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Mechanisms of Modulation of Immune Responses During Blood-Stage Malaria

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

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ABSTRACT

Intraperitoneal infection of resistant C57BL/6 mice with *Plasmodium chabaudi* AS results in moderate levels of peak parasitemia, marked splenomegaly and complete elimination of the parasite and immunity to reinfection. $CD4^+$ T cells play a major role in the development of acquired immunity to this hematoprotozoan parasite. In this thesis, mechanisms of immunoregulation by $CD4^+$ T cells during blood-stage *P. chabaudi* AS infection in C57BL/6 mice were studied. The kinetics of *in vitro* production of the Th1-derived cytokine, IFN-γ, versus the Th2-derived cyokines, IL-4, IL-5 and IL-10, by spleen cells as well as of polyclonal and malaria-specific antibodies in the sera were examined during infection using enzyme-linked immunosorbent assays. Upon antigenic stimulation, spleen cells were found to produce high levels of IFN-γ several days prior to peak parasitemia, while high levels of IL-10 production occurred at the time of peak parasitemia followed by IL-4 and IL-5 later in infection. The levels of polyclonal IgG2a isotype were found to be increased during both the acute and chronic phases of infection, whereas the levels of polyclonal IgM, IgG1 and IgG2b isotypes were found to be increased only during the chronic phase of infection. High titers of malaria-specific IgG2a and IgG1 were detected during the primary as well as secondary infections. Investigation of *in vitro* proliferation of spleen cells to mitogens and malaria specific antigen revealed that the responses of splenic lymphocytes from infected mice to Con A, PHA and LPS were suppressed, with the most severe suppression occurring during the first 14 days post infection. Evidence is provided demonstrating that nitric oxide (NO) and prostaglandins (PG), products of activated macrophages, mediated suppression of lymphocyte proliferation in response to Con A and PHA, whereas only PG were found to suppress LPS-stimulated proliferation. In addition, NO

was found to mediate suppression of proliferation of spleen cells from infected mice in response to parasite antigen. Taken together, results from these studies suggest that immune activation and immunosuppression occur simultaneously during blood-stage malaria with *P. chabaudi* AS infection in C57BL/6 mice.

RÉSUMÉ

L'infection causée par *Plasmodium chabaudi* AS, lorsqu'administré par voie intrapéritoneal aux souris résistantes C57BL/6, résulte en des niveaux modéré de parasitémie, une splénomégalie marquée, l'élimination complète du parasite et l'apparition d'une immunité protectrice contre une éventuelle réinfection avec le parasite. Les lymphocytes T $CD4^+$ jouent un rôle majeur dans le développement de cette immunité acquise. Dans cette thése, les mécanismes d'immunorégulation exercés par les lymphocytes T $CD4^+$ durant la phase sanguine de l'infection a P. chabaudi AS, ont été étudies. La cinétique de production *in vitro* de l'IFN-γ, une cytokine dériveé des cellules Th1, versus les cytokines IL-4, IL-5 et IL-10, dérivées des cellules Th2, ainsi que la cinétique de production des anticorps sériques polyclonaux et spécifiques á la malaria, ont été déterminées á l'aide de tests ELISA. Les résultats obtenus démontrent qu'aprés avoir été stimulées par l'antigène, les cellules spléniques produisent de forts taux d'l'IFN-γ quelques jours précédents la poussée de parasitémie, tandis que la production de forts d'IL-10 a lieu au moment de la poussée de parasitèmie, suivie par l'apparition d'IL-4 et d'IL-5 plus tard en cours d'infection. Les taux d'anticorps polyclonaux d'isotype IgG2a sont accrus autant durant la phase aiguë que la phase chronique de l'infection, tandis que l'augmentation des taux d'anticorps polyclonaux d'isotype IgM, IgG1 et IgG2b n'est observée que durant la phase chronique de l'infection. D'autre part, de hauts titres d'anticorps d'isotypes IgG2a et IgG1, spécifiques à la malaria, sont décelés au cours des infection primaires et secondaires. L'analyse de la prolifération des cellules spléniques *in vitro* en présence de mitogènes ou d'antigènes spécifiques à la malaria a révéle que la capacité des lymphocytes spléniques des animaux infectés à répondre aux mitogènes Con A, PHA et LPS est supprimée; la sévérite de cette suppression étant manifeste durant les 14

premiers jours suivants l'infection. Nos résultats indiquent également que l'oxyde nitrique et les prostaglandines, deux produits des macrophages activés, sont responsable de la suppression de la réponse mitogénique à la Con A et à la PHA, alors que seules les prostaglandines sont impliquées dans la suppression de la proliferation cellulaire stimulée par le LPS. De plus, l'oxyde nitrique est responsable de la suppression de la prolifération des cellules spléniques des animaux infectés en réponse aux antigènes parasitaires. Les différents résultats présentés dans cette thèse suggèrent que l'activation immune et l'immunosuppression observées chez les souris C57BL/6 surviennent stimultanément durant la phase sanguine de l'infection causée par *P. chabaudi* AS.

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LIST OF ABBREVIATIONS

AG	aminoguanidine
Con A	concanavalin A
cpm	counts per minute
ELISA	enzyme-linked immunosorbent assay
FCS	fatal calf serum
HBSS	Hank's balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IFN-γ	interferon-gamma
IL	interleukin
INDO	indomethacin
L-NMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharide
NO	nitric oxide
PG	Prostaglandins
PHA	phytohemagglutinin
PRBC	parasitized RBC
RBC	erythrocyte
SNP	sodium nitroprusside
SIN-1	3-morpholino-sydnonimine-hydrochloride,

PREFACE

In accordance with the regulation of the Faculty of Graduate Studies and Research concerning thesis preparation and approved by the Institute of Parasitology, manuscripts submitted for publication have been incorporated into this thesis. The following is quoted from"Guidelines Concerning Thesis Preparation":

"Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting text, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guideline Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objective of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary".

"Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis".

STATEMENT OF AUTHORSHIP

This thesis consists of two manuscripts (Chapter III and IV) which each has its own Abstract, Introduction, Material and Methods, Results and Discussion. The General Abstract, General Introduction (Chapter I), Literature Review (Chapter II), and General Discussion (Chapter V) relate to the combined research studies presented in this thesis. References appear at the end of each Chapter.

The research work presented in this thesis was performed by the author under the supervision and guidance of Dr. Mary M. Stevenson at the Centre for the Study of Host Resistance, Montreal General Hospital Research Institute. Support for these studies was provided by a grant awarded to Dr. Stevenson from Medical Research Council of Canada. Technical guidance and determination of gamma interferon, interlukin 4 and interlukin 5 in supernatants described in Chapter III was provided by Ms. Mi-Fong Tam, senior technician in Dr. Stevenson's laboratory. Determination of interlukin 10 described in Chapter III was performed by Dr. Isabelle Oswald, NIAID, NIH, Bethesda MD, a coauthor on manuscript I (Chapter III). The author and Dr. Stevenson are coauthors of manuscript II (Chapter IV). The manuscripts appearing in Chapters III and IV have been submitted for publication*.

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Ahvazi, B. C., and M. M. Stevenson. Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria. Submitted to Journal of Leukocyte Biology.

CHAPTER I

GENERAL INTRODUCTION

Malaria is currently the most prevalent infectious disease in the world and continues to cause enormous morbidity and mortality in the tropics. It is not only a public health problem but it also represents a serious obstacle for the socioeconomic development of tropical countries. Approximately 50% of the world's population in more than 100 countries live in areas where malaria is still endemic (WHO, 1991). The estimated annual mortality rate is between two and three million, of which at least 1 million children under the age of five years old die of malaria in Africa alone (Stürchler, 1989). Virtually all deaths result from infections with *Plasmodium falciparum*. The most important risk factors for malaria-related deaths are difficulties in establishing the diagnosis and initiating appropriate therapy.

The history of malaria in this century has been dominated by efforts directed at controlling the vector and at developing potent synthetic anti-malarial agents. The World Health Organization (WHO) was committed to malaria eradication as one of its aims from 1955 until 1976, when it was officially declared a failure. The development of resistance to many insecticides by the vectors and resistance to chloroquine by strains of *P. falciparum* severely impaired the WHO program and contributed to the resurgence of transmission in many areas (Wyler, 1983). Scientists have, therefore, recently begun to direct their research efforts at seeking newer approaches, such as developing vaccines against malaria.

In populations living in endemic areas, acquired immunity to *Plasmodium* is age-dependent, slow to develop and develops only with continual exposure to the parasite. Although much evidence has accumulated over recent years

suggesting that both cell-mediated and humoral immunity contribute to acquired immunity in humans infected with *Plasmodium*, the exact nature of the underlying mechanisms remains to be addressed. It is clear that immune activation (hypergammaglobulinemia and production of malaria specific antibodies) and immunosuppression, in particular suppression of T cell responses, occur concurrently (Druilhe et al, 1983; Ho et al, 1986; Ho and Webster, 1989). However, the mechanism(s) of the concurrence of these divergent responses is unknown.

In research aimed at developing strategies for better understanding and eradication of human malaria, various species of animals are used as laboratory models. In particular, the murine models provide systems to investigate the nature and range of protective host immune responses. Our laboratory has previously defined a model of genetically controlled resistance in inbred mice to infection with the rodent malaria parasite *P. chabaudi* AS (Stevenson et al, 1982). As suggested by others (Langhorne et al, 1989; Tayler-Robinson et al, 1993), this parasite/host combination provides a useful model with many analogues to human malaria, including the following: a) the blood-stage takes place mostly in mature red blood cell; b) many antigens are analogous to those of *P. falciparum*; and c) in most mouse strains, the infection is not lethal, thus allowing the study of a naturally acquired protective immune response. In this thesis, the mechanisms of modulation of immune responses during blood-stage malaria have been analyzed during the course of *Plasmodium chabaudi* AS infection in resistant C57BL/6 mice. In Manuscript I (Chapter III), we investigated the kinetics of *in vitro* cytokine production by spleen cells recovered at various times during infection and determined the levels and isotypes of non-specific and malaria specific antibodies in the serum during infection. In Manuscript II (Chapter IV), the roles of nitric oxide and prostaglandins in suppression of lymphocyte

proliferation during blood-stage infection with *P. chabaudi* AS were investigated.

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CHAPTER II

LITERATURE REVIEW

1. Parasite

Malaria is caused by species of the obligate intracellular protozoa classified in the genus *plasmodium* within the Phylum Apicomplexa, class Sporozoa and suborder Haemosporina. There are more than 100 species of *Plasmodium* which can infect a variety of animals, including man, birds, rodents and non-human primates. Plasmodia species exhibit strictly defined host specificities which depend on the interaction between specific surface receptors on the parasite and those on the surface of erythrocytes which facilitate the invasion of red blood cells (RBC) (Yaeger, 1985).

The parasites are cyclically transmitted to humans by female mosquitoes of the genus *Anopheles*. Four plasmodia species that cause human malaria are *P. malariae*, *P. vivax*, *P. ovale* and *P. falciparum*. Each species has certain morphological characteristics by which the parasite is identified as well as biologic and pathogenic features of clinical importance. The age of erythrocytes is an important determinant of the level of parasitemia. *P. vivax* (benign tertian malaria) and *P. ovale* (mild tertian malaria) tend to invade only reticulocytes while *P. malariae* (quartan malaria) invades senescent erythrocytes. Hence, these species have parasitemias with low densities, resulting in low morbidity. In contrast, *P. falciparum* (malignant tertian malaria) can invade all erythrocytes and parasitemia can reach very high levels. Complications of malaria caused by *P. falciparum* include severe hemolytic anemia and involvement of the cerebral, renal, hepatic, pulmonary, and coagulation systems. Among the different species that infect animals, there are four species (*P. berghei*, *P. yoelii*, *P. chabaudi* and

P. vinckei) which infect rodents, including rats and mice. *P. berghei* and *P. yoelii* invade immature erythrocytes while *P. chabaudi* and *P. vinckei* invade mature erythrocytes. Mouse models offer a number of advantages for studying the disease because of the availability of a wide variety of genetically different inbred strains of mice as well as many immunological reagents such as T cell markers and recombinant cytokines (Mons and Sinden, 1990)

2. Biology and Life Cycle

The life cycle of malaria is complex and certain aspects differ according to the *Plasmodium* species involved (Strickland, 1991). The life cycle includes an asexual phase which occurs in the vertebrate host while maturation and union of male and female gametes followed by production of sporozoites take place only in the mosquito vector (Figure 1, page 8).

2.1. Asexual Phase. Infection occurs when an infected female *Anopheles* mosquito injects saliva containing sporozoites into the blood stream through subcutaneous capillaries. Within a half hour, sporozoites disappear from the blood. The first colonization takes place in parenchymal cells of the liver where they multiply asexually in a process known as *exoerythrocytic schizogony*. There the nucleus undergoes repeated division, resulting in the formation of a schizont containing thousands of uninucleate merozoites. After 6 to 16 days from the time of infection, the hepatic cell containing the tissue schizont ruptures and the merozoites enter the circulation. In infections with *P. falciparum* and *P. malariae*, the tissue schizonts all rupture at about the same time and none persist in the liver. In contrast, *P. vivax* and *P. ovale* have two types of exoerythrocytic forms. A primary type develops and ruptures within 6 to 9 days. In addition, there is a secondary type, the hypnozoite, that may remain dormant in the liver for weeks, months, or up to five years before developing and resulting in relapses of

erythrocytic infection. Merozoites released from tissue schizonts invade erythrocytes (RBC). After a period of growth in the RBC, the schizont stage begins when the parasite undergoes nuclear division and culminates in segmentation to form a second generation of merozoites. This process of asexual multiplication is called *erythrocytic schizogony*. The infected erythrocytes rupture, liberating merozoites which soon actively enter other red cells to repeat the asexual cycle.

Fever is the hallmark of malaria infection and is coincident with the rupture of the infected red blood cells and release of merozoites. With *P. vivax* and *P. ovale*, the erythrocytic cycle from entry of merozoite to rupture of schizonts takes 48 hours and is more or less synchronized in all red cells. With *P. malariae*, the cycle takes about 72 hours but is synchronized so that large numbers of merozoites enter the blood every 72 hours. *P. falciparum* is more irregular. Its erythrocytic cycle takes 36 to 48 hours and synchronization may not be achieved. As stated above, *P. falciparum* is the species that most often causes death. When *P. falciparum* is compared with the 3 other species, the duration of infection is the shortest, exoerythrocytic schizonts release 2.5 to 20 times as many merozoites, and the rate of development of the exoerythrocytic stage is fastest. In contrast, *P. malariae* has the longest duration of infection and is almost a commensal infection in some adults.

2.2. Sexual Phase. From 3 to 15 days after the onset of symptoms, a subpopulation of merozoites differentiates into sexual forms or gametocytes, the female macrogametocytes and male microgametocytes. While feeding on an infected human, the female anopheline mosquito ingests gametocytes, the microgametocytes become exflagellated and the macrogametocytes develop further until fertilization takes place resulting in a motile ookinete. Ookinetes migrate through the stomach wall of the mosquito and develop into the oocyst.

