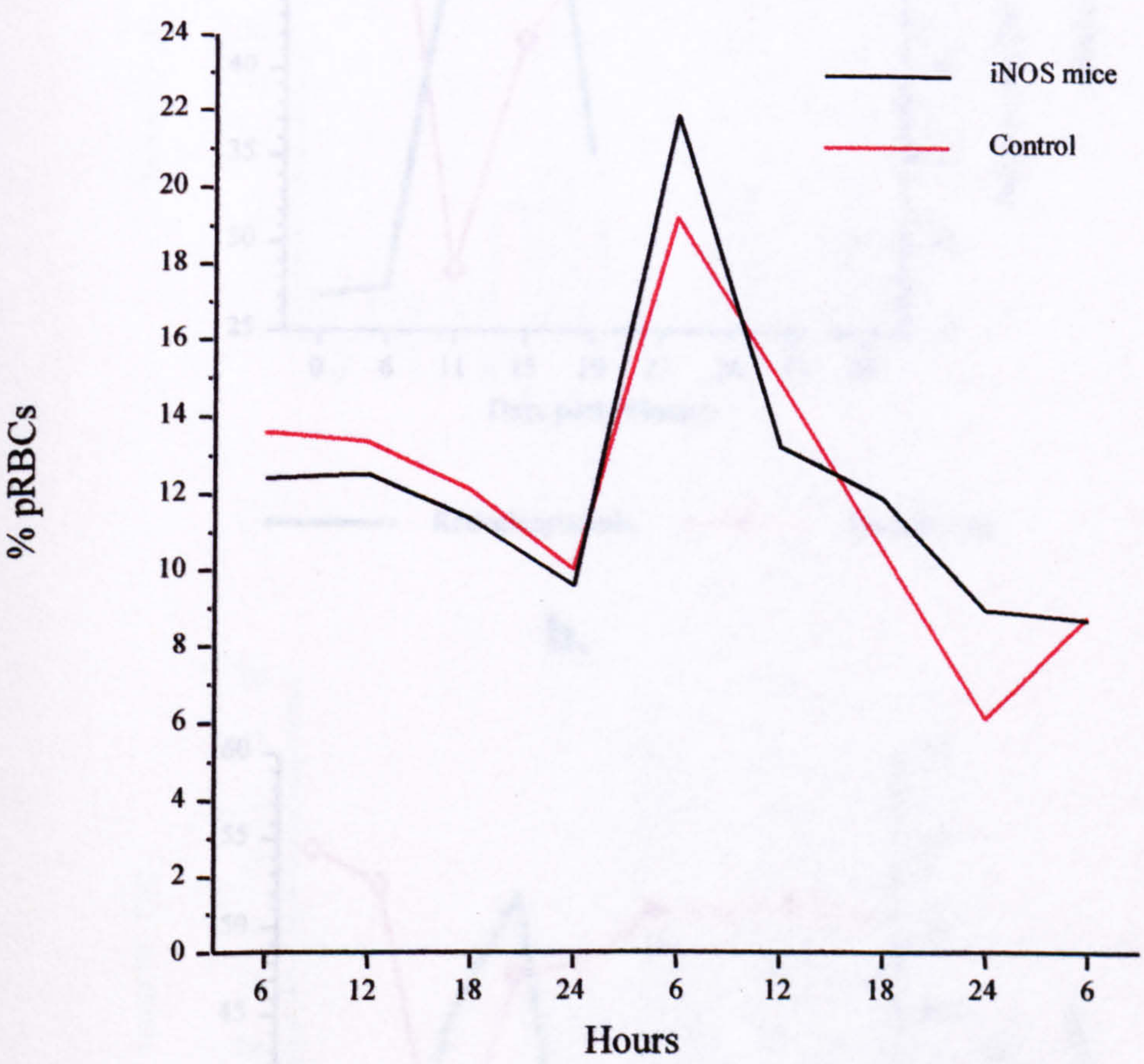
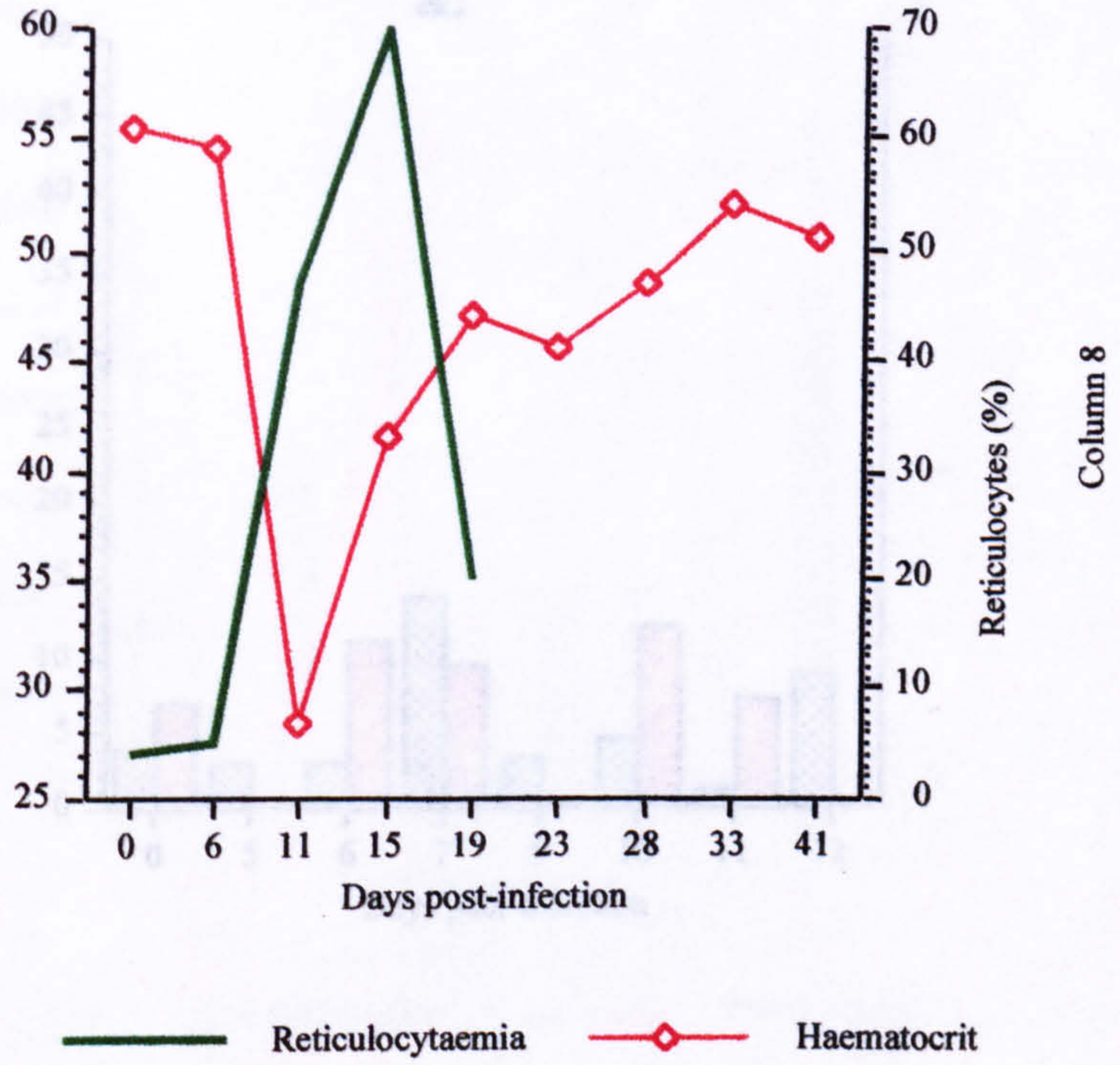


Figure 24.*P. chabaudi* sequestration between day 8 p.i. And 10 p.i. In iNOS depleted mice. Each point represents the average parasitaemia of 6 mice.

a.



b.

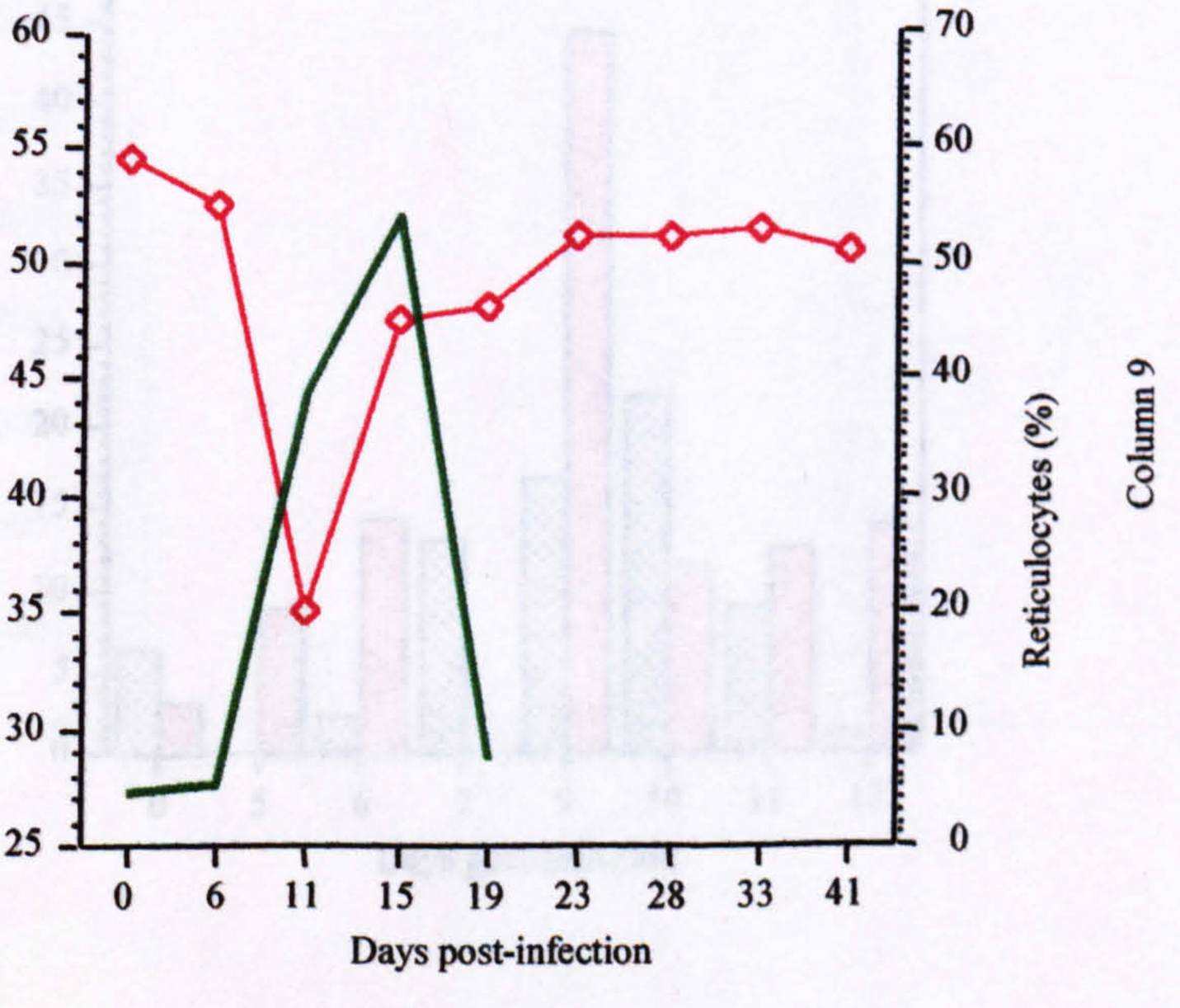


Figure 25. Haematocrit and retyculocitaemia of *P. chabaudi* infected iNOS depleted mice (a) and controls (b). Each point represents the average parasitaemia of 2 mice.