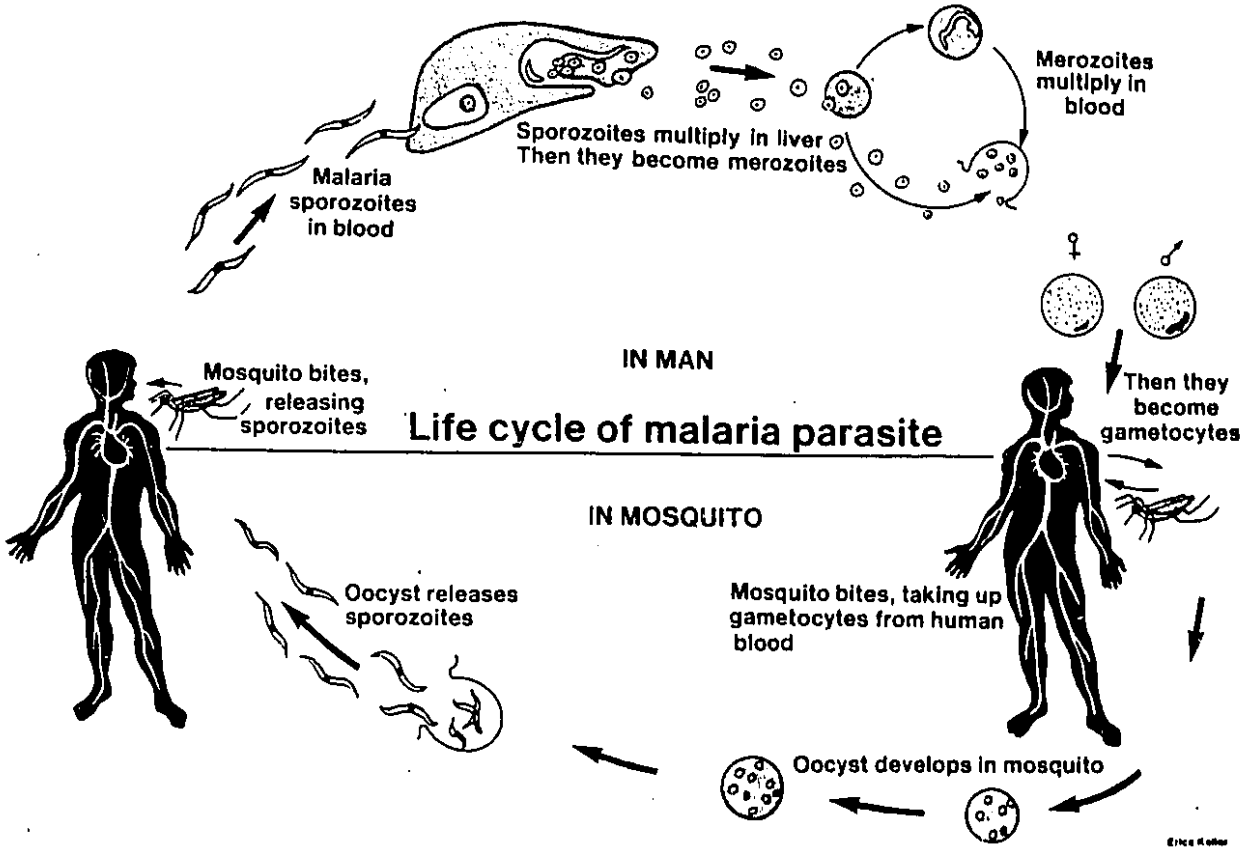

Figure 1. Life cycle of malaria parasite

The oocyst grows rapidly and spindle-shaped sporozoites develop. By the time the sporozoites become mature, the oocyst ruptures, liberating thousands of motile sporozoites into the body cavity; from there they migrate to the salivary glands of the mosquito. When the mosquito takes its next blood meal, sporozoites are injected into the cutaneous blood vessels of the victim and a new infection is initiated.

3. Pathophysiology of Malaria

Pathophysiologic changes in malaria are primarily associated with the impairment of local blood flow resulting from parasitized erythrocytes sticking to the venular endothelium. Anemia is a common complication of malaria due to the destruction of RBC and dysfunction of organs that results from tissue hypoxia caused by vascular obstruction by parasitized RBC. Black water fever or hemoglobinuric fever is a very dangerous complication of malignant malaria due to the extensive intravascular hemolysis of the RBC. A number of other serious complications can occur in malaria infections, including splenomegaly, acute renal failure resulting in elevated urea nitrogen and creatinine, and proteinuria, damage to the pulmonary capillaries occasionally resulting in the adult respiratory distress syndrome (ARDS), pulmonary edema, and cerebral dysfunction. In the affected organs, arterioles and capillaries are distended with sludged red cells, pigment and parasites. RBC infected with the later stages of *P. falciparum* may develop knobs due to changes on the surface of the infected erythrocytes which contain malarial antigens and results in adherence to capillaries deep within organs.

4. Immune Response to Blood-Stage Malaria Infection in Man

4.1. Innate Resistance

The genetic background of the host represents perhaps one of the strongest influences on the magnitude of host response to invasion by *Plasmodium* species. The invasion of RBC by merozoites requires a specific membrane receptor (Pasvol and Wilson, 1982). Thus, for example, individuals whose erythrocytes are Duffy blood-group negative (they lack Duffy a and b determinants) are not susceptible to *P. vivax* infection (Miller et al, 1976). In contrast, *P. falciparum* requires determinants on glycophorin A (the major surface

glycoprotein of RBC) and other sialoproteins for invasion of red cells. Thus, individuals whose RBC are Ena^- are resistant to infection.

A number of other genetically determined erythrocyte characteristics that affect parasite development include hemoglobin composition and enzyme content. The development of *P. falciparum* is suppressed in the presence of fetal hemoglobin, hemoglobin S (HbS), and possibly also in the presence of certain other genetically abnormal hemoglobins, such as hemoglobin C and E (Livingston and Tanaka, 1987). In addition, a number of genetically determined deficiencies of enzymes associated with RBC have been shown to augment protection against malaria. These include glucose-6-phosphate dehydrogenase, glutathione peroxidase and glutathione reductase (reviewed by Stevenson, 1989).

4.2. Acquired Immunity

Immunity to malaria is stage-specific such that immunization with sporozoites protects against sporozoite challenge but not against the blood-stage of infection (Good et al, 1987). Immunity or premunition, which appears to be related to the development of tolerance to plasmodia parasites, has long been known to be acquired with repeated malaria infection in endemic regions. The mechanisms by which this immunity is achieved require both humoral immunity (antibody-dependent) as well as cell-mediated immunity (antibody-independent). If the individual leaves the area or receives malaria chemoprophylaxis, exposure to blood-stage parasites is reduced, and immunoglobulin levels and specific malaria immunity diminish over a period of months to years. Upon reinfection, the individual is susceptible to a severe bout of clinical malaria (Melancon-Kaplan and Weidanz, 1989).

4.2.1. Humoral Immunity

In general, infection with *Plasmodium* rapidly induces an increase in immunoglobulin synthesis. The most pronounced changes are found in IgG and IgM levels but IgA levels are also increased. Moreover, not all malarial antibody is associated with protective immunity. Protection is associated with antibodies of IgG class while results for and against the protective value of IgM have been reported (reviewed by Taylor, 1989). Direct evidence from *in vivo* and *in vitro* studies suggest that antibody plays an important role in protective immunity (Nussenzweig and Nussenzweig, 1986). Antibodies enhance parasite recognition and phagocytosis by macrophages by binding to sporozoites, free merozoites, parasitized RBC, or the macrophages themselves (Taylor, 1989). Complement and antibody-mediated lysis do not appear to play a role in parasite destruction, since complement levels appear to be depressed in acute malarial infection (Taylor, 1989). Merozoites coated with antibody are inhibited in their ability to penetrate RBC. Antibody-mediated phagocytosis of merozoites during their brief extracellular period is probably the major means of reducing parasitemia in individuals with partial immunity. Protective immunity to malaria in human is mediated in part by antibodies, and develops after a long time of repeated exposure to the parasite.

It has been demonstrated that immunity can be passively transferred across the placenta to protect neonates against malaria (Blacklock and Gordon, 1925). Similar findings for passive transfer of protection by immune sera have also been reported. African children suffering from acute malaria were cured and protected after receiving purified immunoglobulin from adult immune sera (Cohen et al, 1961). Subsequent studies in Thailand have also demonstrated that administration of the gamma-globulin fraction obtained from immune adult Africans can passively transfer protective immunity to individuals with severe *P.*

falciparum malaria (Bouharoun-Tayoun et al, 1990). Recent findings have demonstrated that 88% of individuals with cerebral malaria had significant IgG, IgM and IgA anti-malaria antibodies in cerebrospinal fluid (CSF) (Mitra et al, 1991). However, the levels of antibodies in CSF did not correlate with either the degree of parasitemia or the severity of coma but did correlate significantly with duration of coma suggesting a role for these antibodies in the immunopathology associated with cerebral malaria. Thus, results from the above studies not only demonstrate that antibodies contribute to the prevention or elimination of blood-stage parasitemia during acute malaria but also point to a significant role for humoral immunity in pathology associated with severe malarial infection.

4.2.2. Cell-Mediated Immunity

T cells play an essential role in host immune responses to malaria. In addition to their effector cell function as cytotoxic cells, they provide help to B cells and other T cells and, thus, serve to regulate these responses. Some researchers are bound to question the role of T cells in man in protection against blood-stage malaria. For ethical reasons, critical experiments to address this issue obviously cannot be performed. However, immunity in the absence of protective antibody has been demonstrated for murine malarias as will be described below.

T cell responses are both activated and suppressed during acute blood-stage *P. falcipaurm* malaria. Activation is indicated by the presence of T cell-dependent malaria-specific as well as non-specific antibodies (Ballet et al, 1987; Kabilan et al, 1987). In a study from the Gambia, it was shown that supernatants from malaria antigen-activated T cells could induce adherent mononuclear cells to kill parasites *in vitro* in the absence of antibody (Brown and Nossal, 1986). Furthermore, *in vitro* lymphocyte proliferation and gamma interferon (IFN-γ) production by T cells to blood-stage antigen have been demonstrated in adults

from an endemic area and individuals recovered from acute malaria infection (Ho et al, 1986; Riley et al, 1988). Observations of elevated levels of circulating soluble IL-2 receptor (IL-2R) and IFN-γ in the serum of some patients with acute *falciparum* malaria also is indicative of T cell activation. Using synthetic peptides representing T cell epitopes from the antigen Pf155, or ring-infected erythrocyte surface antigen (RESA), it was shown in a separate study that peripheral blood T cells from patients with malaria could be induced *in vitro* to secrete IL-2, IL-4 and IFN-γ (Troye-Blemberge et al, 1985; Troye-Blemberge et al, 1990; Riley et al, 1990). IL-4 production did not correlate with either T cell proliferation or IFN-γ production but did correlate with anti-peptide antibody (IgG) levels. Increased numbers of gamma delta T cells have also been reported in both the peripheral blood (Ho and Webster, 1990) and spleens of patients with *P. falciparum* malaria (Bordessoule et al, 1990). However, it is not known whether these cells contribute to protection against blood-stage infection with *P. falciparum.*

T cell responses to blood-stage antigens were also found to be suppressed during malarial infection. Patients with acute *falciparum* malaria exhibit antigen-specific immunosuppression (Ho and Webster, 1990). The mechanisms of the immunosuppression in malarial infection are complex, and many hypotheses have been proposed, such as, defects in both IL-2 production and IL-2R expression in response to malaria-specific antigen (Ho et al, 1986). Both $CD4^+$ and $CD8^+$ T cell numbers were found to be reduced with no consistent alterations in the $CD4^+/CD8^+$ ratio during clinical disease. Humans infected with *P. falciparum* exhibit depressed responses to tetanus toxoid, *Salmonella typhi* antigen and meningococcal vaccine (McGregor and Barr, 1962; Greenwood et al, 1972; Williamson and Greenwood, 1978). It has been reported that, in contrast to peripheral blood T cells derived from uninfected individuals, T cells from patients with acute *P. falciparum* malaria failed to prevent B lymphocytes infected with

Epstein-Bar virus (EVB) from proliferating abnormally (Whittel et al, 1984). This finding, which was attributed to decreased numbers of $CD4^+$ cells, prompted Weidanz and Long (1988) to suggest an explanation for the relationship between Burkitt's lymphoma and malaria (Whittel et al, 1984). Thus, there is a situation in which T cell activation and immunosuppression occur concurrently in patients with *P. falciparum.* Nevertheless, the mechanism of T cell-mediated immunity in malaria as well as the requirements for its induction in vaccination are still poorly understood (Good, 1990).

5. Immune Response to Blood-Stage Malaria Infection in Mice

5.1. Innate Resistance

The level of host resistance to infection in inbred mice with the rodent malaria species *P. berghei, P. yoelii* and *P. chabaudi* AS, has been observed to be genetically controlled (reviewed by Stevenson, 1989). In 1950, Greenberg and colleagues were the first to describe genetic differences in resistance among inbred strains of mice to infection with *P. berghei.* Analysis of different inbred strains and F1 hybrid combinations demonstrated that C57BL and C57L strain mice survived *P. berghei* infection while DBA/2 and A/LN mice succumbed to the infection. Resistance was found to be genetically controlled by a single, dominant, autosomal gene or closely linked set of genes. In contrast to *P. berghei*, the genetic control of susceptibility to *P. yoelii* appears to depend on multiple genes. The genetic control of resistance in inbred mice to infection with the rodent malaria parasite *P. chabaudi* AS was defined in our laboratory. C57BL/6 mice were found to be resistant while A/J mice were found to be susceptible. Analysis of hybrid and backcross progeny derived from the strain combination of resistant C57BL/6 and susceptible A/J mice demonstrated that the trait of resistance is controlled by an autosomal, dominant, non H-2 linked gene

called *Pchr*. Linkage analysis of the traits of resistance and the degree of splenomegaly in AXB/BXA recombinant inbred (RI) mouse strains showed that these traits are genetically linked (Stevenson et al, 1982; Stevenson and Skamene, 1985).

5.2. Acquired Immunity

Studies using experimental models of malaria in animals have shown that resistance to blood-stage infection is mediated by protective antibodies and T cell-dependent, cell-mediated mechanisms of immunity. Depending upon the infecting species of *Plasmodium* and prior experience of the host, either humoral or cell-mediated immune mechanisms predominate. Resolution of blood-stage infection with *P. yoelii* requires humoral immunity while control of acute *P. chabaudi* has been shown to require T-cell mediated immunity (Grun and Weidanz, 1981). The pathways operating during an *in vivo* immune response to *Plasmodium* are dependent upon several factors, including the nature of the immune response at a particular time point, and the susceptibility of the parasite to macrophage-derived mediators, such as reactive oxygen or nitrogen intermediates (Weidanz and Long, 1988).

5.2.1. Humoral Immunity

The role of antibodies in rodent malaria immunity is complex, as both the kinetics and nature of antibody production, and the protective value of antibodies may vary with the host and species of parasite. Successful passive transfer experiments have been performed in rodent models with immune serum and with monoclonal antibodies specific for parasite or infected red cell surface structures (reviewed by Dean and Cohen, 1983; Yoshida et al, 1980; Freeman et al, 1980). A number of studies support the *in vivo* protective role of antibodies

against blood-stage infection with *P. berghei* and *P. yoelii*. The passive administration of antibodies to *P. berghei*-infected rats results in delayed, reduced peak parasitemia and enhanced survival (Zuckerman and Golenzer, 1970; Lourie and Dunn, 1972). Moreover, T cell deficient rats infected with *P. berghei* were found to be protected by passive transfer of hyperimmune serum, suggesting that antibodies alone are sufficient for the control of a primary infection with this species (Lourie and Dunn, 1972). Evidence from experiments with μ-suppressed mice infected with *P. yoelii* suggests a role for B cells in the development of immunity (Weinbaum et al, 1976). It was also demonstrated that B cell deficient mice succumbed to avirulent *P. yoelii* infection (Grun and Weidenz, 1981). In addition, passive transfer of polyclonal as well as monoclonal antibodies protected intact mice against the lethal variant of *P. yoelii* (Jayawardena et al, 1978; Freeman et al, 1980). However, in T cell-deprived mice, the transfer of antibodies does not prevent infection, suggesting the importance of T cells in the generation of the protective immunity (Jayawardena et al, 1978). Studies by Taylor et al (1988) demonstrated that the isotypes of malaria-specific antibodies produced in response to *P. yoelii* varied among 11 inbred strains of mice. More specifically, while all strains produced IgM, IgG2a, and IgG3 antibodies, only 3 of 11 strains produced anti-*P. yoelii* of IgG1 isotype. Subsequently these investigators have demonstrated that passive transfer of IgG2a fractioned hyperimmune sera resulted in protective immunity in mice infected with *P. yoelii* (White et al, 1991). Blood-stage *P. yoelii*, thus, represents a model of *plasmodium* infection which provides evidence for the requirement of B cells and their products, antibodies, in immunity to malaria. In contrast, antibody titers do not always correlate well with protection against *P. chabaudi* infection, although antibodies are required for the eventual clearance of infection. Langhorne et al (1989) demonstrated that the presence of malaria-specific IgM antibodies does

not suffice to control *P. chabaudi* infection, whereas control of subpatent parasitemia in the later phase of infection correlates with appearance of IgG antibodies. The above studies would suggest, but do not prove, that anti-malaria antibodies play a role in the final elimination of blood-stage infection with *P. chabaudi*. The kinetics of antibody responses during infection with *P. chabaudi* AS in C57BL/6 mice are presented in Chapter III.

5.2.2. Cell-Mediated Immunity

It is now clear that T cells play an important role in the development of protective immune responses in the mouse to the blood-stage *Plasmodium* which appears to be independent of their role as helper cells for antibody production (reviewed by Langhorne et al, 1989). This is substantiated by evidence from many experimental models showing that parasitemia cannot be controlled in T cell-deficient animals (Brown et al, 1968; Weinbaum et al, 1976; Jayawardena et al, 1977). B cell-deficient mice infected with *P. yoelii* or *P. chabaudi adami* were observed to develop acute malaria. However, *P. chabaudi adami* infected mice resolved the infection with kinetics of parasitemia similar to those seen in immunologically intact mice instead of dying as did mice infected with *P. yoelii* (Grun and Weidanz, 1981). B cell-deficient mice have also been found to resolve acute blood-stage infection caused by *P. vinckei petteri* or *P. chabaudi chabaudi* (Cavacini et al, 1990). Furthermore, athymic nude mice were unable to resolve the infection with *P. chabaudi adami* and eventually died (Grun and Weidanz, 1981). In addition, protection against *P. chabaudi* and *P. chabaudi adami* can be achieved by adoptive transfer of malaria specific T cells from immune animals to naive animals (McDonald and Phillips, 1978; Brinkmann et al, 1985; Cavacini et al, 1986). Brake et al (1988) demonstrated that adoptive transfer of a T cell clone derived from a protective T cell line by limiting dilution

was protective against *P. chabaudi adami*. These cells expressed the $CD4^+$ phenotype, proliferated in response to specific antigen in an MHC-restricted manner, and secreted both IFN-γ and IL-2. The results of several studies confirm the importance of $CD4^+$ T cells in acquired immunity to acute infection with *P. chabaudi* (Süss et al, 1988; Podoba and Stevenson, 1991). In these studies, mice depleted of $CD4^+$ T-cells *in vivo* by monoclonal antibody treatment were not able to resolve the infection. In contrast, depletion of $CD8^+$ T cells had no effect on the early course of parasitemia, although these mice experienced two recurrent bouts of parasitemia during the late stage, and required more than five weeks to eliminate the infection (Podoba and Stevenson, 1991).

Analysis of murine $CD4^+$ T cell clones have revealed that they can be further divided into at least two subsets based on their profile of lymphokine production (Mosmann et al, 1986). Upon activation, Th1 cells secrete IL-2, IFN-γ and lymphotoxin (TNF-β) whereas Th2 cells secrete IL-4, IL-5 and IL-10. Functionally, Th1 cells mediate delay type hypersensitivity (DTH) and macrophage activation while Th2 cells provide help for B cells in generation of antibody production (Cherwinski et al, 1987; Mosmann and Coffman, 1989). Evidence for cross-regulation of Th1 and Th2 clone has been obtained. IFN-γ inhibits the proliferation of Th2 clones whereas IL-10 inhibits the cytokine production by Th1 clones (Gajewski and Fitch, 1988; Fiorentino et al, 1989). The importance of Th1 versus Th2 subsets in determining the outcome of parasitic diseases has recently been appreciated (Sher and Coffman, 1992). The contribution of these subsets to protection versus pathology is best understood in murine *Leishmania major* infection. Infection of C57BL/6 mice with *L. major* is associated with the development of Th1 cells with strong DTH responses, and resistance to infection. In contrast, infection of BALB/C mice by *L. major* is

accompanied by the expansion of Th2 cells correlated with high titers of IgG1 and IgE antibodies, and disease progression.

Several studies have demonstrated a role for both Th1 and Th2 cells in resolution of blood-stage infection with *P. chabaudi* (Langhorn et al, 1990; Stevenson and Tam, 1993). In these studies, the induction of Th subsets during the infection was assessed by the cytokine profiles of $CD4^+$ T cells from *P. chabaudi* (AS) infected mice in *in vitro* spleen cell cultures and by limiting dilution assay. Results from these studies have shown that the mechanisms which resolve the primary acute parasitemia are Th1 cell-dependent whereas Th2 cells are required for the eventual clearance of infection and sterilizing immunity. The kinetics of *in vitro* cytokine production by spleen cells from *P. chabaudi* AS infected C57BL/6 mice are presented in Chapter III.

It would appear that $CD4^+$, particularly Th1 cells, also mediate blood-stage immunity to other *Plasmodium* infections by the production of cytokines, such as IL-2 and IFN-γ. *In vitro* studies have shown that ConA-stimulated spleen cells from *P. yoelii* and *P. berghei* infected mice secrete high levels of IL-2 during acute infection (Lelchuk et al, 1984). In contrast, during chronic infection, blast cells from spleen cells induced by ConA responded to IL-2 but did not secrete this lymphokine, suggesting a defect at the level of IL-2 synthesis (Lelchuk and Playfair, 1985). IFN-γ activates macrophages to produce metabolites toxic for intraerythrocytic parasites. Upon activation, macrophages are capable of destroying *Plasmodium* parasites *in vitro* via oxgen-dependent and independent mechanisms (Ockenhouse and Shear, 1984a; Ockenhouse et al, 1984b; Stevenson et al, 1992). In addition, Stevenson et al (1989a) demonstrated that *in vivo* depletion of macrophages results in enhanced susceptibility to infection, higher parasitemia and death.

Numerous observations suggest that the process of parasite killing occurs in the spleen where parasites filter through a network of T cells as well as monocyte-macrophages (Kumar et al, 1989; Good, 1990; Yap and Stevenson, 1994). The spleen plays a major role in immunity to malaria and splenectomy usually enhances the severity of disease (Kumar et al, 1989; Yap and Stevenson, 1994). During infection diverse structural and physiological changes occur in the spleen (Stevenson and Kraal, 1989b). The spleen participates in the filtration, removal and destruction of aged, damaged or infected RBC. It also provides a suitable microenviroment for the interaction of antigen presenting cells, mainly macrophages, and immune cells leading to the development of acquired immunity. Moreover, it is well known that T cells increase in number in the spleens of mice infected with plasmodia (Jayawardena et al, 1975). Thus, the presence of architecturally an intact spleen is required for $CD4^+$-dependent, antibody-independent immunity (Grun et al, 1985).

6. Nitric Oxide Biosynthesis

Nitric oxide (NO) is one of the smallest biological molecules which is produced by, and acts on almost every cell type in various tissues of the body. NO is a relatively short-live molecule with a half life of five seconds. Because of both its low molecular weight and lipophilic nature, NO can diffuse freely across cell membranes in an isotropic fashion (Nathan, 1992). The formation of NO in mammalian cells is catalyzed by the enzyme nitric oxide synthase (NOS). This enzyme requires a specific nitrogen atom from the amino acid L-arginine in order to synthesize nitric oxide. The oxygen atom, on the other hand, is derived from molecular dioxygen. The reaction also requires an electron donor, nictotinamide adenine dinucleotide phosphate (NADPH), and a cofactor, tetrahydrobiopterin, which is tightly bound to NOS and stabilizes the enzyme. During the synthesis of

nitric oxide, the removal of the nitrogen atom from L-arginine produces the amino acid L-citrulline. Furthermore, NO can react with superoxide (O_2^-) to form either of two, unreactive anions, nitrite (NO_2^-) and nitrate (NO_3^-), which are stable endproducts of NO synthesis and can be measured in plasma or urine as total reactive nitrogen intermediates (Stuehr and Griffith, 1992; Marletta, 1993). Alternatively, NO can diffuse into surrounding tissues where it reacts with iron-sulfur or heme-containing proteins, resulting in inhibition of certain enzymes, including aconitase, an enzyme involved in the tricarboxylic acid cycle, and complex I and complex II of the mitocondrial electron transport chain. Moreover, it has been shown that NO inhibits ribonucleotide reductase, the enzyme that converts ribonucleotides to the deoxyribonucleotides necessary for DNA synthesis. By inhibiting deoxyribonucleotide synthesis, NO depletes the precursors necessary for DNA synthesis in cells such as susceptible tumor target cells (Moncada et al, 1991; Nathan, 1992).

NO controls and influences a number of critical physiological processes, including regulation of blood pressure and neurotransmission (Bredt and Bredt, 1992). In addition to these activities, it appears that high levels of NO are also involved in inflammatory response-induced tissue injury, mutagenesis and neurotoxicity (Moncada et al, 1991). The magnitude and duration of NO production determine whether its actions are physiologic or pathologic. To date, two major forms of NOS have been identified, the constitutive form and inducible form. The constitutive and inducible forms of NOS differ in ways other than the mechanisms by which they are activated which will be described below. In particular, they are differentially inhibited by analogues of arginine, such as N^G-monomethyl-L-arginine (L-NMMA) or aminoguanidine (AG), respectively. This finding is based on studies demonstrating that L-NMMA is more potent than AG at inhibiting constitutive NOS isolated from rat brain, whereas AG was found to

be more potent than L-NMMA at inhibiting inducible NOS in endotoxin-stimulated cultured macrophages (Tilton et al, 1993). The inducible form of NOS also produces much greater amounts of NO than the constitutive form, a factor that is key to their differing roles in the body.

6.1. Constitutive Nitric Oxide Synthase: Biological Role

Constitutive NOS (cNOS) is activated by the regulatory protein calmodulin, which itself is activated by an influx of calcium ions into the cell. In the presence of elevated calcium, calmodulin binds tightly to constitutive NOS which results in immediate and transient synthesis of small quantities of NO. The best characterized examples of physiological processes controlled by calcium-activated NOS derived NO include vasodilation and regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, and opening and closing of ion channels. Constitutive NO has been implicated in the regulation of blood pressure, in part by endothelial cells that line the blood vessels. Several biological molecules including hormones and cytokines can activate NOS found in these cells, by binding to the appropriate receptor on the endothelial cell membrane (Brizzolara and Burnstock, 1991). This binding causes calcium channels in the membrane to open, leading to an influx of calcium into the cells. The calcium binds to calmodulin and activates cNOS. NO synthesized by the endotheial cells diffuses freely into both the interior of the blood vessel and into the underlying smooth muscle cells. In the blood vessel, NO enters blood platelets and decreases their aggregation with each other and their adhesion to endothelial cells. Within smooth muscle cells, NO activates the enzyme guanylyl cyclase, causing an increase in cyclic guanosine monophosphate levels, which decreases the amount of free calcium in the muscle cell, causing the muscle to

relax. Muscle relaxation allows the vessel to dilate, which lowers the blood pressure (Moncada et al, 1991).

6.2. Inducible Nitric Oxide Synthase: Its Role in Immunity and Pathology

In contrast, inducible NOS (iNOS) is not dependent upon the presence of calcium, rather the enzyme is synthesized as a new protein in response to cytokines, such as IFN-γ, TNF and IL-1α, or lipopolysacchharide (LPS), a component of the cell wall of gram negative bacteria (Xie et al, 1992). Thus, once the protein has been synthesized, NO synthesis will occur vigorously as long as cytokine is available and the NOS remains active. Cytokine indirectly enhances the activity of iNOS by increasing the intracellular level of tetrahydrobiopterin. Recent studies have demonstrated that the inducible form of NO serves as a protective molecule against many parasitic and bacterial infections (James and Nacy, 1993). However, as described below, overproduction of this molecule is also associated with immunosuppression and pathology (Albina et al, 1991).

A number of studies have documented that activated murine macrophages produce large amounts of NO in response to cytokine and/or LPS stimulation resulting in inhibition of growth of intracelluar organisms, such as *Leishmania major* (Green et al, 1991), *Toxoplama gondii*, *Mycobacterium leprae* (Adams et al, 1990; Adams et al, 1991) and *Plasmodium berghei* (Mellouk et al, 1991), or extracelluar organisms such as *Cryptococcus neoformans* (Granger et al, 1988), *Schistosomas mansoni* (James and Glaven, 1989), and *Entamoeba histolytica* (Lin and Chadee, 1992). The proposed mechanism of induction of macrophage NO synthesis during parasitic infection is via Th1 activation with production of cytokines, particularly IFN-γ, which results in induction of macrophage NO synthesis. Studies by Oswald et al (1992) demonstrated that IL-10 synergized

with IL-4 and transforming growth factor beta (TGF-β) to inhibit IFN-γ-induced NO mediated killing of *S. mansoni*. Thus, activation of Th1 cells with production of specific cytokines which up-regulate NO production plays an important role in resolution of infection with a variety of microorganisms. On the other hand, activation of Th2 cells results in the production of cytokines which down regulate this anti-microbial mechanism.

Although the data described above support the hypothesis that the inducible form of NO plays a major role in the function of the immune system as a macrophage cytotoxic effector molecule, other experimental results suggest the extreme pathological consequences of NO production in the infected host. High levels of NO generated by cytokine-stimulated cells were found to be toxic to parasites and tumor cells, but ultimately to damage the NO-producing cells and, indiscriminately, the surrounding healthy cells and tissue (Kolb and Kolb-Bachofen, 1992) through inactivation of complex I and complex II of the electron transport chain and the Kerbs cycle enzyme aconitase (Nathan, 1992). NO production via cytokine regulation was found to be the inhibitor of neutrophil chemotaxis (Kaplan et al, 1989) and leukocyte adhesion (Kubes et al, 1991), a mediator of tissue injury caused by deposition of immune complexes (Mulligan et al, 1991) and to play a role in suppression of lymphocyte proliferation (Albina and Henry, 1991). NO was found to be a potent inhibitory molecule in the *in vitro* rat splenocyte mixed-lymphocyte culture system, resulting in inhibition of allospecific proliferation and of induction of cytolytic T cells (Langrehr et al, 1991). NO production by macrophages in spleen cell populations from mice infected with *Trypanosoma brucei* (Sternberg and McGuigan, 1991) and *T. rhodesiense* (Schleifer and Mansfield, 1993) was shown to suppress proliferative splenocyte in response to ConA. NO was also found to inhibit T cell reactivity to staphylococcal enterotoxin (Isobe and Nakashima, 1992). These results suggest

that NO may play a role in immunosuppression that has been reported in several parasitic systems to be mediated by adherent cells. The role of nitric oxide in suppression of lymphocyte proliferation during infection with *P. chabaudi* AS is the main theme of Chapter IV.

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CHAPTER III

Regulation of Antibody Responses by T helper Cell Subsets During Blood-Stage *Plasmodium chabaudi* AS†

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Abstract

The kinetics of *in vitro* production of the Th1 cytokine, IFN-γ, and of the Th2 cytokines, IL-4, IL-5 and IL-10, by spleen cells were investigated during blood-stage *P. chabaudi* AS infection in C57BL/6 mice. Spleen cells were found to produce high levels of IFN-γ on day 5, two days before peak parasitemia, in response to parasite antigen. In contrast, increased IL-10 production occurred on day 7 followed by significant production of IL-4 and IL-5 between days 14 and 28 post infection. The kinetics of both polyclonal and malaria-specific antibody production of the IgM, IgE and IgG subclasses were also investigated in the sera of *P. chabaudi* AS infected mice. The levels of total IgG2a isotype were found to be increased during the acute phase and high levels persisted during the chronic phase of infection. In contrast, the levels of total IgM, IgG1 and IgG2b isotypes were found to be increased only during the chronic phase of infection. Determination of malaria-specific antibody levels revealed high titers of IgG2a and IgG1 antibodies on days 10 and 21 post infection. In addition, analysis of sera from hyperimmune mice demonstrated that while all of animals had high titers of malaria-specific IgG2a antibody, only 50% had high titers of IgG1 antibody. Neither total nor malaria-specific IgE was detected in the sera of *P. chabaudi* AS infected mice at any time. These results confirm our previous studies which demonstrated that sequential activation of Th1 followed by Th2 cells occurs during blood-stage infection with *P. chabaudi* AS. In addition, these results indicate that *P. chabaudi* AS infection in mice induces a poly-isotypic hypergammaglobulinemia which has also been described in man infected with *Plasmodium* species.