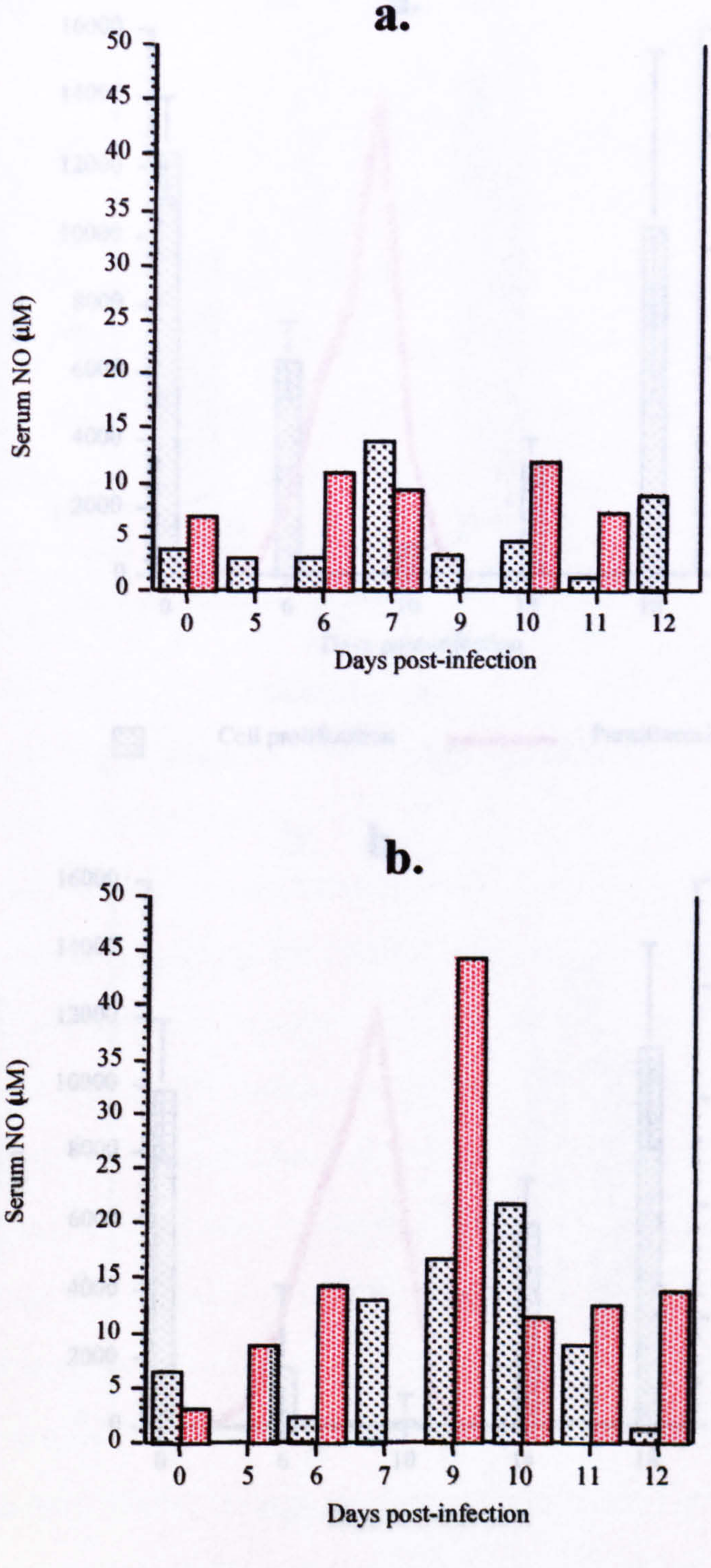


Figure 26.Serum nitric oxide production of *P. chabaudi* infected iNOS depleted mice (a) and controls (b). Each bar represents one individual. A peak of parasitaemia was observed at day 10 p.i.

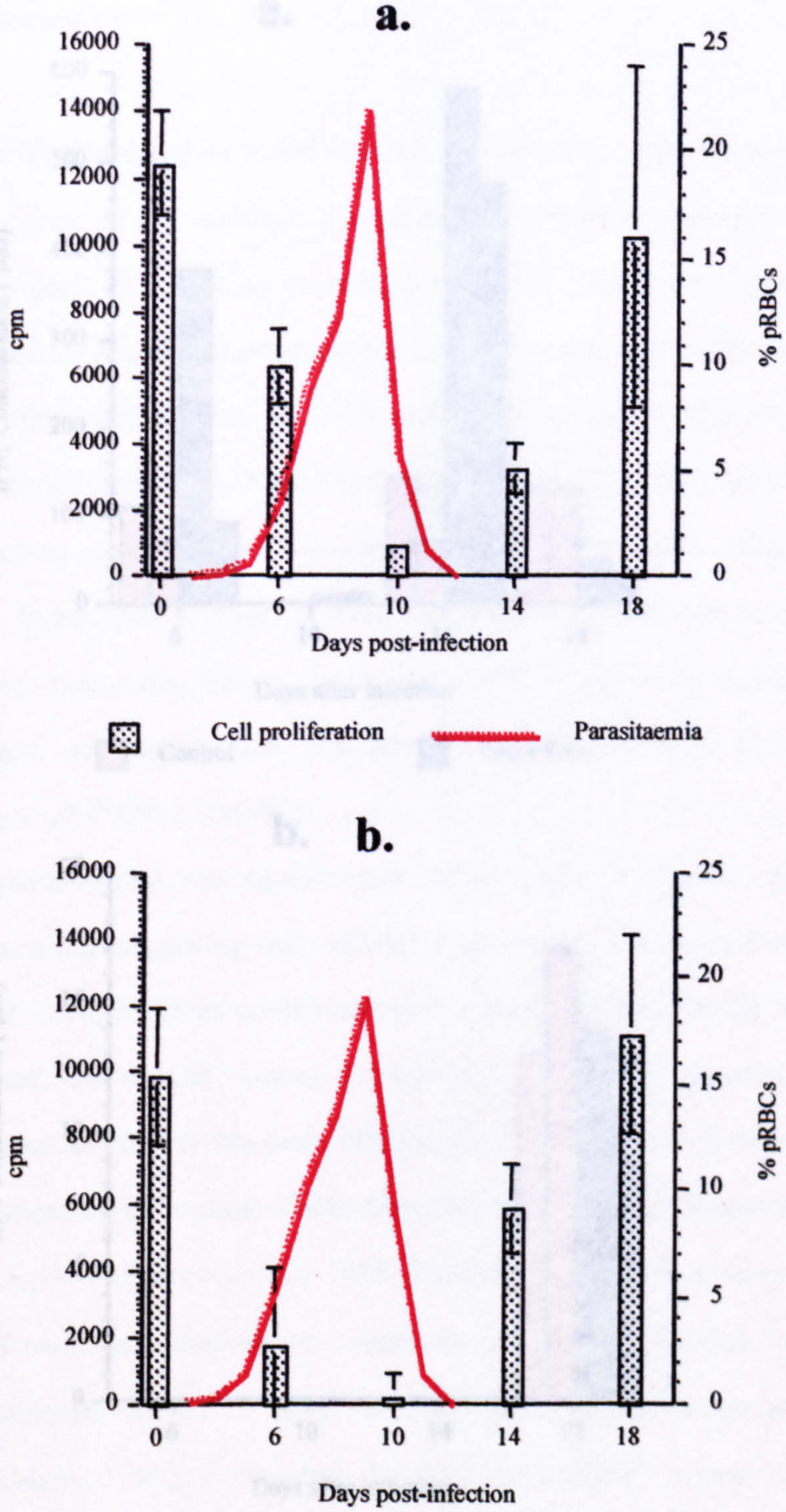


Figure 27.*In vitro* proliferation of spleen cells of *P. chabaudi* infected iNOS depleted mice (a) and controls (b). Cells were stimulated with ConA. Each bar represents the average of two mice.