Introduction

An accumulating body of evidence suggests that both cell-mediated and humoral immunity contribute to the resolution of blood-stage malaria infections in man and animals (1,4). This is substantiated by many experiments which defined the role of T and B cells in acquired immunity to *Plasmodium chabaudi*. Recent studies have shown that J_HD mice, which are genetically B-cell deficient, were able to control acute *P. chabaudi chabaudi* and *P. chabaudi adami* infections to subpatent levels (33). However, these animals were not able to eliminate the parasite completely. These results confirm earlier studies by Grun and Weidanz (8) and point to a significant role for antibody production by B cells in complete clearance of the parasite. Furthermore, previous studies by our laboratory (23) and others (31) demonstrated that $CD4^+$ T cells play a central role in acquired immunity in mice infected with *P. chabaudi* (*chabaudi*) AS. In these studies, mice depleted of $CD4^+$ T cells *in vivo* by monoclonal antibody treatment were unable to control acute parasitemia and suffered chronic infection. In addition, several studies have provided evidence that acquired immunity to *P. chabaudi* (*chabaudi*) AS is established via activation of Th1 cells followed by subsequent activation of Th2 cells (12,29). In these studies, cytokine production by splenic $CD4^+$ T cells was determined by limiting dilution analysis or in bulk cultures either during the acute phase, when parasite multiplication and peak parasitemia occur, or during the chronic phase, when parasitemia declines to sub-patent levels.

In recent years, $CD4^+$ T cells have been classified into two distinctive subsets, based upon the repertoire of cytokines produced (17). Upon antigenic stimulation, Th1 cells produce IL-2, IFN-γ, and tumor necrosis factor beta (TNF-β), mediate delayed-type hypersensitivity and macrophage activation, whereas Th2

cells produce IL-4, IL-5, and IL-10, and provide help to B cells for antibody production. Differential production of cytokines by $CD4^+$ T cell subsets during an immune response has been shown to have important regulatory effects on the nature of the antibody response (26). In mice, immune responses in which IFN-γ production predominates are characterized by IgG2a production, whereas responses in which substantial IL-4 but little IFN-γ production occurs induce IgG1 and IgE with little IgG2a (4). It is likely that cytokine production and selective immunoglobulin isotypes distribution reflect direct effects of IL-4 and IFN-γ on B lymphocytes (7).

In the present report, we investigated the kinetics of *in vitro* production of the Th1 cell-derived cytokine, IFN-γ, and the Th2 cell-derived cytokines, IL-4, IL-5 and IL-10, by spleen cells harvested at various time during infection. In addition, the levels and isotypes of total and malaria-specific immunoglobulins in the serum collected from animals during blood-stage infection with *P. chabaudi* AS were determined.

Materials and Methods

Mice. C57BL/6 mice, 8-12 weeks old, were age-and sex-matched in all experiments. Mice were either purchased from Charles River (St. Constant, Quebec, Canada), or bred in our facilities.

Parasite. *P. chabaudi* AS was obtained from Dr. D. Walliker (University of Edinburgh, Edinburgh, Scotland). The parasite was maintained by weekly passage in female C57BL/6 mice. After 12 passages, a fresh inoculum was prepared and a new passage was initiated from frozen stock cultures which were stored at -70°C. For passage or infection of experimental animals, blood was

collected via the retro-orbital plexus from two infected C57BL/6 mice and pooled. Total erythrocyte (RBC) counts were determined. The percent parasitemia was determined by counting the percentage of parasitized RBC (PRBC) per 100 RBC on duplicate, Dif-Quik (American Scientific Products, McGaw Park, IL) stained thin blood smears. RBC, diluted in sterile phosphate-buffered saline (PBS), were adjusted to the desired concentration of PRBC and injected intraperitoneally (i.p.) into passage or experimental mice. For passage, mice were injected with a dose of 10^7 PRBC. Experimental infections were initiated with a dose of 10^6 *P. chabaudi* AS PRBC.

Determination of parasitemia. To determine the course of infection, blood samples were collected from experimental mice by bleeding via the tail vein at the times indicated. Duplicate thin blood smears were prepared and stained with Dif-Quik. Parasitemia was determined by counting the percentage of infected cells per 100 PRBC per slide. The parasitemia is expressed as mean percent PRBC ± standard error of the mean (SEM) for each group of mice.

Malaria antigens. Mice with high parasitemia (>50% PRBC) were bled by cardiac puncture. RBC were washed twice in Hanks' balanced salt solution (HBSS) without phenol red (GIBCO Laboratories, Grand Island, NY) to remove serum proteins. Packed RBC were lysed in 0.2% NaCl and then restored to isotonicity by addition of 1.6% NaCl. The preparation was sonicated in a Fisher Sonic Dismembrator (Fisher Scientific, Canada, Montreal, Quebec, Canada) until membranes were disrupted. Protein concentration was determined with Bio-Rad reagents (Bio-Rad Laboratories, Richmond, CA), using hemoglobin as a standard.

Spleen Cell Preparation. Spleens were aseptically removed and perfused with 10 ml of RPMI 1640 (Flow Laboratories, Inc., Mississauga, Ontario, Canada) supplemented with 5% heat inactivated fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah), 2% HEPES buffer (Flow) and 0.12% gentamicin. Cell suspensions were centrifuged at 350 x *g* for 10 minutes. RBC were lysed with cold NH_4Cl (0.17 M) and the cells were washed two times in fresh medium. Membrane debris was removed by filtering the cell suspension through sterile gauze. The viability was determined by trypan blue exclusion and was always greater than 90%. Spleen cells were adjusted to a final concentration of 5 x 10^6 cells/ml in complete RPMI 1640 prepared as described above. Aliquots of 3 ml in triplicate were incubated in 6 well flat-bottom tissue culture plates (Flow) with 10^6/ml washed PRBC. Control cultures were unstimulated. Forty eight hours later, supernatants were collected and centrifuged at 350 x g for 10 minutes. The supernatants were stored at -20°C until they were assayed for IFN-γ, IL-4, IL-5 or IL-10.

Cytokine ELISAs. Two-site sandwich enzyme-linked immunosorbent assays (ELISAs) were performed to quantify cytokines in the spleen cell culture supernatants. IFN-γ was detected as described previously (27), the coating antibody (Ab) was DB-1, a murine anti-rat monoclonal antibody (mAb), generously supplied by Dr. P. van der Meide, TNO Primate Centre, Rijswijk, The Netherlands. The detecting Ab was a polyclonal monospecific rabbit anti-mouse IFN-γ prepared in our laboratory by standard methods (27). Horseradish peroxidase conjugated (HRP) goat anti-rabbit IgG (Bio-Rad) was used as a secondary antibody and the reactions were visualized by incubating the plates with the substrate, 2, 2'-azino-di (3-ethyl-benz-thiazoline-6-sulfonic acid) (ABTS; Bio-Rad). IL-4 was detected as described by Mosmann et al (17). The two

antibodies used were rat anti-mouse IL-4 mAb BVB-4-1D11 (Pharmingen, San Diego, CA), and a biotinylated rat anti-mouse IL-4 mAb BVD-6 (Pharmingen). IL-5 was detected according to the protocol described by Schumacher et al (26). The two antibodies used were the rat anti-mouse IL-5 mAb TRFK-5 as the coating antibody and, as the secondary reaction site, biotinylated TRFK-4. IL-10 was detected as described by Mosmann et al (17), using immunobilized rat mAb anti-IL-10 (SXC-2) and biotinylated rat anti-IL-10 (SXC-1) as first and second mAbs, respectively. For IL-4, IL-5 and IL-10, streptavidin-horseradish peroxidase conjugate (GIBCO) was used as the secondary layer and the reactions were visualized with ABTS. Plates were read using an SLT Lab Instruments ELISA reader at 405 nm. Cytokine concentrations were calibrated from standard curves calculated on the basis of a known concentration of recombinant murine IFN-γ (Genzyme, Boston, MA) or murine IL-4 (Genzyme). Murine IL-5 was prepared in our laboratory from Con A stimulated spleen cells from mice infected with *Schistosoma mansoni* (a generous gift of Dr. J. Smith, Institute of Parasitology, McGill University, Ste. Anne de Bellevue, Quebec) and standardized by Dr. P. Scott (University of Pennsylvania, Philadelphia, PA). Recombinant murine IL-10 was obtained from COS-7 cells transfected with the IL-10 gene as described by Oswald et al (20).

Determination of serum immunoglobulin levels. Blood was collected from mice via cardiac puncture and allowed to clot for 10 minutes at room temperature. Sera was removed by microcentrifugation at 300 X *g* for 5 minutes, and individual samples were stored at -20°C. A sandwich ELISA was used to determine the total levels and *P. chabaudi AS*-specific isotypes (IgM, IgE and IgG subclasses) in the sera collected at various times after infection. Immune mice were

rechallenged two weeks after recovering from primary infection and hyperimmune sera obtained one week later.

Total immunoglobulin antibody ELISA. Twelve serial 10-fold dilutions, starting at 1:1000, of either purified immunoglobulin antibody standards (1µg/ml; Cedarlane, Hornby, Ontario, Canada) of IgM IgE and IgG subclasses or, serum samples in PBS (50 µl) were added to Immunol II plates (Dynatech, Quebec, Montreal, Canada) in duplicate, and incubated overnight at 4°C. Plates were washed three times with PBS-0.1% Tween 20 and blocked with 5% skim milk in d.d.H_2O and incubated for 1 hr at room temperature. After washing, 50 µl of goat anti-mouse immunoglobulin (Cedarlane) diluted 1:1000 for IgM, 1:2000 for IgG2a, and 1:4000 for IgG2b, rabbit anti-mouse IgG1 (Cedarlane), diluted 1:10, 000, or alkaline phosphatase conjugated rat anti-mouse IgE (Cedarlane), diluted 1:4000, in 1% BSA-PBS buffer were added per well and incubated for 1 hr at room temperature. Plates were washed as before, and 50 µl of a 1:3000 dilution in 1% BSA-PBS buffer of HRP-conjugated rabbit anti-goat IgM, IgG2a or IgG2b, (Cedarlane), or 1:3000 HRP-conjugated goat anti-rabbit IgG1 (Cedarlane) were added per well and incubated for 1 hr at room temperature. The specificity of each reagent was confirmed prior to use. Plates were washed as before, and 100 µl of ABTS substrate for IgM and IgG subclasses or p-nitrophenyl phosphate substrate for IgE added per well and optical density read using an ELISA reader at 405 nm.

***P. chabaudi* AS-specific antibody ELISA.** Immunolon II plates (Dynatech, Quebec, Montreal, Canada) were coated with 50 µl of malaria Ag (O.D 280 = 0.05) in bicarbonate buffer pH 9.6 and incubated overnight at 4°C. Plates were washed three times with PBS-0.1% Tween 20 and blocked with 5% skim milk in

d.d.H_2O and incubated for 2 hrs at room temperature. Aliquots of 50 µl 1:10, 1:100, 1:500, 1:1000, and 1:5000 dilutions of mouse sera in 1% BSA-PBS buffer were added and incubated for 2 hrs at room temperature. Plates were washed as before, and 50 µl of HRP-conjugated goat anti-mouse isotypes (Southern Biotechnology Associates, Inc., Birmingham, AL) at a 1:250 dilution for IgG1 and a 1:100 dilution for IgG2a in 1% BSA-PBS were added per well, and incubated for 1 hr at room temperature. The specificity of each reagent was confirmed prior to use. Plates were washed as before, the color was developed by adding 100 µl of ABTS substrate added per well, and the optical density read using an ELISA reader at 405 nm.

Statistical Analysis. Differences between control and experimental groups were analyzed by the Student *t* test. A probability of less than 0.05 was considered significant.

Results

Course of *P. chabaudi* AS infection.

The course of infection in *P. chabaudi* AS infected C57BL/6 mice can be divided into two phases, acute and chronic, according to the percentage of PRBC present in the peripheral blood (Figure 1). The acute phase of infection is characterized by appearance of the parasites (<1%) by day 3, followed by parasite multiplication and peak parasitemia of approximately 35-40% between day 7-10. Parasitemia declines by day 14. During the chronic phase, parasitemia is maintained at sub-patent levels through day 21 before being eliminated by day 28.

Production of Th1 and Th2 cytokines by spleen cells during infection.

The kinetics of cytokine production by spleen cells from *P. chabaudi* AS infected mice were investigated. Spleen cells harvested from groups of either normal or infected mice at various times were cultured *in vitro* with specific antigen, and supernatants were tested for the presence of the cytokines, IFN-γ, IL-4, IL-5 and IL-10. Previous studies from our laboratory demonstrated that PRBC stimulated spleen cells from *P. chabaudi* AS infected C57BL/6 mice produced maximum levels of IFN-γ several days before the peak parasitemia (28), while significant levels of IL-5 were produced on days 21 and 28 post infection (30). As shown in Figure 2A, following stimulation with PRBC, spleen cells from normal mice produced 1.22 ± 1.22 ng/ml of IFN-γ. Remarkably, on day 5 post infection, the spleen cell supernatants contained IFN-γ at levels approximately 50-fold higher than those of normal mice (60 ± 22.1 ng/ml; $P<0.001$). IFN-γ production declined by day 7 and remained at background levels on day 10 through day 28 post infection (Figure 2A). In this experiment, peak IFN-γ production occurred on day 5 which was 2 days before the animals exhibited a peak parasitemia.

The kinetics of production of IL-4 and IL-5 by PRBC-stimulated spleen cells from infected mice differed greatly from those observed for production of IFN-γ. Spleen cells from normal mice produced 3.17 ± 0.31 U/ml IL-4 and 0.157 ± 0.061 ng/ml IL-5 (Figure 2B and 2C). Production of IL-4 and IL-5 by spleen cells from infected mice was not significantly greater than that of normal mice during the acute phase of infection ($P>0.05$). However, IL-4 production was found to be slightly but significantly elevated during the chronic phase of infection, on days 14 (1.6-fold; $P<0.002$), and 21 (1.5-fold; $P< 0001$), but returned to normal levels on day 28 post infection (Figure 2B). As demonstrated in Figure 2C, IL-5

production was significantly increased on days 21 (2.5-fold; $P<0.005$) and 28 (7.5-fold; $P<0.001$) post infection.

The levels of IL-10 production by PRBC-stimulated spleen cells was also determined in supernatants of *P. chabaudi* AS infected mice at 7, 14 and 21 days post infection. As shown in Figure 3, upon antigenic stimulation, spleen cells from normal mice produced 3.66 ± 1.6 U/ml IL-10. At 7 days post infection, however, spleen cells from infected mice produced levels of IL-10 more than 6-fold higher than the spleen cells from normal mice ($P<0.001$). In contrast, at 14 and 21 days post infection, the supernatants from infected mice contained only background levels of IL-10 similar to that spleen cells from normal mice.

Kinetics of immunoglobulin isotype production during infection.

The levels of total IgG2a, IgM, IgG1 and IgG2b immunoglobulin isotypes in the sera of *P. chabaudi* AS infected C57BL/6 mice were determined during the acute and chronic phases of infection (Figure 4). In this experiment, peak parasitemia occurred on day 10 post infection. As shown in Figure 4A, analysis of the total IgG2a levels in sera of infected mice demonstrated significantly increased production as early as day 5 ($P<0.0001$) and peak production on day 7 post infection ($P<0.0001$). Total IgG2a levels were decreased by day 10 but remained significantly higher than normal ($P<0.01$) and persisted at this level throughout the chronic phase of infection ($P<0.0001$). As demonstrated in Figures 4B and 4C, the levels of total IgM and IgG1 in sera of infected mice during the acute stage of infection were not significantly different from those of controls ($P>0.05$), except for IgG1 on day 10 post infection ($P<0.002$). However, significantly higher levels of total IgM and IgG1 were observed during the chronic phase of infection ($P<0.0001$). In striking contrast, the total IgG2b levels (Figure 4D) were significantly lower during the acute phase of infection as the

percentage of PRBC became sharply elevated (P<0.0001). However, the levels of total IgG2b returned to normal by day 14 but were significantly higher than normal on days 21 and 28 post infection (P<0.0001). IgE antibody was not detectable in the sera of either normal or infected C57BL/6 mice at any time during blood-stage *P. chabaudi* AS.

The titers of malaria antigen-specific IgG2a and IgG1 antibodies were determined in the sera from either infected mice during primary infection at two time points, the peak parasitemia on day 10 (35-40% PRBC), and on day 21 when animals had almost cleared the infection (2-3% PRBC), or from hyperimmune mice. As shown in Figure 5, malaria antigen-specific IgG2a and IgG1 antibodies levels were detected during primary infection on day 10. The results show that 5 of 8 mice (62.5%) have high titers (1:5000) of *P. chabaudi* AS specific IgG2a and IgG1 isotypes. Furthermore, malaria-specific IgG2a and IgG1 antibodies were still present at high titers in the sera of a majority of animals (75%) on day 21 post infection. In contrast, upon rechallenge infection, 100% (8 of 8) of the mice had high titers of malaria-specific IgG2a antibodies, while only 50% (4 of 8) of mice had high titers of IgG1 (Figure 5).

Discussion

Previous studies from our laboratory have demonstrated that IFN-γ and IL-5 are reciprocally produced during blood-stage *P. chabaudi* AS in resistant C57BL/6 mice (30). The results of these studies showed that activation of Th1 cells with production of IFN-γ by spleen cells in response to Con A and malaria antigen followed by activation of Th2 cells with production of IL-5 occurs during *P. chabaudi* AS infection in C57BL/6 mice. Furthermore, these studies clearly delineate splenic $CD4^+$ T cells as the source of IFN-γ, while both $CD4^+$ and

$CD8^+$ T cells were found to produce IL-5 upon *in vitro* and *in vivo* depletion of T cell subsets. The general conclusion to be drawn from these studies and those previously reported by other investigators (12) is that resolution of primary infection with *P. chabaudi* AS occurs via activation of Th1 cells during the acute phase and subsequent activation of Th2 cells during the chronic phase of infection.

In the present study, we have extended these findings by characterizing production of other Th2 derived cytokines, IL-4 and IL-10, during infection in the supernatants of spleen cells stimulated with parasite antigens. Our results show that spleen cells produced significantly high levels of IL-4 at 2 and 3 weeks post infection while high levels of IL-10 were produced early, that is, at 1 week post infection, in response to parasite antigens. IL-4 known as B cells growth factor, stimulates both proliferation and differentiation of B cells, and induces isotype switching to IgG1 and IgE (4). IL-10, which is produced by B cells (18), and macrophages (15) as well as Th2 cells (16), likewise augment B cell proliferation and differentiation and also inhibits antigen-specific proliferation of Th1 cells and their cytokine production and, therefore, favours differentiation of Th2 cells (15). IL-10 down-regulates certain macrophage functions, such as, expression of major histocompatibility complex class II molecules and the antigen presenting function of these cells and, thus, indirectly inhibits IFN-γ production by Th1 cells (5,15). In addition, Oswald et al (20) have shown that IL-10 inhibits the microbicidal activity of IFN-γ activated macrophages against *Schistosoma mansoni*. While the cellular source of IL-10 was not investigated in this study, we speculate that macrophages and B cells contribute to production of this cytokine, since these cells have been shown to be activated, at least during the acute phase of infection. In this regard, previous studies from our laboratory demonstrated that splenic macrophages are activated during the acute phase of *P. chabaudi* AS

infection and this activation is mediated by Th1 cell-derived IFN-γ (29). Moreover, as demonstrated here and in other studies (11), B cells are activated resulting in the production of polyclonal and malaria-specific antibodies. Furthermore, recent studies have shown that B-cell depleted (anti-μ treated) mice infected with *P. chabaudi chabaudi* (AS) display a Th1 response and are deficient in the production of IL-10 in comparison to normal mice (33). These investigators suggested that B-cell derived IL-10 may be an essential component for the shift from a Th1 toward a Th2 response that takes place during infection in normal mice. Thus, in the present study, it appears that production of high levels of IL-10 by spleen cells early in infection (Figure 3) down-regulates production of the Th1 derived cytokine, IFN-γ (Figure 2A), and initiates a Th2 response as demonstrated by production of IL-4 and IL-5 later in infection (Figure 2B and 2C).

It is of interest to note that IL-10 has recently been reported to be elevated in the plasma of patients with severe or mild malaria (22). In this study, the circulating levels of IL-10 among patients with severe *P. falciparum* infection were found to be higher than patients with mild infection. Nevertheless, the levels of circulating IL-10 decreased markedly in patients after anti-malarial treatment. The authors concluded that there is a correlation between the presence of circulating IL-10 and the appearance of clinical symptoms.

The data described here also demonstrate that *P. chabaudi* AS infection in C57BL/6 mice induces poly-isotypic hypergammaglobulinemia. As demonstrated in Figure 4, during the acute phase of infection when rapid parasite multiplication and peak parasitemia occur, the total IgG2a isotype predominated and thereafter persisted at high levels during the chronic phase of infection. In contrast, significant increases of all the other isotypes occurred two weeks post infection during the chronic phase, when parasitemia declined and remained at sub-patent

levels. Neither total nor malaria-specific IgE antibody was detectable in the sera of infected C57BL/6 mice (data not shown). Our results are similar to those recently reported that IgE is undetectable in *P. chabaudi* infected mice (33). However, IgE have been detected in individuals infected with *P. falciparum* (2).

A number of studies have extensively described polyclonal activation of B lymphocytes in a variety of *Plasmodium* infections in man and in experimental rodent models (6,9,10,11). Polyclonal immunoglobulin production during blood-stage malaria was found to be T cell-dependent (9,10) and to vary with the species of *Plasmodium* (10,11,23). Primary infection in mice with the virulent strain of *P. yoelii* (17XL) was found to induce high levels of all immunoglobulin isotypes (32). In contrast, infection by the avirulent strain of *P. yoelii* (17XNL) or *P. chabaudi adami* was found to induce a highly polyclonal activation of IgM and IgG subclasses, in which the IgG2a isotype was shown to predominate (10,11). The poly-isotypic hypergammaglobulinemia observed to occur in the present study during the course of *P. chabaudi* AS infection in C57BL/6 mice is consistent with previous studies by Langhorne et al (11) which demonstrated polyclonal B cell activation in *P. chabaudi adami* infected mice.

Titers of malaria-specific IgG2a and IgG1 isotypes were found to be high in sera obtained during primary infection as well as from hyperimmune mice. While the frequency of malaria-specific IgG2a and IgG1 was equally distributed among animals during the acute and chronic phases of infection, the pattern of distribution of these isotype differed in animals undergoing rechallenge infection. High titers of malaria-specific IgG2a were found in all animals, whereas only 50% of animals had high titers of IgG1. Furthermore, our results obtained with hyperimmune mice are consistent with earlier studies by Langhorne et al (10) which demonstrated the presence of malaria-specific IgG1 and IgG2a in the sera of hyperimmune C3H mice infected with *P. chabaudi adami*. Our results, and

those reported by Langhorne et al (10), also demonstrate the presence of malaria-specific IgG2a during the entire course of infection. In contrast, in their studies, malaria-specific IgG1 was low or almost absent. However, the presence of IgG1 isotype in the sera of mice infected with *P. chabaudi* has been reported elsewhere (33).

Previous studies by Taylor et al (32) demonstrated that antibody responses to *P. yoelii* 17XNL in 11 inbred strains of mice varied in their isotypic response to the parasite. More specifically, while all strains produced IgM, IgG2, and IgG3 antibodies, only 3 of 11 strains produced detectable anti-*P. yoelii* of the IgG1 isotype during primary infection with this parasite. The disparity in our results and those obtained by Langhorne et al (10) in IgG1 antibody production may be explained by the difference in the mouse strains or dose of infectious inoculum used. Indeed, in studies reported here, C57BL/6 mice were used and infected with a dose of 10^6 *P. chabaudi* AS, whereas in studies obtained by Langhorne et al (10) C3H mice were used and infected with a dose of 10^4 *P. chabaudi adami.*

The results of the present study may reflect the effect of T cell subset derived cytokines on B cells for enhanced antibody production during infection with *P. chabaudi* AS. A Th1 response with high levels of IFN-γ production correlates with enhanced production of both polyclonal and malaria-specific IgG2a during the acute phase of infection. However, significant levels of IgG2a persisted during the chronic phase of infection in spite of a Th2-biased response by spleen cells. $CD4^+$ T cells in other organs, such as the liver and lymph nodes, may have produced IFN-γ which stimulated B cells for production of IgG2a during the chronic phase of infection. In this regard, evidence for differential induction of $CD4^+$ T cell subsets by different lymphoid organs has been reported (24). Another possibility could be due to the contribution of $CD8^+$ T cells, natural killer cells (3), or T cells with gamma delta receptors (13) which have been shown

to be increased during the course of infection. Previous studies by our laboratory demonstrated the importance of $CD8^+$ T cells during the chronic phase of infection, such that CD8-depleted mice had prolonged and significant levels of parasitemia during the chronic phase of infection (23). Alternatively, polyclonal and specific B-cell stimulation during *P. chabaudi* AS infection may be mediated by mechanisms other than the effect of cytokines on B cells for antibody production as reported in murine model of *Trypanosomasis* (14). Interestingly, rechallenge of immune mice results in enhanced titers of malaria-specific IgG2a, suggesting the preferential synthesis of IgG2a in hyperimmune mice. More recently, it has been shown that passive transfer of fractionated hyperimmune sera significantly modulated the course of infection with *P. yoelii* (35). Results of this study demonstrated that the fraction containing the IgG2a isotype but not the IgG1 isotype is important for passive transfer of protective immunity in mice infected with *P. yoelii*. It would be interesting to investigate which type of T helper subset predominate in C57BL/6 mice rechallenged with *P. chabaudi* AS and to further identify the protective immunoglobulin isotype in hyperimmune sera.

In summary, the results of the present study confirm previous observations that resolution of *P. chabaudi* AS blood-stage malaria is mediated by activation of Th1 cells during the acute phase of infection and subsequent activation of Th2 cells during the chronic phase. Furthermore, these results suggest that early production of IL-10 by spleen cells may be a key factor in switching from a Th1 to a Th2 response during the course of infection. Our data also demonstrate that activation of both Th1 and Th2 cells is associated with poly-isotypic hypergammaglobulinemia during blood-stage *P. chabaudi* AS malaria in C57BL/6 mice.

Acknowledgment

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Figure 1. Course of blood-stage *P. chabaudi* AS infection in C57BL/6 mice. Groups of 4-6 C57BL/6 mice in two experiments were infected i.p. with 10^6 *P. chabaudi* AS PRBC. Parasitemia was determined by quantitating the percent PRBC in the peripheral blood on the days indicated. The parasitemia is expressed as the Mean percent PRBC of data pooled from the two experiments. Standard Error of the Mean (SEM)<10%.

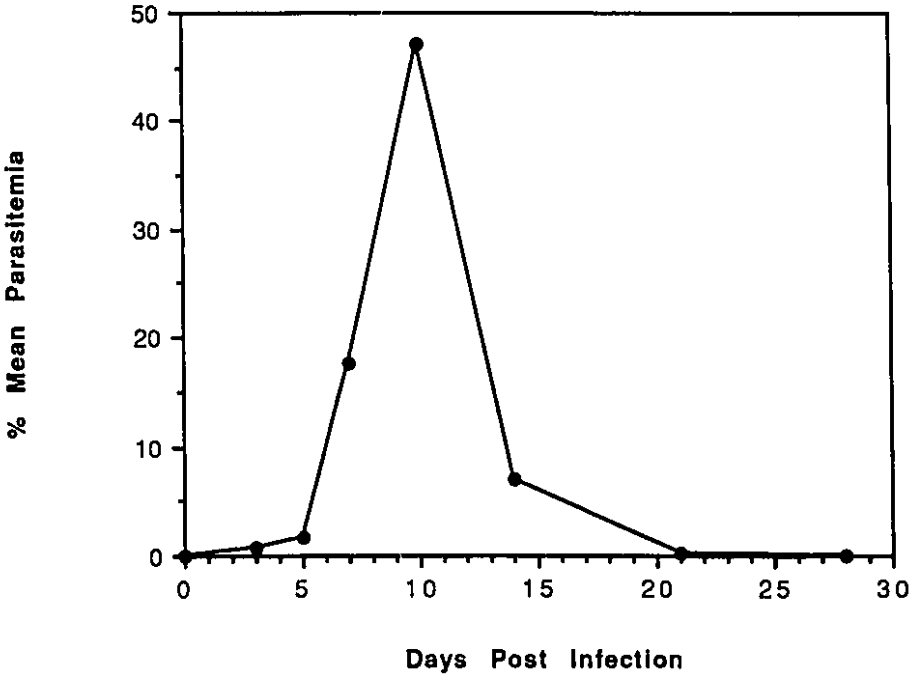

% Mean Parasitemia
50
40
30
20
10
0
0
5
10
15
20
25
30
Days Post Infection

Figure 2. *In vitro* cytokine production by spleen cells from *P. chabaudi* AS infected C57BL/6 mice. Single cell suspensions of spleen cells from normal or *P. chabaudi* AS infected mice were stimulated *in vitro* with malaria antigen (10^6 PRBC/ml). Supernatants were collected 48 hrs later and assayed for IFN-γ (A), IL-4 (B), and IL-5 (C) by sandwich ELISA. Data represent the Mean ± Standard Error of the Mean (SEM) for 5-6 individual mice per time point in 2 separate experiments.

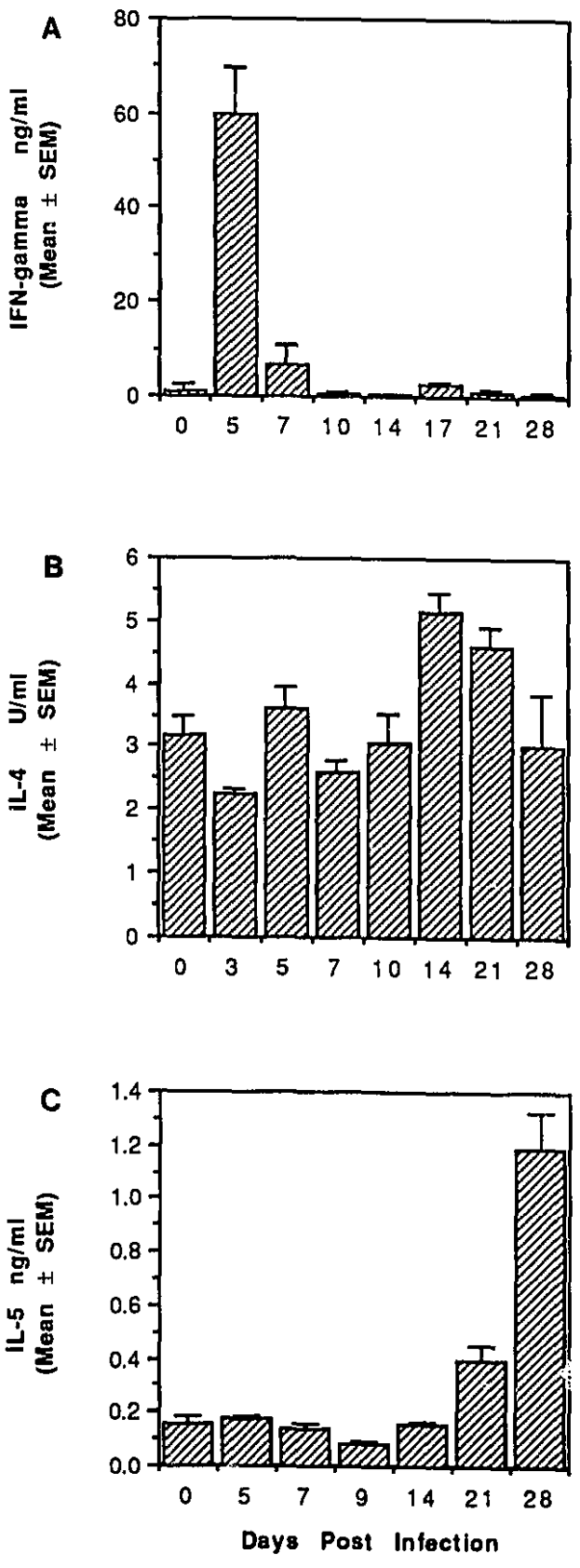

A
IFN-gamma ng/ml
(Mean ± SEM)
80
60
40
20
0
0 5 7 10 14 17 21 28
B
IL-4 U/ml
(Mean ± SEM)
6
5
4
3
2
1
0
0 3 5 7 10 14 21 28
C
IL-5 ng/ml
(Mean ± SEM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 5 7 9 14 21 28
Days Post Infection

Figure 3. *In vitro* production of IL-10 by spleen cells from *P. chabaudi* AS infected C57BL/6 mice. Single cell suspensions of spleen cells from normal or *P. chabaudi* AS infected mice were stimulated *in vitro* with malaria antigen (10^6 PRBC/ml). Supernatants were collected 48 hrs later and assayed for IL-10 by sandwich ELISA. Data represent the Mean ± Standard Error of the Mean (SEM) for 5-6 individual mice per time point in 2 separate experiments.