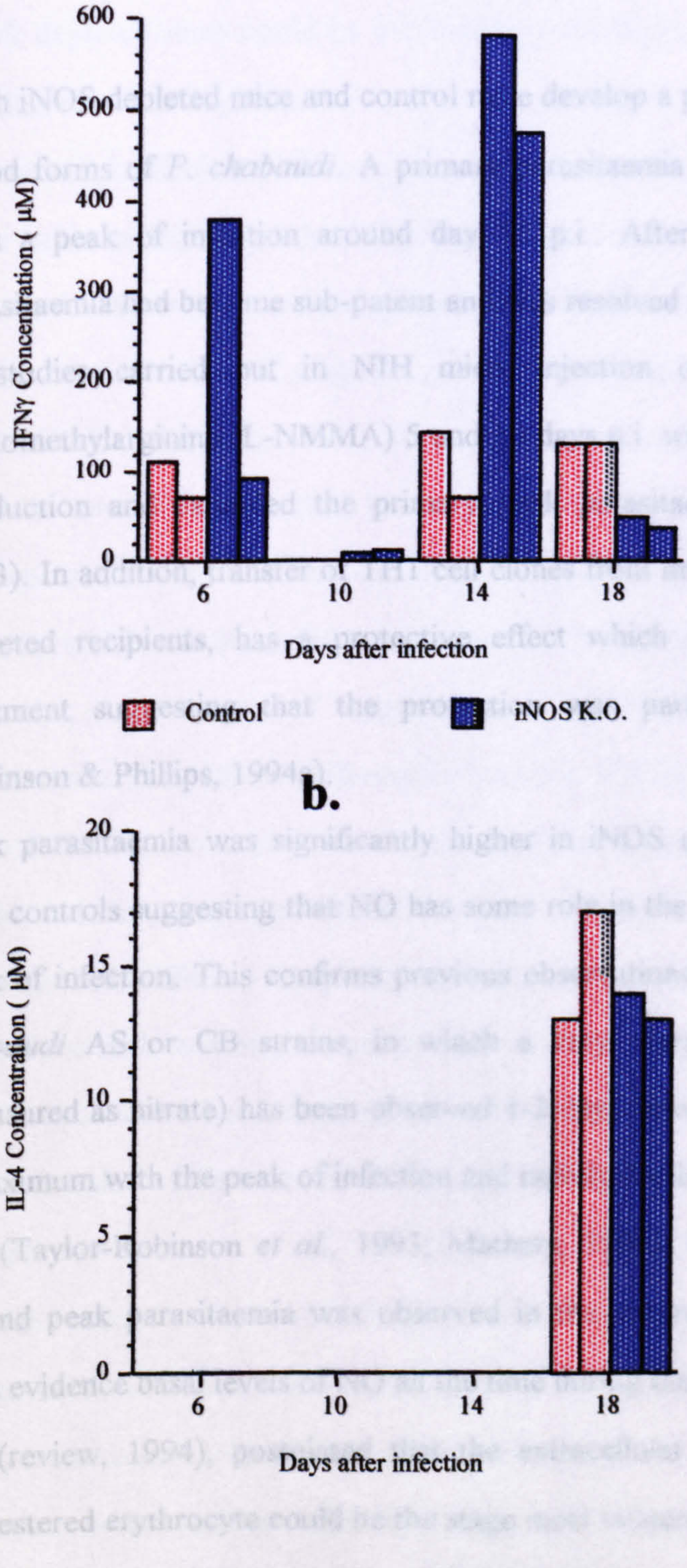


Figure 28.IFNγ (a) and IL-4 (b) production by spleen cells stimulated with ConA of iNOS depleted mice and controls infected with *P. chabaudi*. Each bar represents one individual. A peak parasitaemia was observed at day 9 p.i.

7.3. Discussion

Both iNOS depleted mice and control mice develop a parasitaemia after injection with blood forms of *P. chabaudi*. A primary parasitaemia was observed from day 3 p.i. with a peak of infection around day 10 p.i.. After two weeks of infection, the parasitaemia had become sub-patent and was resolved from around 4 weeks p.i..

In studies carried out in NIH mice, injection of the NOS inhibitor L-Nγ-monomethylarginine (L-NMMA) 5 and 10 days p.i. with *P. chabaudi*, prevented NO production and extended the primary peak parasitaemia (Taylor-Robinson *et al.*, 1993). In addition, transfer of TH1 cell clones from immune donors into $CD4^+$T cell-depleted recipients, has a protective effect which was reduced after L-NMMA treatment suggesting that the protection was partially NO-dependent (Taylor-Robinson & Phillips, 1994a).

Peak parasitaemia was significantly higher in iNOS depleted mice when compared with controls suggesting that NO has some role in the control of the parasite around peak of infection. This confirms previous observations in NIH mice infected with *P. chabaudi* AS or CB strains, in which a very sharp increase in NO production (measured as nitrate) has been observed 1-2 days before peak parasitaemia, reaching a maximum with the peak of infection and rapidly declining to background levels after this (Taylor-Robinson *et al.*, 1993; Mathers, 1994). This same pattern of NO rise around peak parasitaemia was observed in the control mice, while iNOS depleted mice evidence basal levels of NO all the time during the course of infection. Phillips *et al.*, (review, 1994), postulated that the extracellular merozoite released from the sequestered erythrocyte could be the stage most vulnerable to NO. Since *P. chabaudi* sequesters in the liver where NO would be expected to be produced by Kupffer cells and hepatocytes, sequestered parasites may be exposed to relatively high local concentrations of NO. This would not be the case in iNOS depleted mice, and, therefore, an impairment of the control of the parasite can be expected.

A lower haematocrit and higher proportion of reticulocytes in the peripheral blood of iNOS depleted mice could be explained by the higher parasitaemia they suffered in comparison with controls.

Vasodilatory effects of NO may result in changes in the pattern of sequestration in mice with impaired production of the molecule. Cytosolic guanylate cyclase is activated when nitric oxide displaces the iron from the porphyrin ring plane. This results in generation of a cyclic guanosine monophosphate (reviewed by Ignarro, 1992; Clark & Rockett, 1996). Through this mechanism NO is a major controller of vascular tone. Therefore, NO produced around the peak of malaria infection would be expected to produce vasodilatation of deep tissue capillaries and post-capillary venules which would make conditions for parasite sequestration less favourable. However, assessment of sequestration by following changes in peripheral parasitaemia showed no differences between the experimental mice and controls. Several factors can explain this result. First, the changes in the parasitaemia may be subtle and the assay used is not sensitive enough to detect them. Second, several other factors, different from NO, are known to be involved in sequestration and the role of the molecule might only be accessory. Third, a vasodilatory effect secondary to NO production has been demonstrated in the brain and has been associated with cerebral malaria (Clark *et al.*, 1991), but a generalised vasodilatory effect is not yet proven.

Six days after *P. chabaudi* infection, iNOS depleted mice exhibited a significantly higher level of proliferation of their spleen cells in comparison with controls and under stimulation with ConA. This confirms previous observations in *L. major* infection, where spleen cells from infected iNOS mice had a higher index of stimulation when cultured with specific and non-specific antigens (Wei *et al.*, 1995). During the peak of malaria infection, spleen cells from iNOS depleted mice and from the control group showed suppression of *in vitro* proliferation . Immuno-suppression during acute malaria infection has been documented by several authors. A failure of lymphocyte proliferation in response to asexual blood stage malarial antigens has been

associated with acute *P. falciparum* malaria (Ballet *et al.*, 1981; Brasseur *et al.*, 1983; Troye-Blomberg *et al.*, 1983). Evaluation of spleen cells supernatants for *in vitro* production of IFNγ showed that production by cells from iNOS depleted mice was significantly increased in response to ConA stimulation at days 6 and 14 p.i.. Cells from both groups of mice recovered on day 10 p.i. did not produce IFNγ. By day 18 p.i., IFNγ production was significantly reduced in the iNOS depleted mice and was not significantly different from that of the controls. High production of IFNγ by spleen cells of iNOS depleted mice has also been reported in *L.major* infection in response to leishmanial antigen (Wei *et al.*, 1995). In the *Leishmania* model, IL-4 production has been found to be reduced when compared with controls. However, when infected with *P. chabaudi*, production of IL-4 was only detected at day 18 p.i. in both experimental and control groups. This confirms previous observations suggesting that a sequential activation of TH1 and TH2 cells subsets are involved in the control of *P. chabaudi* infection (reviewed by Phillips *et al.*, 1994). Taylor-Robinson *et al.*, (1994), demonstrated that expansion of malarial-specific TH1 clones, but not TH2, were inhibited by NO, and suggested that the molecule may down regulate TH1 cells and precede the development of a TH2 specific response. This has been confirmed by the experiments presented in this chapter, in which in iNOS depleted mice infected with *P. chabaudi*, the absence of NO resulted in higher peak parasitaemia and was associated with high *in vitro* production of IFNγ but did not affect IL-4 production.