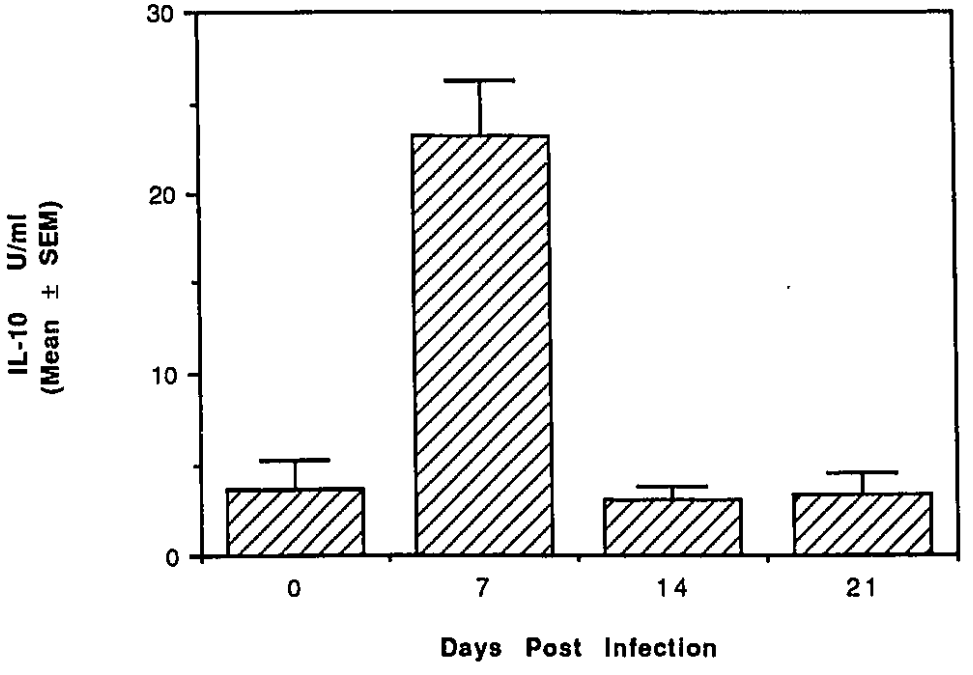

30
20
10
0
IL-10 U/ml
(Mean ± SEM)
0
7
14
21
Days Post Infection

Figure 4. Kinetics of total immunoglobulin isotype production during infection with *P. chabaudi* AS infection in C57BL/6 mice. Sera were collected on the days indicated and total IgG2a (A), IgM (B), IgG1 (C), and IgG2b (D) levels were determined by ELISA. The values represent the Mean ± Standard Error of the Mean (SEM) of 4-6 individual mice per time point.

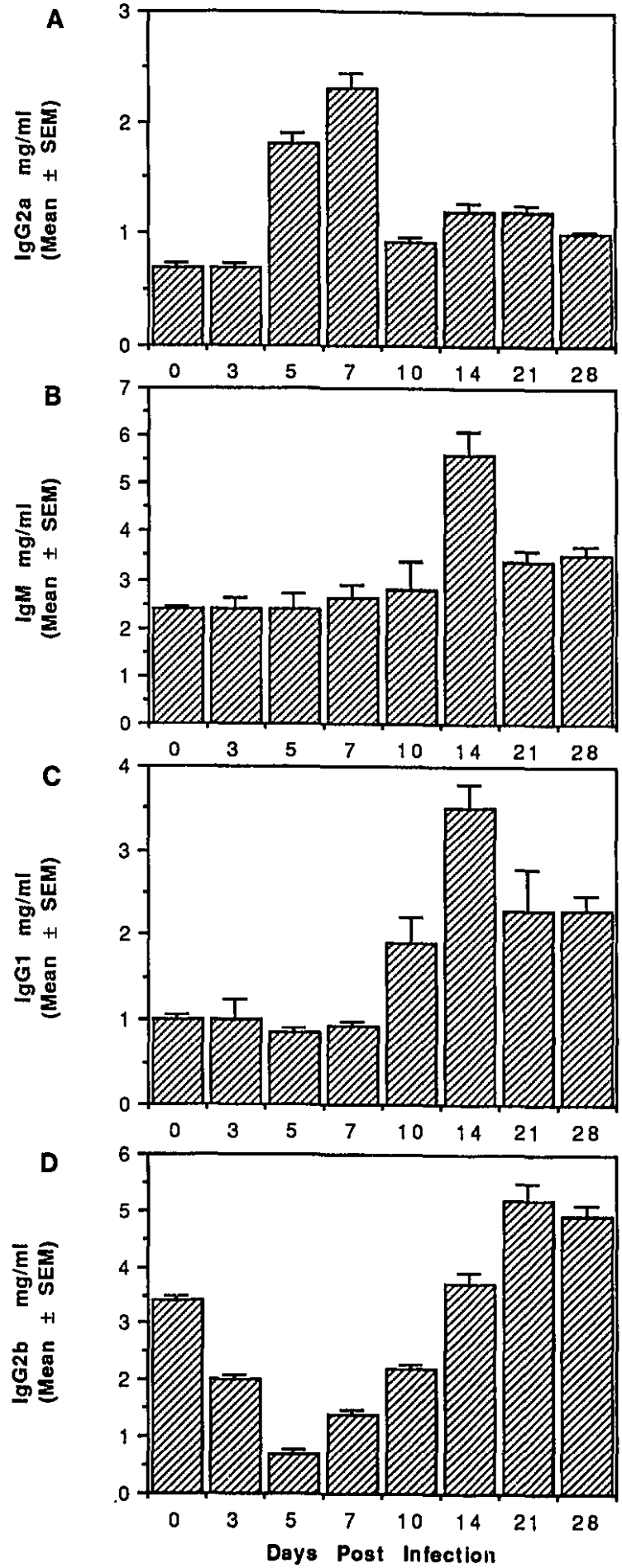
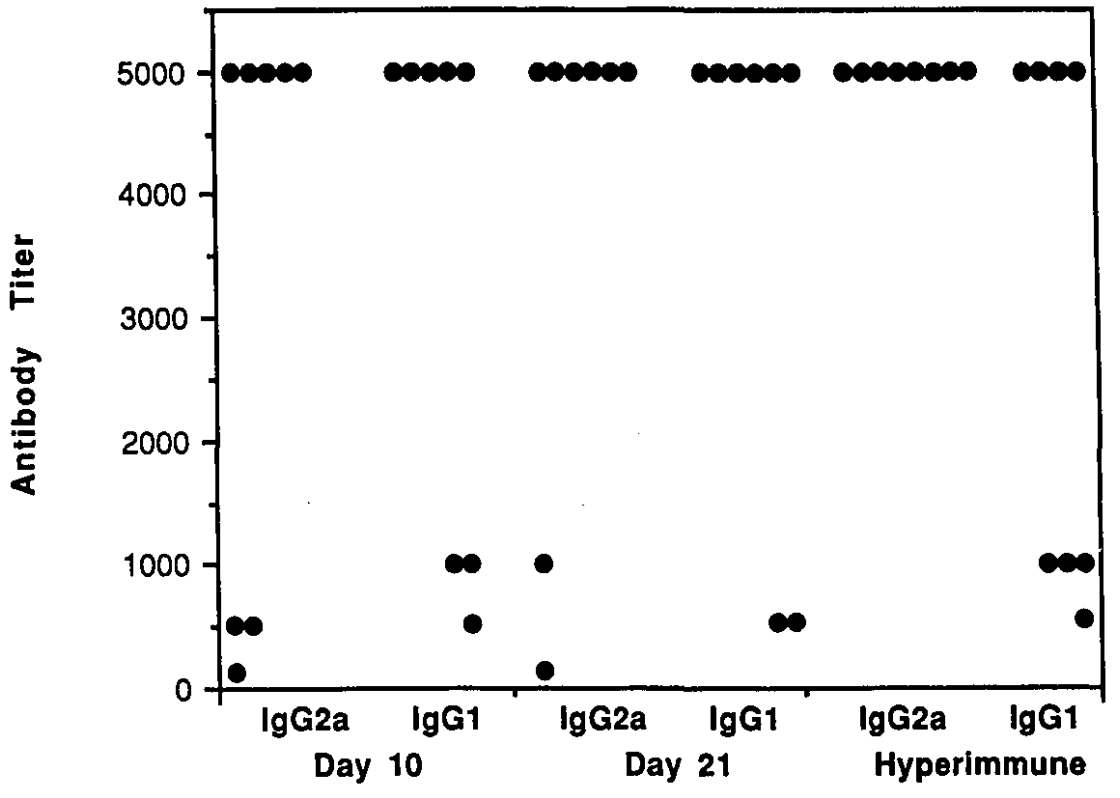

A
IgG2a mg/ml
(Mean ± SEM)
3
2
1
0
0 3 5 7 10 14 21 28
B
IgM mg/ml
(Mean ± SEM)
7
6
5
4
3
2
1
0
0 3 5 7 10 14 21 28
C
IgG1 mg/ml
(Mean ± SEM)
4
3
2
1
0
0 3 5 7 10 14 21 28
D
IgG2b mg/ml
(Mean ± SEM)
6
5
4
3
2
1
0
0 3 5 7 10 14 21 28
Days Post Infection

Figure 5. Kinetics of malaria-specific immunoglobulin isotype production during *P. chabaudi* AS infection in C57BL/6 mice. Sera were collected on day 10, at the time of peak parasitemia (35-40% PRBC), and on day 21 (2-3% PRBC), or from hyperimmune mice. Malaria-specific antibodies were determined by ELISA. The titers of antibodies in sera from individual mice are expressed as the reciprocal of the highest serum dilution at which the optical density was above that of normal mice. Sera from 8 individual mice were used per each time point.


Antibody Titer
5000
4000
3000
2000
1000
0
IgG2a
IgG1
IgG2a
IgG1
IgG2a
IgG1
Day 10
Day 21
Hyperimmune

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CONNECTING STATEMENT

Studies in Chapter III demonstrate that distinct host immune responses are regulated during *P. chabaudi* AS infection, including production of the Th1-derived cytokine, IFN-γ. The following study investigates the contribution of nitric oxide and prostaglandins, products of IFN-γ activated macrophages, in suppression of non-specific and specific immune responses during the acute phase of infection.

CHAPTER IV

Role of Macrophage-Derived Nitric Oxide in Suppression of Lymphocyte Proliferation During Blood-Stage Malaria†

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Running Title: NO and Malarial Immunosuppression

Key Words: *Plasmodium chabaudi* AS, mice, immunosuppression

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Abstract

Examination of the proliferative responses *in vitro* to mitogens (Con A, PHA, LPS) of spleen cells recovered from C57BL/6 mice during blood-stage *Plasmodium chabaudi* AS infection revealed that the most severe suppression occurred during the first 14 days post infection. This time period is coincident with the acute phase of infection and with *in vitro* production of high levels of NO by peritoneal and splenic macrophages. Therefore, the roles of NO as well as prostaglandins, molecules previously found to mediate immunosuppression during parasitic infections, were investigated in the suppression of proliferation to mitogens and specific antigen of spleen cells from 7 and 14 day *P. chabaudi* AS infected mice. Addition of either 0.5 mM N^G-monomethyl-L-arginine (L-NMMA) or 0.5 mM aminoguanidine (AG), specific inhibitors of NO synthase, or 10 μg/ml indomethacin (INDO), a prostaglandin inhibitor, partially but significantly abrogated the suppression in response to Con A and PHA. Only the addition of INDO significantly increased the responses to LPS. Addition of L-NMMA or AG in combination with INDO partially but significantly abrogated the suppression to Con A and completely abrogated the suppression in response to PHA. The addition of L-NMMA or AG also significantly increased proliferation in response to parasite antigen. The contribution of NO to suppression of lymphoproliferation was confirmed by adding sodium nitroprusside (SNP) or 3-morpholino-sydnonimine-hydrochloride (Sin-1), chemical generators of NO, to CON A- or PHA-stimulated splenocyte cultures prepared from normal mice. The mechanism of NO mediated suppression was investigated in co-culture experiments using spleen cell from normal mice and peritoneal macrophages from either normal or day 7 infected mice. The addition of 5-10 x 10^4 peritoneal macrophages from infected mice significantly and consistently suppressed Con A- or PHA-stimulated proliferation of normal splenocytes. Moreover, suppression

correlated with production of NO and could be reversed by the addition of L-NMMA or AG. These results suggest that, in addition to PG, increased NO production by macrophages within the first 2 weeks after infection with *P. chabaudi* AS contributes to immunosuppression associated with blood-stage malaria.

Introduction

Malaria, due to infection with hematoprotozoan parasites of the genus *Plasmodium*, is acknowledged to be by far the most important tropical parasitic disease, causing enormous morbidity and mortality throughout the Third World. Suppression of non-specific and specific immune responses is a common occurrence during acute blood-stage malaria and both T and B cell mediated responses are suppressed. It has been known for many years that children with acute *P. falciparum* malaria have impaired antibody responses to unrelated antigens and are more susceptible to gastrointestinal and respiratory infections than normal children (1-3). Numerous studies have reported suppression of *in vitro* lymphoproliferative responses in individuals with acute *P. falciparum* infection to malaria antigens and mitogens as well as decreased lymphoproliferative responses and delayed type hypersensitivity responses to unrelated antigens (4-7). Similar observations concerning immunosuppression have been reported in experimental rodent models of malaria. Antibody responses to sheep red blood cells and unrelated antigens have been shown to be severely reduced in malaria infected mice (8-11). Spleen cells from malaria infected rats and mice are deficient in their ability to proliferate in response to mitogens (12,13). In addition, delayed type hypersensitivity responses to unrelated antigens are impaired in *P. berghei* infected mice (10).

Although the mechanism(s) of malaria-induced immunosuppression is not yet fully understood, macrophages have been implicated to play an important role (14-17). It has been proposed that macrophages from infected patients or animals contribute to malarial immunosuppression due to defective antigen presentation, altered production of soluble immunoregulatory factors and production of soluble inhibitory factors, such as prostaglandins (14,18-20). Nitric oxide (NO), an L-arginine-derived, cytotoxic and anti-microbial product of cytokine-activated

macrophages, has also been demonstrated to contribute to suppression of lymphoproliferation in response to the mitogen, Con A, in normal rats and mice (21-23). Furthermore, recent evidence has shown that NO plays a role in suppression of lymphocyte proliferation in mice infected with a variety of pathogenic microorganisms including *Trypanosoma brucei* (24,25), *Listeria monocytogenes* (26), *Salmonella typhimurium* (27) and *Toxoplasma gondii* (28).

Previous results from our laboratory demonstrated that antibody responses to sheep erythrocytes are severely suppressed in C57BL/6 mice during blood-stage infection with *P. chabaudi* AS (11). In this report, we extended this observation by examining the proliferative responses *in vitro* to mitogens of spleen cells recovered from these mice during infection. Since the most severe suppression was found to occur during the acute phase of infection, we investigated the underlying mechanisms of suppression of spleen cell proliferation during this time. In a separate study in our laboratory, peritoneal and splenic macrophages from *P. chabaudi* AS infected C57BL/6 mice were found to produce high levels of NO during the first 2 weeks post infection, with the maximal production occurring at the time of peak parasitemia (Jacobs, P., Yap, G.S., Radzioch, D., Stevenson, M.M. Manuscript in preparation). Therefore, we investigated the roles of NO and prostaglandins. Our results confirm previous observations concerning the role of prostaglandins in inhibiting lymphoproliferation during acute blood-stage malaria and demonstrate a role for macrophage-derived NO in malaria-induced suppression of lymphocyte proliferation to mitogens and specific antigen.

Materials and Methods

Mice

C57BL/6 mice, 8-12 weeks old, were age- and sex-matched in all experiments. Mice were either purchased from Charles River (St. Constant, Quebec, Canada) or were bred in our animal facility from breeding pairs obtained from Jackson Laboratories (Bar Harbor, MN).

Parasite

P. chabaudi AS was maintained by weekly passage as previously described (29). For passage or infection of experimental animals, blood was collected via the retro-orbital plexus from two infected C57BL/6 mice and pooled. Total erythrocyte (RBC) counts were determined. The percent parasitemia was determined by counting the percentage of parasitized RBC (PRBC) per 100 RBC on duplicate, Dif-Quik (American Scientific Products, McGaw Park, IL) stained thin blood smears. RBC, diluted in sterile phosphate-buffered saline (PBS), were adjusted to the desired concentration of PRBC and injected intraperitoneally into passage or experimental mice. For passage, mice were injected with a dose of 10^7 PRBC. Experimental infections were initiated with a dose of 10^6 *P. chabaudi* AS PRBC.

Determination of parasitemia

To determine the course of infection, blood samples were collected from experimental mice by bleeding via the tail vein at the times indicated. Duplicate thin blood smears were prepared and stained with Dif-Quik. Parasitemia was determined by counting the percentage of infected cells per 100 PRBC per slide. The parasitemia is expressed as mean percent PRBC ± standard error of the mean (SEM) for each group of mice.

Spleen cell proliferation assay

Spleens were aseptically removed at various time after infection and perfused with 10 ml of RPMI 1640 (Flow Laboratories, Inc., Mississauga, Ontario) supplemented with 5% heat-inactivated fetal calf serum (Hyclone Laboratories, Inc., Logan, UT), 2% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Flow), and 0.12% gentamicin (Schering Canada Inc., Montreal, Quebec, Canada). Cell suspensions were centrifuged at 350 x *g* for 10 m. RBC were lysed with cold NH_4Cl (0.17 M), and the cells were washed twice in fresh medium. Membrane debris was removed by filtering the cell suspension through sterile gauze. The viability was determined by trypan blue exclusion and was routinely >90%. Spleen cells were adjusted to a final concentration of 2.5 x 10^6 cells per ml in RMPI 1640 supplemented as described above. Aliquots of 0.1 ml of cell suspensions in triplicate or sextuplicate were placed into 96-well flat-bottom tissue culture plates (Linbro; Flow), and 0.1 ml of medium alone or medium containing either 1 μg of concanavalin A (Con A; Calbiochem, La Jolla, Calif) per ml, a 1:10 dilution of phytohemagglutinin (PHA; Difco, Detroit, MI), 50 μg of *Escherichia coli* lipopolysaccharide (LPS; Difco) per ml, or 10^6 washed PRBC per ml was added to triplicate wells and incubated for 72 hr at 37°C, 5% CO_2 in moist air. During the last 16 h of culture, 1 μCi of [^{3}H] thymidine (specific activity, 1 Ci/mmol) in 10 μl of complete medium was added to each well. Cells were harvested with an automatic cell harvester (Flow). Incorporated radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments, Inc., California). Data are expressed as mean counts per minute (cpm) ± SEM. In some experiments, the stimulation index was calculated by dividing the mean cpm for replicate cultures of spleen cells stimulated with mitogen or antigen by the mean cpm of cells cultured with medium alone.

Preparation of macrophage-spleen cell co-cultures

Resident peritoneal macrophages were harvested from normal or day 7 infected mice by peritoneal lavage. Cells were suspended in Dulbecco's modified Eagle medium (GIBCO Laboratories, Grand Island, New York) containing 10% fetal calf serum (HyClone) and 0.12% gentamicin. Total and differential counts were determined and the cells were adjusted to the desired concentration of macrophages. Aliquots of 0.1 ml of cell suspensions at a final concentration of 5, 25, 50 or 100 x 10^3 macrophages were added in triplicate to replicate 96-well flat-bottom tissue culture plates (Linbro; Flow), and allowed to adhere for 2 h at 37°C in an atmosphere of 5% CO_2 in air. Nonadherent cells were removed by washing 3 times with warm Hank's balanced salt solution (HBSS; GIBCO). Aliquots of 0.1 ml of spleen cells, harvested from normal mice as described above and adjusted to 2.5 x 10^6 cells per ml, and 0.1 ml of medium or mitogens, as described above, were added to the macrophage monolayers. One plate was used to determine spleen cell proliferation. Supernatants from the second plate were harvested at 72 h to determine NO_2^- accumulation as described below.

Reagents

Experiments were carried out either in the absence or in the presence of 0.5 mM N^G-monomethyl-L-arginine (L-NMMA; Calbiochem Corp., LaJolla, CA), a specific inhibitor of nitric oxide synthase (NOS), 0.1-0.5 mM aminoguanidine (AG; Sigma Chemical Co., St Louis, MO), a specific inhibitor of the inducible NOS (30) or 10 μg/ml indomethacin (INDO; Sigma), an inhibitor of prostaglandins. Exogenous NO was generated by the addition of sodium nitroprusside (SNP; Sigma) or 3-morpholino-sydnonimine-hydrochloride (SIN-1; 31), a generous gift from Dr. I. Oswald, NIAID, NIH, Bethesda MD, at the concentrations indicated.

NO_2^- determination

Aliquots of 50 µl of supernatants collected from macrophage monolayers were transferred to a 96 well flat-bottom plate and allowed to react with 50 µl of Greiss reagent for 10 m at room temperature. Greiss reagent was prepared by mixing equal volumes of 0.1% *N*-(1-naphthyl) ethylenediamine (Sigma) and 1% sulfanilamide (Sigma) in 5% orthophosphoric acid. Absorbance at 550 nm was measured using a microplate reader (Flow). For each experiment, a standard curve was prepared using $NaNO_2$.

Statistical Analysis

Differences between control and experimental groups were analyzed by the Student *t* test. A probability of less than 0.05 was considered significant. All experiments were repeated one or more times with similar results.

Results

Kinetics of Immunosuppression During *P. chabaudi* AS Infection

Following intraperitoneal infection with 10^6 *P. chabaudi* AS PRBC, C57BL/6 mice exhibit a course of infection characterized by a prepatent period of approximately 3 days, when there are <1% PRBC in the peripheral blood, followed by rapid parasite multiplication and a moderate peak parasitemia (35-40%) occurring between days 8-10 post infection (Figure 1A). In this mouse strain, which is resistant to blood-stage *P. chabaudi* AS, crisis occurs and there is a rapid decrease in parasitemia to sub-patent levels (32). The parasite is cleared by 3-4 weeks post infection and the animals are immune to challenge infection.

The *in vitro* proliferation of spleen cells from C57BL/6 mice in response to the mitogens Con A, PHA and LPS was determined at various times during *P.*

chabaudi AS infection. Data are presented in Figure 1B as per cent normal based on a comparison of the stimulation indices of spleen cells from normal and infected mice. Between days 5 and 14 post infection, there was severe suppression of proliferation to both T and B cell mitogens such that the responses were less than 20% of normal. Despite the fact that the parasites had almost been totally cleared from the peripheral blood by day 21, the responses were approximately half the normal response. The responses were, however, returning to normal levels by day 28 post infection.

Role of NO in Immunosuppression During Blood-Stage Malaria

As described above, the most severe suppression of proliferative responses to mitogens was observed between days 5 and 14, during the acute phase of infection. This time period is coincident with peak parasitemia, crisis and the rapid decrease in parasitemia levels to sub-patent levels as well as with high levels of NO production by splenic and peritoneal macrophages (Jacobs, P., Yap, G.S., Radzioch, D., Stevenson, M.M. Manuscript in preparation). To investigate the role of NO in the suppression of lymphocyte proliferation during acute blood-stage malaria, the effect of L-NMMA on spleen cell proliferation to Con A, PHA and LPS was examined. The effect of INDO, a prostaglandin inhibitor, was also examined, either alone or in combination with L-NMMA. The results of a representative experiment for spleen cells from normal and day 7 or day 14 infected mice are shown in Figure 2. As described above, proliferation to Con A, PHA and LPS of spleen cells from mice recovered at these times post infection was significantly less ($P<0.005$) than the responses of normal mice in the control cultures with medium plus mitogen (Figure 2A-C). The addition of L-NMMA or INDO, either alone or in combination, significantly increased ($P<0.005$) the proliferative responses to Con A of spleen cells from infected mice while only the

addition of INDO in combination with L-NMMA significantly increased ($P<0.001$) the response of spleen cells from normal animals (Figure 2A). Similarly, the addition of either L-NMMA or INDO significantly increased ($P<0.001$) the response to PHA of spleen cells from infected mice (Figure 2B). The addition of a combination of L-NMMA and INDO was found to fully restore the PHA response of cells from infected animals to normal levels. Although we used doses of L-NMMA and INDO which did not effect the proliferation of normal cells to Con A (22,23), we consistently observed that the addition of L-NMMA or INDO, alone or in combination, significantly decreased the response of normal spleen cells to PHA in comparison to the medium control. The addition of INDO but not L-NMMA significantly increased ($P<0.005$) the response to LPS of spleen cells from infected mice such that the level of proliferation was comparable to that of cells from normal mice cultured with medium and LPS. In this experiment, the addition of INDO to cultures of spleen cells from normal mice was also observed to significantly increase ($P<0.005$) proliferation to LPS.

The effect of AG, a specific inhibitor of inducible NOS (30), on proliferation of spleen cells from *P. chabaudi* AS infected mice was also examined. Preliminary studies demonstrated that AG was effective in significantly increasing the proliferative response to Con A or PHA of spleen cells from infected mice over a range of doses from 0.1 mM to 1.0 mM, doses which did not significantly alter the responses of cells from normal mice (data not shown). As we observed with L-NMMA, the addition of 0.5 mM AG to spleen cell cultures from day 7 infected mice significantly increased proliferation in response to both Con A and PHA (Table 1).

To confirm the role of NO in suppression of lymphocyte proliferation to Con A or PHA, NO was generated *in vitro* by the addition of SNP to splenocyte cultures prepared from normal mice. Proliferation in response to Con A was

completely inhibited in the presence of SNP at concentrations ≥0.01 mM ($P<0.008$; Figure 3A). A similar SNP dose response was obtained in PHA stimulated spleen cell cultures (data not shown). Proliferation was also examined in the presence of another NO releasing agent, SIN-1. Unlike SNP, Sin-1 does not contain a cyanide group which may have been cytotoxic and has been shown to be non-toxic (I. Oswald, personal communication). As shown in Figure 3B, Sin-1 marginally inhibited proliferation in response to PHA at 0.01 mM ($P<0.05$), significantly inhibited the response at 0.1 mM ($P<0.01$) and completely inhibited the response at 1.0 mM ($P<0.001$). Similar results were obtained when spleen cells were stimulated with Con A in the presence of SIN-1 (data not shown).

L-NMMA or AG Significantly Increases Antigen Specific Proliferation

The effect of L-NMMA or AG on the proliferation of splenocytes from infected mice to malaria antigen was also investigated. As shown in Table 2, the addition of either NO inhibitor to spleen cells from day 7 infected mice significantly increased proliferation in response to PRBC approximately 1.5 fold. It is of interest to point out that the addition of AG significantly increased the spontaneous proliferation of spleen cells from infected mice cultured in medium without mitogen. Although for the experiment shown here, the addition of L-NMMA did not cause a similar effect, this effect was observed in other experiments. The divergence of results with L-NMMA may be related to variations in the course of infection from experiment to experiment, that is, the peak parasitemia can occur between 8 to 10 days post infection (32). Nevertheless, the observation of significantly increased antigen specific as well as spontaneous proliferation by spleen cells from infected mice in the presence of

NO inhibitors suggests that *in vivo* NO may down-regulate lymphocyte proliferation during malaria and, thus, limit immune-mediated pathology (33).

Macrophages Mediate NO-Dependent Suppression of Proliferation During Blood-Stage Malaria

To investigate the cellular mechanism of suppression of lymphocyte proliferation during blood-stage *P. chabaudi* AS, co-cultures containing spleen cells from normal mice and various numbers of peritoneal macrophages from normal or day 7 infected mice were prepared and stimulated with Con A. There were no significant changes in proliferation to Con A in co-cultures containing 5-100 x 10^3 macrophages from normal animals (Figure 4). In contrast, proliferation was significantly decreased ($P<0.01$) in co-cultures containing high numbers of macrophages from infected mice, in the case of this experiment, 50 or 100 x 10^3 macrophages. Furthermore, in a separate experiment, the addition of AG (Figure 5A) to co-cultures containing 25, 50 or 100 x 10^3 macrophages from infected mice resulted in significant increases ($P<0.01$, $P<0.001$ or $P<0.001$, respectively) in Con A-induced proliferation. Indeed, the responses in the presence of AG were restored to levels comparable with that of control spleen cell cultures stimulated with Con A in the absence of both the NO inhibitor and infected macrophages. Determination of NO production in co-cultures without AG demonstrated that suppression of spleen cell proliferation correlated with production of high levels of NO (Figure 5B). Moreover, the addition of AG significantly reduced NO production in these co-cultures ($P<0.001$). Thus, inhibition of NO production by AG in co-cultures containing spleen cells from normal animals and macrophages from *P. chabaudi* AS infected mice correlated with restoration of spleen cell proliferation to Con A to normal levels. Similar results were obtained in co-cultures of spleen cells and macrophages from infected mice stimulated with PHA

and in co-cultures stimulated with Con A or PHA when L-NMMA was used to inhibit NO (data not shown).

Discussion

Malaria-induced immunosuppression appears to be complex. Suppression of both antibody and cell-mediated responses occurs during blood-stage malaria and there are alterations in both non-specific and specific immune responses. The most extensively studied aspect of suppression during acute blood-stage malaria, which has been documented in both humans and experimental animals, is the down-regulation of lymphocyte proliferation *in vitro* to malaria antigens, mitogens and unrelated antigens (4-7,12,13). Various mechanisms have been proposed as the basis of the altered lymphoproliferative responses, including defects at the level of either the lymphocyte or the antigen presenting cell. Studies in humans have demonstrated peripheral blood lymphocytes from individuals with acute falciparum malaria produce decreased levels of the immunoregulatory cytokine, interleukin-2, as well as express decreased levels of cell bound IL-2 receptors in response to stimulation with malaria specific antigens or non-specific antigens suggesting that defects in IL-2 responses contribute to malarial immunosuppression (34,35). Decreased production of IL-2 during acute malaria was also observed in mice (20). Alternatively, defects in the antigen presenting capacity of macrophages and altered production of soluble immunoregulatory factors or soluble inhibitory factors, such as prostaglandins, by these cells have been suggested to contribute to malarial immunosuppression (14,18-20).