The results presented in this chapter confirm that mice deficient in iNOS provide an important tool which could help to understand the involvement of NO in immunity against *P. chabaudi* malaria. However, the mechanisms mediated by NO which contribute to parasite killing remain unclear. Further work with this model of *P. chabaudi* AS infection should include study of *in vitro* proliferative responses against *P. chabaudi* antigen, as well as *in vitro* cytokine production at different stages of infection, since specific responses against malarial antigens may differ from those observed in response to ConA. Expression of TH1 and TH2 cytokines and NO

should be assessed in samples from liver and spleen and compared with levels of the molecules in the circulation. Finally, changes in sequestration in this model of infection should be further explored and histological samples, in addition to blood smears, should illustrate them better.

CHAPTER EIGHT

GENERAL DISCUSSION

8. General Discussion

Resistance to blood stages of *P. chabaudi chabaudi* AS is largely $CD4^+$ T lymphocyte dependent. Both TH1 and TH2 cell subsets produce soluble mediators that have been associated with protection. The aim of the work reported in this thesis was to contribute to the understanding of the role of these T cells in the acquisition of immunity against the murine infection with asexual blood stages of *P. c. chabaudi* AS. To this end the murine cytokines IFNγ, TNFα, TGFβ and IL-4 were delivered *in vivo* using a *S. typhimurium* expression system and their effects on the malaria infection were studied. The contribution of NO in the development of protective immunity against malaria was further assessed by studying the course of the infection in iNOS deficient mice.

The level of resistance to blood-stage infection with *P. chabaudi* varies among inbred strains of mice and is dependent on the strain of the parasite. Host protective mechanisms appear to be different in resistant and susceptible hosts. Resistant NIH mice infected with *P. chabaudi* AS strain experience a primary patent parasitaemia and one or two recrudescences before the infection is eliminated. TH1 cells seem to be dominant in the control of the primary parasitaemia, while mainly TH2 appear to mediate the control of recrudescences and challenge infections (Von Der Weid & Langhorne, 1993a; Taylor-Robinson & Phillips, 1994). In contrast, susceptible A/J had an enhanced TH2 type response early during *P. chabaudi* malaria infection, which led to severe malaria disease and death (Stevenson& Gadhirian , 1989; Jacobs *et al.*, 1996a & b).

The immune effector mechanisms which may contribute to the better control of malaria infection in resistant mice start with the rising parasitaemia (Figure 29). Initially, antigens are taken up by monocytes/macrophages and are processed and presented to T cells. An equally important role of macrophages during early stages of malaria infection is to produce IL-12. This cytokine has pleiotropic effects on both NK and T cells (Kobayashi *et al.*, 1989). IL-12 induces IFNγ and TNFα

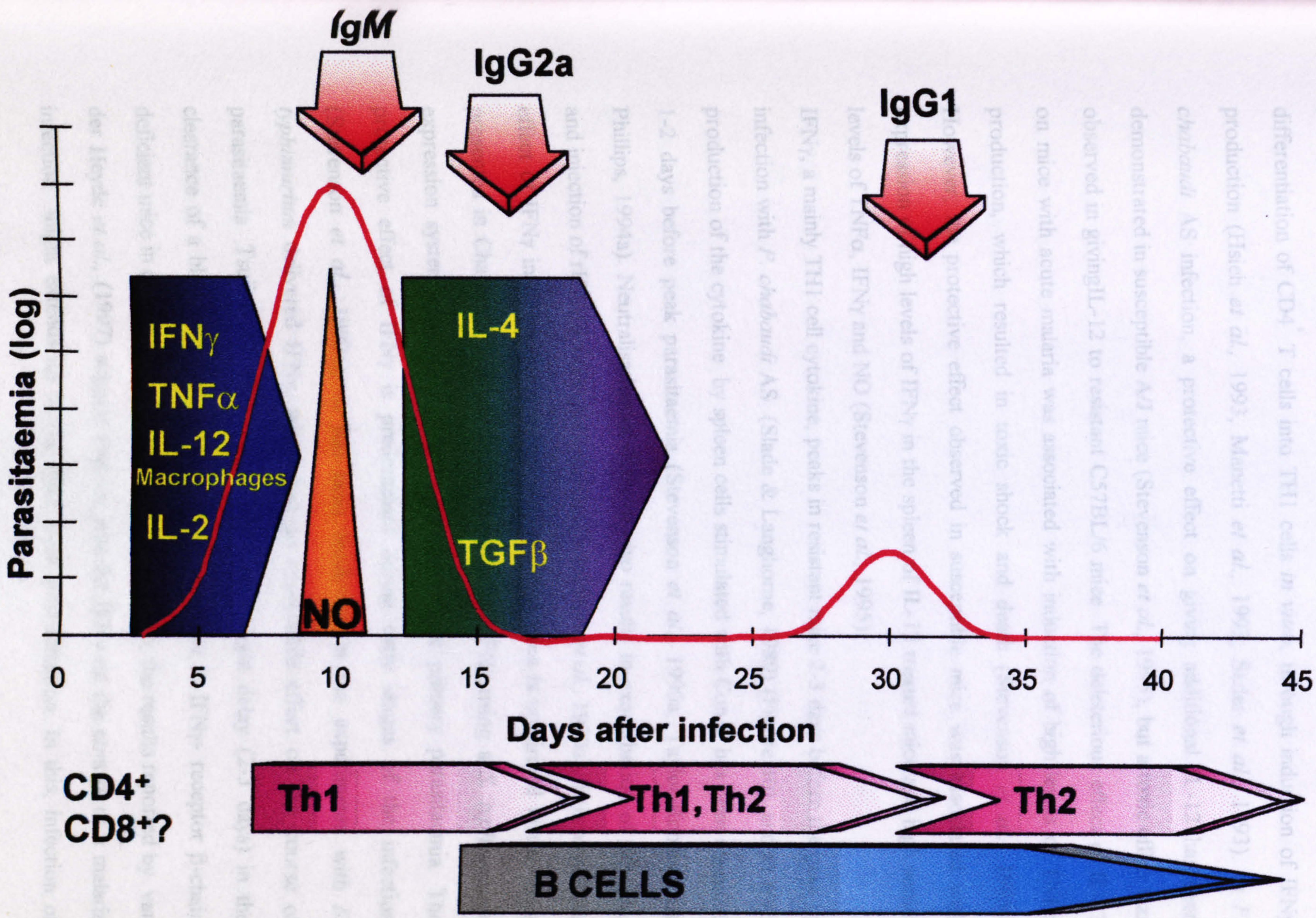


Figure 29.Immune response in NIH mice during *P. chabaudi* infection.

production by these cells, enhances their cytotoxic activity and stimulates their proliferation (when in the presence of IL-2). Additionally, IL-12 promotes the differentiation of $CD4^+$ T cells into TH1 cells *in vitro*, through induction of IFNγ production (Hsieh *et al.*, 1993, Manetti *et al.*, 1993; Seder *et al.*, 1993). In *P. chabaudi* AS infection, a protective effect on giving additional IL-12 has been demonstrated in susceptible A/J mice (Stevenson *et al.*, 1995), but a toxic effect was observed in givingIL-12 to resistant C57BL/6 mice. The deleterious effect of IL-12 on mice with acute malaria was associated with induction of high levels of TNFα production, which resulted in toxic shock and death (Stevenson *et al.*, 1995). However, the protective effect observed in susceptible mice was associated with expression of high levels of IFNγ in the spleen of IL-12 treated mice and high serum levels of TNFα, IFNγ and NO (Stevenson *et al.*, 1995).

IFNγ, a mainly TH1 cell cytokine, peaks in resistant mice 2-3 days before the peak of infection with *P. chabaudi* AS (Slade & Langhorne, 1989) (Figure 30), and *in vitro* production of the cytokine by spleen cells stimulated with ConA, has been observed 1-2 days before peak parasitaemia (Stevenson *et al.*, 1990a, Taylor-Robinson & Phillips, 1994a). Neutralisation of IFNγ *in vivo* results in exacerbation of infection and injection of rIFNγ depress parasitaemia (Stevenson *et al.*, 1990a). This protective effect of IFNγ in *P. chabaudi* infection in resistant mice is confirmed in the work presented in Chapter 3, in which administration of IFNγ using a *S. typhimurium* expression system resulted in a better control of the primary parasitaemia. The protective effect of IFNγ is predominant during early stages of the infection (Stevenson *et al.*, 1990a), as has been confirmed in the experiments with *S. typhimurium* delivered IFNγ, which had an appreciable effect on the course of parasitaemia. Tsuji *et al.* (1995) have reported a slight delay (2-3 days) in the clearance of a blood infection with *P. chabaudi adami* in IFNγ- receptor β-chain deficient mice in comparison with controls. Furthermore, the results reported by van der Heyde *et al.*, (1997) suggest that the role for IFNγ on the control of a malaria infection might extends up to the third week post-infection. In this, infection of

IFNγ-deficient mice with blood forms of *P. chabaudi adami*, resulted in a higher peak parasitaemia and prolonged infection when compared with controls.

TH1 cells and IFNγ activated macrophages produce reactive nitrogen intermediates, of which NO has been implicated in the control of infectious agents (James & Hibbs, 1990). In malaria, NO may be effective in controlling blood forms of *P. chabaudi* AS and *P. chabaudi* CB infection in NIH mice. A very sharp increase of NO production which rises from 1-2 days before the peak of infection, reaches a maximum simultaneously with peak parasitaemia and decreases rapidly afterwards (Taylor-Robinson *et al.*, 1993; Mathers, 1994). Moreover, inhibition of NO synthase by L-NMMA has resulted in higher and extended primary parasitaemias compared with controls (Taylor-Robinson *et al.*, 1993). The way NO contributes to the control of blood forms of malaria is not completely understood. A direct cytotoxic effect of NO has been observed on asexual erythrocytic stages of *P.falciparum* treated with NO derivatives *in vitro* (Rockett *et al.*, 1991). In addition, a cytostatic effect on *P.falciparum* blood forms has been observed *in vitro* when cultures were treated with SNAP (an NO producer) in order to reach physiological concentrations of NO (Balmer *et al.*, 1995). Mice inoculated with the *S. typhimurium*/IFNγ construct controlled a *P. chabaudi* AS infection better than controls, although no significant increase in NO production could be detected in their serum during the primary parasitaemia. Therefore it can be speculated that IFNγ is responsible for the better control of the infection through mediation of NO independent mechanisms (see Figure 30). Alternatively NO production during malaria infection may depend on some threshold levels of antigen loading. This has been also suggested in an NIH- *P. chabaudi* CB model of infection, in which treatment with chloroquine 1-2 days before peak parasitaemia resulted in lower parasitaemias and basal serum NO during the infection (unless chloroquine itself inhibits NO production) (Phillips *et al.*, 1994). Therefore increased NO production during acute malaria infection may have a protective role, but might not be necessary if the parasitaemia is not high enough to compromise the survival of the host. Such a protective action of NO has been

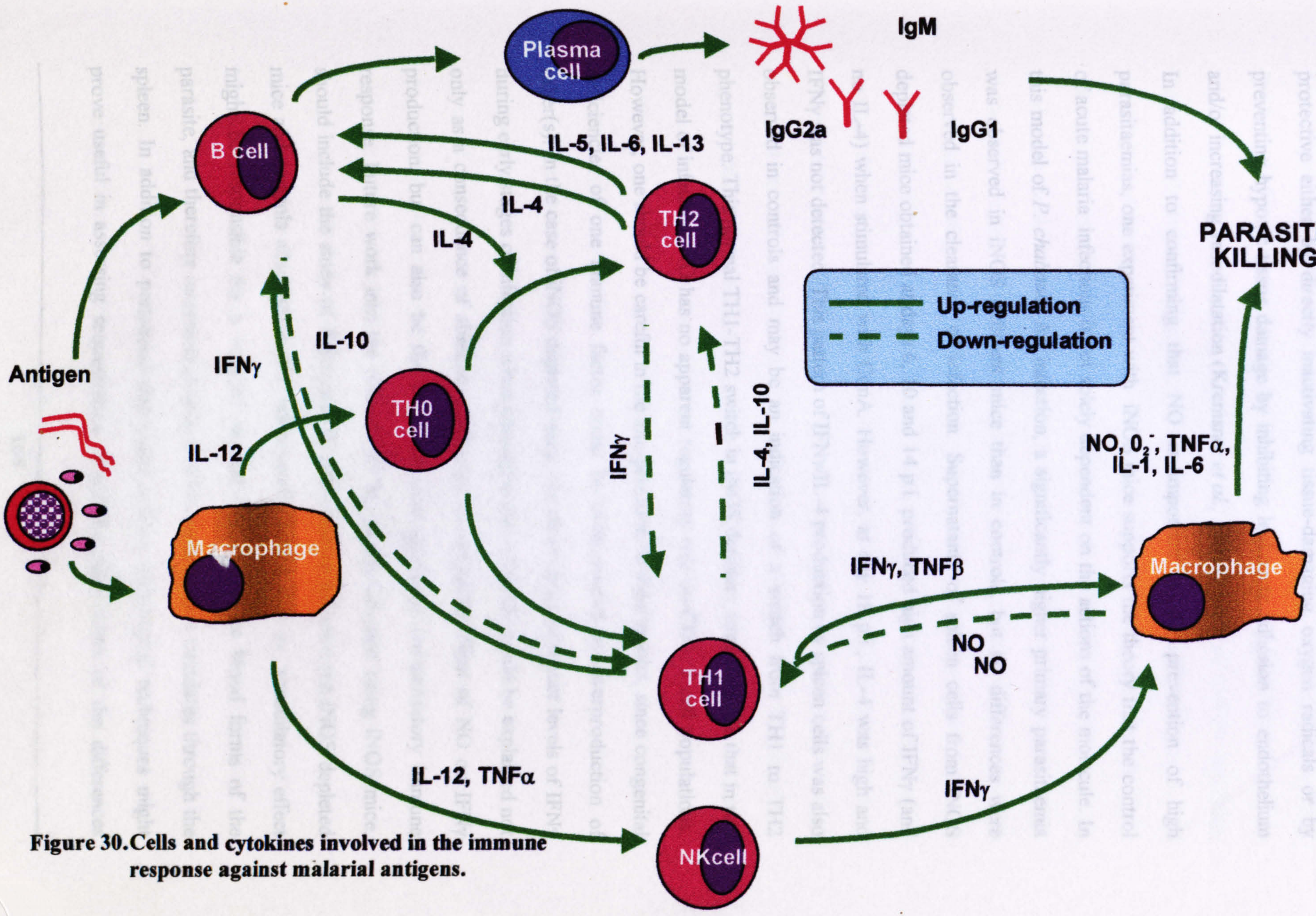


Figure 30. Cells and cytokines involved in the immune response against malarial antigens.

demonstrated in murine infection with *P. vinckei,* in which the molecule was protective either by directly inactivating tissue-damaging oxygen radicals or by preventing hypoxic tissue damage by inhibiting leukocyte adhesion to endothelium and/or increasing vasodilatation (Kremsner *et al.,* 1993).

In addition to confirming that NO is important in the prevention of high parasitaemias, one experiment with iNOS mice supports the theory that the control of acute malaria infections is not solely dependent on the actions of the molecule. In this model of *P. chabaudi* AS infection, a significantly higher primary parasitaemia was observed in iNOS deficient mice than in controls, but no differences were observed in the clearance of infection. Supernatants of spleen cells from iNOS depleted mice obtained at days 6, 10 and 14 p.i, produced high amount of IFNγ (and no IL-4) when stimulated with ConA. However, at day 18 p.i., IL-4 was high and IFNγ was not detected. This pattern of IFNγ/IL-4 production by spleen cells was also observed in controls and may be an indication of a switch from TH1 to TH2 phenotype. This normal TH1-TH2 switch in iNOS deficient mice suggests that in this model of infection, NO has no apparent regulatory role on $CD4^+$ T cell populations. However, one should be careful in the interpretation of these results, since congenital deficiencies of one immune factor could be compensated by overproduction of other(s). In the case of iNOS depleted mice, the observation of higher levels of IFNγ during early stages of infection when compared with controls, could be explained not only as a consequence of absence of the down-regulatory effect of NO on IFNγ production, but can also be the result of an exaggerated compensatory immune response. Future work into the role of NO in malaria infection using iNOS mice, should include the study of differences in sequestration between the iNOS depleted mice and controls which produced NO normally. NO due to its vasodilatory effect might be responsible for a reduced sequestering of mature blood forms of the parasite, and therefore increased clearance of the parasite as it circulates through the spleen. In addition to peripheral thin blood smears, histological techniques might prove useful in assessing sequestration. Since the significance of the differences

observed in the course of parasitaemia are affected by the number of mice evaluated (see chapter 7), all studies should be performed in groups of more than six mice. Evaluation of proliferative responses of spleen cells stimulated with malarial antigens, which was tried unsuccessfully, would be a better parameter of specific immunity, therefore a *P. chabaudi* antigen should be standardised to be used in this assays. This would also aid in elucidating if any difference in cytokine production exists between the malaria infected iNOS depleted mice and controls. In addition, *in vitro* cytokine production should be correlated with serum levels of cytokines during the infection. Evaluation of cytokine expression *in situ* in the liver and the spleen might contribute to the understanding of the phenomena responsible for the better control of infection in NO producing mice, as it would provide a better picture of in cytokine production between iNOS depleted mice and controls. For this, samples of these organs taken at different time points of the malaria infection are stored in N_2 (-196_C) have been preserved and training in PCR techniques has started. Finally, other models of NO depleted mice have been reported and it would be interesting to compare the course of *P. chabaudi* infection observed in them with the one reported here.

TNFα is an important cytokine produced by macrophages during infection. Its important role in mediation of immunity against malaria has been illustrated in experiments where susceptible A/J mice were treated with rTNFα. They survived *P. chabaudi* AS infection and had a significant decrease in the peak parasitaemia when compared with untreated mice, which died after developing high parasitaemias (Stevenson & Ghadirian, 1989). In the same study, treatment of C57BL/6 resistant mice with rTNFα had no effect on the course of the parasitaemia. A/J mice given the *S. typhimurium*/TNFα construct had a significantly decreased patent parasitaemia, while in resistant NIH mice this treatment had no effect on the course of infection. The first conclusion that can be drawn from these results is the confirmation that the TNFα construct is expressed and active *in vivo* (McSorley, personal communication) as it has a significant effect on reducing the peak of parasitaemia in A/J mice. Secondly, the differences in the role of TNFα among the different strains of

mice observed by other researchers, can be reproduced using this delivery system. Jacobs *et al.*(1995) have attributed the differences in the response to TNFα treatment between the different strains of mice, to early changes in the pattern of the cytokine production during acute *P. chabaudi* AS infection. Mice with high levels of TNFα mRNA expression in the liver and increased levels of serum TNFα during late stages of infection showed susceptibility to malaria. On the other hand, resistance to infection has been observed in mice with high TNFα mRNA expression in the spleen during early stages of infection. Administration of TNFα using a *S. typhimurium* delivery system might result in an early increase of TNFα production in the spleen of A/J mice compared with control mice, thereby increasing resistance to infection. Since TNFα might already been produced in the spleen of NIH during early stages of the infection, administration of the construct did not change the pattern of production of the cytokine and therefore had no effect in the course of infection. Moreover, it could be expected that excessive production of TNFα might result in toxic shock (and possible death). This was the case in the experiments using rTNFα at very high doses and reported above. However, mice inoculated with the TNFα construct showed no evidence of more severe illness nor did they die. A possible explanation, is that the increased systemic levels of TNFα which are associated with severe disease, were not achieved after inoculation with the construct. Previously, Ianaro *et al.* (1995) have demonstrated that in a similar cytokine delivery system (*S. typhimurium*/TGFβ), oral administration of a *S. typhimurium* construct did not result in an increase of serum basal levels of the cytokine.

TNFα production has been inversely correlated with TGFβ levels in human malaria. *P. falciparum* infected individuals who have high TNFα serum levels, showed a decreased TGFβ production which can be restored after antimalarial treatment (Wenisch *et al.*, 1995). That study it was suggested that TGFβ might have a protective effect against malarial disease through down regulation of TNFα. However TGFβ production can have a immunosupressive effect due to down regulation of macrophage activation and inhibition of production of oxygen free

radicals (Tsunawaki *et al.*, 1988) and NO (Ding *et al.*, 1990; Nelson *et al.*, 1991). Knowledge of the role TGFβ in the acquisition of immunity against malaria remained limited. The experiments reported in Chapter 5 are preliminary and indicate that administration of TGFβ using a *S. typhimurium* as a delivery vehicle has no effect in the course of parasitaemia in both susceptible A/J or resistant NIH mice. Nevertheless, the importance of TGFβ in down regulation of TH1-like immune responses (Ianaro *et al.*, 1995) should be further explored and the *S. typhimurium* might be an useful instrument. Since it constitutes an affordable and easy way to deliver TGFβ *in vivo* in malaria infected mice.

As mentioned above, during a *P. chabaudi* AS infection in NIH mice, a sequential activation of TH1 and TH2 cells appear to be involved in the control of the infection. As the primary parasitaemia goes into remission, a dominant TH2 response is initiated. In susceptible A/J mice however, high levels of TH2 cytokines (IL-4, IL-5 and IL-10) are produced by spleen cells of mice during the first week of infection (Stevenson & Tam, 1993). These latter results suggest that an early TH2 cell activation might lead to severe disease and death. In the experiments where resistant NIH mice were given the *S. typhimurium*/IL4 construct, no differences were observed in the course of infection and the mice did not become more susceptible to the parasite. Thus, IL-4 administered in this way might not have any effect on a strong TH1 type response. However, as occurred with the *S. typhimurium*/TGFβ construct, the experiments with the IL-4 construct were very limited and preliminary and further experiments evaluating its effect on the immune response against *P. chabaudi* AS should be performed. An important parameter which must be evaluated in the future when working with this IL-4 construct is its effect on B cells and antibody production during malaria infection.

During the experiments carried out with the *S. typhimurium* mutants, it was observed that the control mutant provided some degree of protection against the malaria infection. Although inconsistent, this protection was in most cases evident as a lower peak parasitaemia in comparison with *P. chabaudi* controls. *S. typhimurium* BDR509

has been shown to induce TH1 and TH2-type cytokines in the gut associated lymphoid tissue (GALT), after oral administration (Karem *et al.*, 1996). Moreover, TH1-type responses are seen in organs such as spleen, following oral immunisation with *S. typhimurium* mutants (Klimpel *et al.*, 1995). These results suggest that the potential for a TH1 or TH2 polarised immune response after oral administration of the bacteria does exist, and that upon stimulation with *P. chabaudi* antigens an enhanced TH1 may be dominant and help with the control of the infection during early stages.

The results presented in this thesis showed that *S. typhimurium* mutants expressing cytokine genes are useful in modulating the immune response against blood forms of *P. chabaudi* AS when administered orally. However the mechanism(s) involved in this modulation need to be explored further. *S. typhimurium*/IFNγ had an important effect in the control of the parasite during the primary parasitaemia in NIH mice, but no clear mechanism of action could be elucidated. NO does not seem to be involved in the process. However iNOS expression in liver and spleen should be evaluated since serum NO does not reflect increased *in situ* production of the molecule. For this, tissue samples from liver and spleen of NIH mice inoculated with *S. typhimurium*/IFNγ 8 days before malaria infection, were collected from different time points after malaria infection. The samples will also be valuable for obtaining information of the type of cytokines produced, initially to confirm the *in vivo* expression of IFNγ by *S. typhimurium*, and later to establish a dominant pattern of cytokine expression which might explain the differences observed between experimental and controls groups. Tissue samples from spleen and liver were also fixed for evaluation with conventional histopathological techniques. No histopathological differences were observed after evaluation of hematoxylin-eosin stained sections of liver and spleen (data not shown). Samples were also cryo-preserved and a technique for detection of α-naphthyl acetate esterase has been standardised for the detection of macrophages. Once performed it would allow evaluation of differences in macrophage migration in the different groups.

The important observation that TNFα delivered by a *S. typhimurium* vector contributed significantly to the control of the parasite in A/J mice during acute infection, also requires further investigation. When using this construct in malaria infection, assessment of the pattern of cytokine production in liver and spleen is essential in understanding the immune mechanisms evoked by the cytokine, since others have demonstrated that increased levels of TNFα expression in spleen are protective against severe *P. chabaudi* infection (Jacobs *et al.*, 1996b). Furthermore, research into the iNOS expression in liver of resistant and susceptible mice would confirm previous observations made by others, which indicate that NO is involved in TNFα-dependent protection (Jacobs *et al.*, 1996a).

The work carried out with the TGFβ and IL-4 *S. typhimurium* constructs was preliminary. Further exploration on their effects in the course of infection in resistant and susceptible models of *P. chabaudi* infection should be performed by changing the timing between the *S. typhimurium* administration and the malaria infection. This in order to identify first the optimal time for the cytokines to have any effect on the course of the malaria infection.

Taken together the experiments presented in this thesis demonstrate that the outcome of a *P. chabaudi* AS blood-stage infection involves a complex interaction of cells and factors (i.e. cytokines, NO, ROI) of the immune system. In addition, the *S. typhimurium* mutants provide an excellent tool for the delivery of cytokines and for the study of their effects in the development of immunity against *P. chabaudi* AS in resistant and susceptible mice. *S. typhimurium* has shown to be useful for the delivery, *in vivo*, of murine cytokines in malaria infected mice. This would prove valuable in the research of malaria vaccines either by the use of *S. typhimurium* to coexpress cytokines and malarial antigens or by inoculating *S. typhimurium* expressing cytokine genes into immunised individuals. Finally, the iNOS depleted model of *P. chabaudi* AS infection can also contribute in further understanding the role of the molecule in the control the infection.

APPENDIX

GIEMSA'S BUFFER

3.0 g NA_2HP_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 two following solutions:

(a) 0.1% alpha-naphtyl-amine (Sigma) in distilled water.

(b) 1% sulphanilamide (Sigma) in 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, pH 7.5

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 freshly diluted in 450 µl distilled water.

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 1l with distilled water.

Buffer

40 ml stock, made up to 1l with 0.9 % saline and adjusted to pH 7.2.

RPMI

Malaria Stock medium

10.39 g	RPMI powdered medium (with L-glutamine) (Gibco).
5.94 g	(25mM) N_2-hydroxyethylpiperazine-N'- ethane sulphonic acid (HEPES) (Sigma).

Made up to 960 ml with distilled water and filter-sterilised (Millipore/Gelman filter size 0.22 μm).

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)

Cell culture Stock medium

10.39 g	RPMI powdered medium (with L-glutamine) (Gibco).
5.94 g	(25mM) N_2-hydroxyethylpiperazine-N'- ethane sulphonic acid (HEPES) (Sigma).

Made up to 960 ml with distilled water and filter-sterilised (Millipore/Gelman filter size 0.22 μm), pH adjusted to 7.2.

Cell culture Incomplete

Stock supplemented with the following:

11 ml	L-glutamine (Gibco)
5.5 ml	$NaHCO_3$ (3.5%)
0.55 ml	2-Mercaptoethanol (0.1 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

0.17M	Tris(hydroxymethyl)aminomethane (20.6 g/l)
0.16M	ammonium chloride (8.3 g/l)
10 ml	Tris added to 90 ml ammonium chloride and the 0.83% stock solution adjusted to pH 7.4.

MOPS GEL

10 ml	10X MOPS
73 ml	RNAse free dH_2O
1.2 gm	agarose (1.2%)

Boil for about 5 min or until agarose is completely dissolved. Cool to 55°C. In fumehood, add 17 ml formaldehide (17%). Mix and pour.

10X MOPS

0.2M.	Morpholinopropanesulphonic acid
0.05M	Sodium acetate
0.01M	EDTA pH 7

Make up to 400ml with RNAase free dH_2O.

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