In the present investigation, we examined proliferative responses to mitogens *in vitro* of spleen cells recovered from C57BL/6 mice at various times

during blood-stage *P. chabaudi* AS. Our observation that the most severe suppression in response to Con A, PHA or LPS occurred between days 5 and 14 post infection, during the acute phase coincident with peak parasitemia, together with our previous observation that macrophages from *P. chabaudi* AS infected mice produce high levels of NO during the first 2 weeks post infection (Jacobs, P., Yap, G.S., Radzioch, D., Stevenson, M.M. Manuscript in preparation) prompted us to investigate the role of macrophage-derived NO in suppression of lymphocyte proliferation in this model. We also investigated the role of prostaglandins which have previously been found to contribute to suppression of lymphoproliferation during acute falciparum malaria (19). Addition of the NO inhibitor L-NMMA to cultures of spleen cells recovered from day 7 or day 14 infected mice stimulated with Con A or PHA, T cell stimulants, but not LPS, a B cell mitogen, resulted in significantly increased proliferation while the addition of INDO was found to significantly increase the proliferative response of spleen cells from infected mice to all three mitogens. Previous observations by Albina et al (23) demonstrated that macrophage-mediated suppression of LPS induced spleen cell proliferation is dependent upon prostaglandins but occurs independently of NO. We also observed that addition of a combination of L-NMMA and INDO to spleen cells from infected mice significantly increased the response to Con A and fully restored the response to PHA to the level of spleen cells from normal, uninfected animals. In addition, L-NMMA significantly increased the response of spleen cells from day 7 infected mice in response to malaria-specific antigen. Moreover, a role for NO in suppression of lymphoproliferation was confirmed by our finding that the addition of SNP or SIN-1, agents which generate NO *in vitro*, resulted in decreased responsiveness of spleen cells from normal mice to Con A or PHA. Our observation that the addition of an inhibitor of NO synthesis significantly increased the proliferative response of spleen cells from infected mice to Con A,

PHA or specific antigen provides strong evidence that NO contributes to suppression during acute blood-stage malaria. A similar conclusion was reached in recent studies by Rockett and his colleagues (36) who demonstrated that the addition of L-NMMA significantly increased the responses of spleen cells from *P. vinckei* infected mice to Con A.

To determine if cytokine inducible NO was responsible for the observed suppression, we performed proliferation assays in the presence of AG, an NOS inhibitor reported to inhibit the cytokine-inducible form of this enzyme (30). Indeed, we observed that AG was effective in significantly increasing the proliferation of spleen cells from day 7 *P. chabaudi* AS infected mice in response to both Con A and PHA as well as parasite antigen. Since macrophages are considered to be a major source of cytokine inducible NO (21), we performed co-culture experiments using peritoneal macrophages from day 7 infected or normal mice and spleen cells from normal mice stimulated with Con A. These experiments demonstrated that peritoneal macrophages from infected mice suppressed the proliferative responses of spleen cells from normal animals. Furthermore, the suppression could be reversed by the addition of AG or L-NMMA and restoration of the proliferative responses to normal levels was found to correlate with inhibition of NO production in the co-cultures.

The concept that macrophages play a role in malarial immunosuppression is attractive given that these cells play a central role in immune responses as antigen presenting cells, by cytokine production, and as potent effector cells of cell-mediated immunity. Although not much is known concerning the antigen presenting function of macrophages during malaria, macrophages from malaria infected animals produce high levels of cytokines, such as TNF-α (40). Macrophages have been shown to be activated during blood-stage malaria, presumably via IFN-γ, the major cytokine regulating NO production by these

cells, which is produced by malaria stimulated T cells (21,34,37-40). It has been demonstrated by our laboratory and others that during acute infection with *P. chabaudi* AS, peak parasitemia is controlled via a cell-mediated immune response involving Th1 cells and macrophages (41-43). *In vitro* and *in vivo* evidence suggest that NO is an important anti-plasmodial molecule (44,45). The results of the present study, thus, argue that macrophage-derived NO may play dual roles during blood-stage malaria in suppression of T cell proliferation and parasite killing. NO mediated suppression by macrophages of lymphoproliferation *in vivo* during blood-stage malaria may, therefore, contribute to the down-regulation of immune responses not only to other antigens but also to parasite antigens and thereby limit malarial pathology which appears to be mediated primarily by $CD4^+$ T cells (33,46).

Acknowledgements

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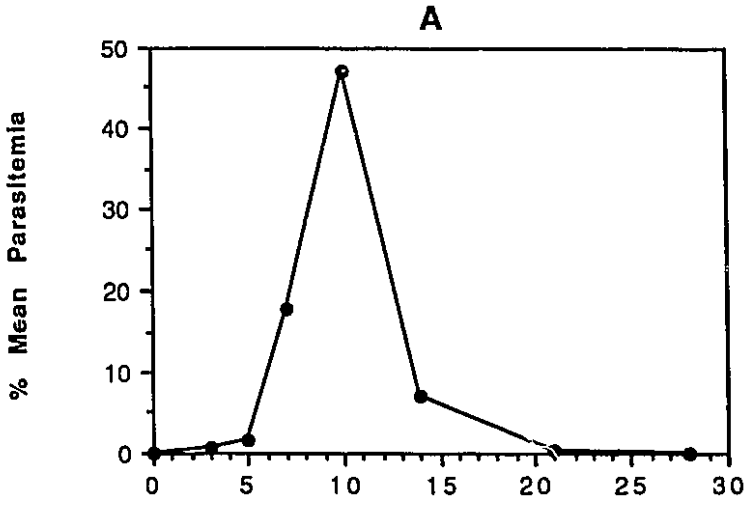
Figure 1. (A) Course of blood-stage *P. chabaudi* AS infection in C57BL/6 mice. Parasitemia was determined on duplicate blood smears collected from groups of 4-6 individual mice in two separate experiments. Parasitemia is expressed as the Mean percent PRBC of pooled data from two experiments. Standard Error of the Mean (SEM) <10%. (B) Kinetics of suppression of spleen cell proliferation in response to mitogens during *P. chabaudi* AS infection. Spleen cells harvested from normal or infected mice on the days indicated were stimulated *in vitro* with Con A, PHA, LPS or medium.. At the end of 72 hrs culture period, incorporation of [^{3}H]-thymidine was determined. Stimulation indices were obtained by dividing the response of the stimulated by the non-stimulated cells x 100. The percentage of normal responses was determined by dividing the stimulation indices of infected mice by that of normal mice. The values are expressed as the Mean percent normal response ± SEM of data pooled from 4-6 individual mice per time point in two separate experiments.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% Mean Parasitemia
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40
30
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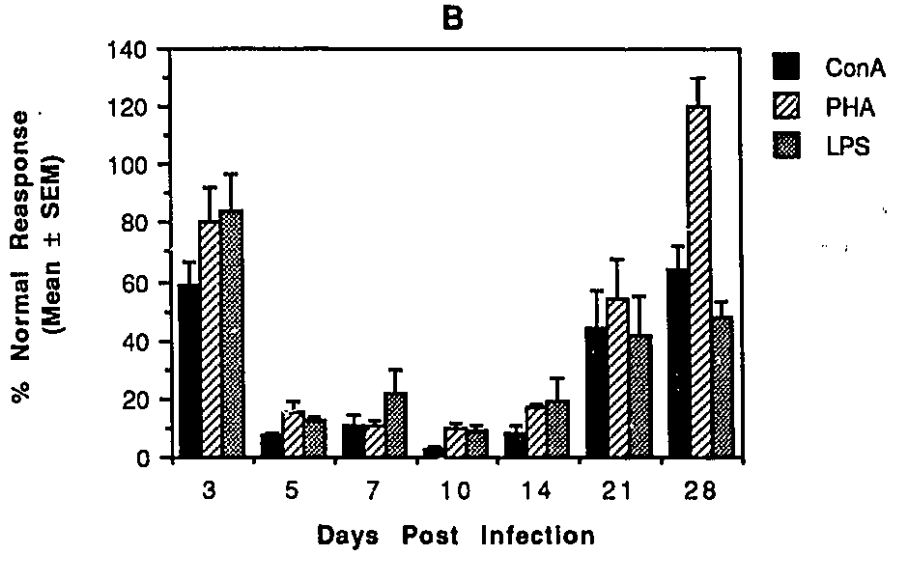
10
15
20
25
30


B
% Normal Reasponse
(Mean ± SEM)
140
120
100
80
60
40
20
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3
5
7
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14
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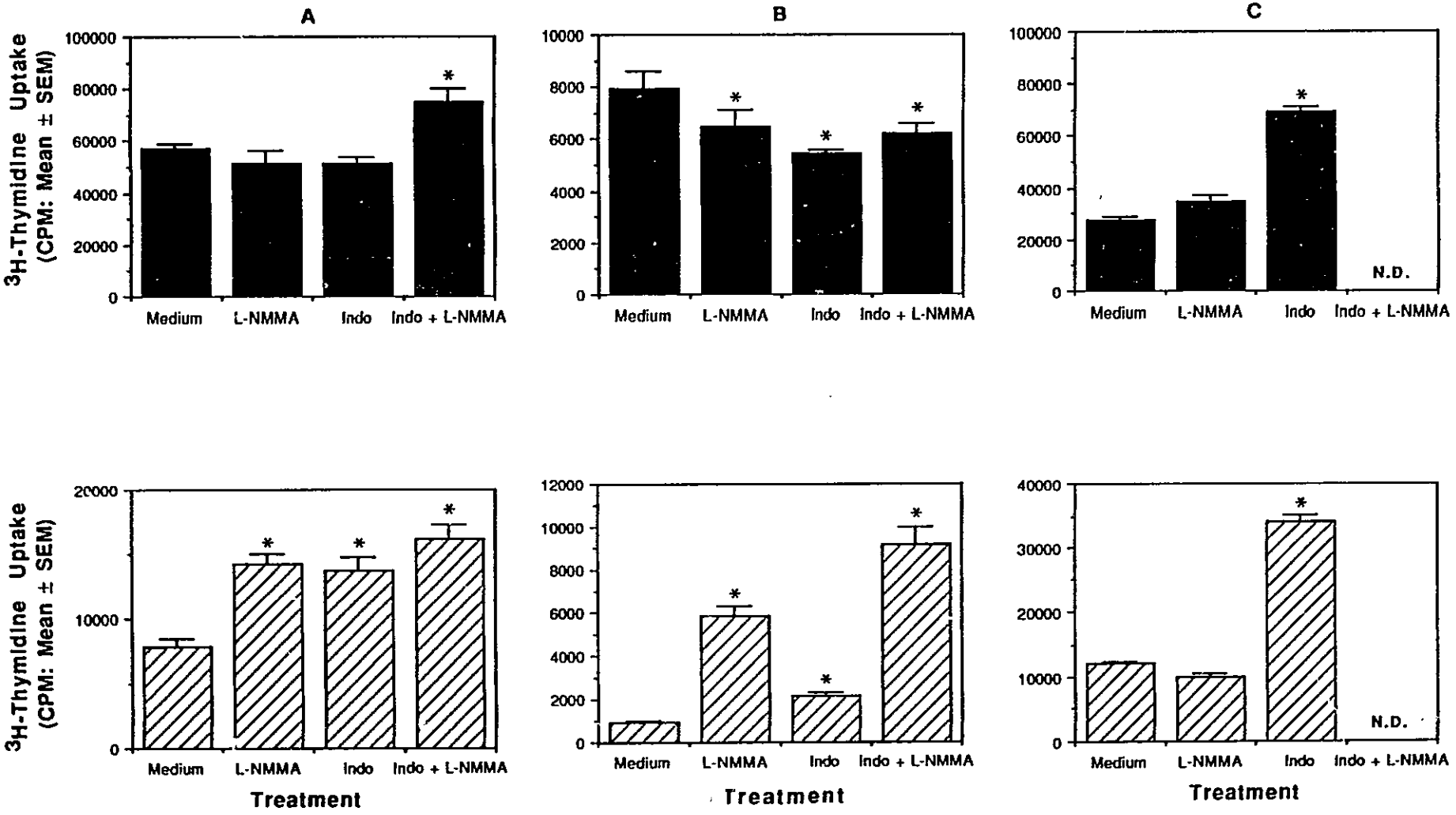
28
Days Post Infection
ConA
PHA
LPS

Figure 2. Effect of L-NMMA and INDO on mitogen-stimulated proliferation of spleen cells from *P. chabaudi* AS infected mice. Spleen cells harvested from normal (top panel), from day 7 infected mice (bottom panel) were stimulated *in vitro* with Con A (A), PHA (B) or from day 14 infected mice with LPS (C) in the absence or presence of 0.5 mM of L-NMMA and/or 10 μg/ml INDO. At the end of 72 hrs culture period, incorporation of [^{3}H]-thymidine was determined. Data represent the Mean cpm ± Standard Error of the Mean (SEM) of sextuplicate samples from one of three similar experiments. * indicates $P<0.05$ vs no L-NMMA and/or INDO added. N.D., not done.


A
B
C
^{3}H-Thymidine Uptake (CPM: Mean ± SEM)
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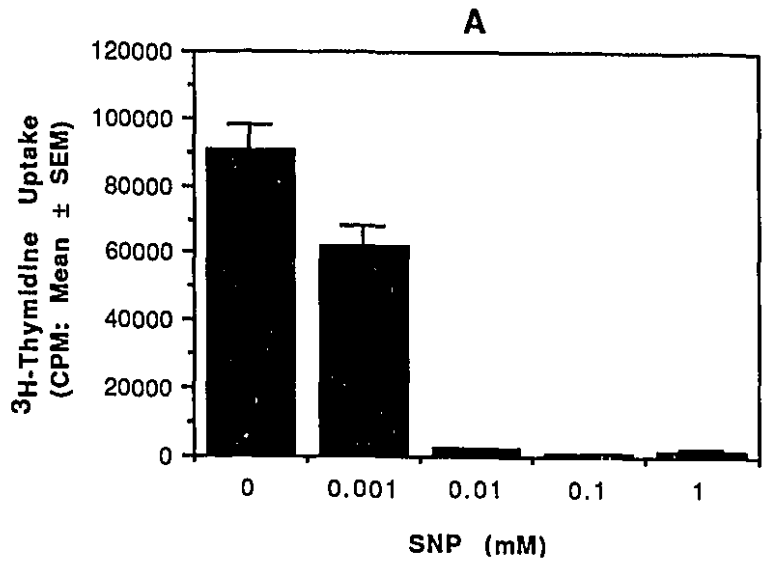
80000
60000
40000
20000
0
10000
8000
6000
4000
2000
12000
30000
*
N.D.
Medium
L-NMMA
Indo
Indo + L-NMMA
Treatment

Figure 3. Effect of chemically generated NO on mitogen-stimulated proliferation of spleen cells from normal mice. Spleen cells harvested from normal mice were stimulated *in vitro* with either ConA (A) or PHA (B) in the absence or presence of various concentrations of SNP (top panel) or SIN-1 (bottom panel) as indicated. At the end of 72 hrs culture period, incorporation of [^{3}H]-thymidine was determined. Data represent the Mean cpm ± Standard Error of the Mean (SEM) of triplicate samples.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^{3}H-Thymidine Uptake
(CPM: Mean ± SEM)
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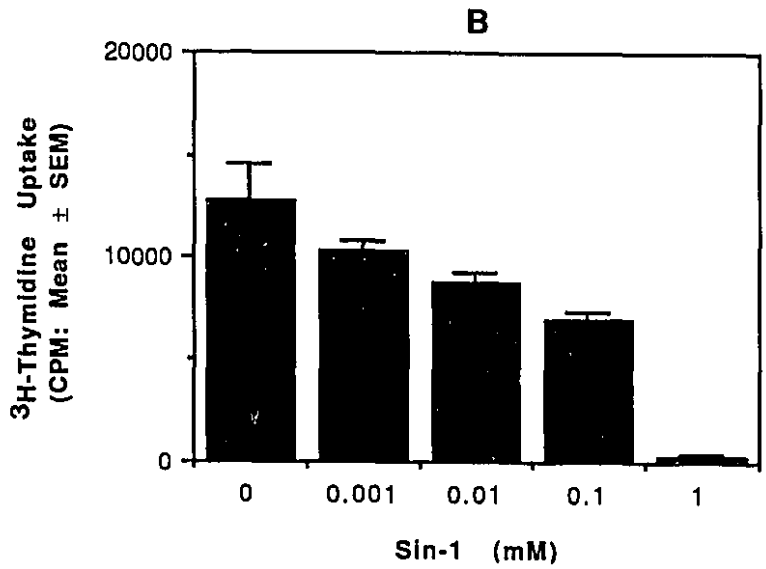
1
SNP (mM)


B
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^{3}H-Thymidine Uptake
(CPM: Mean ± SEM)
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0.001
0.01
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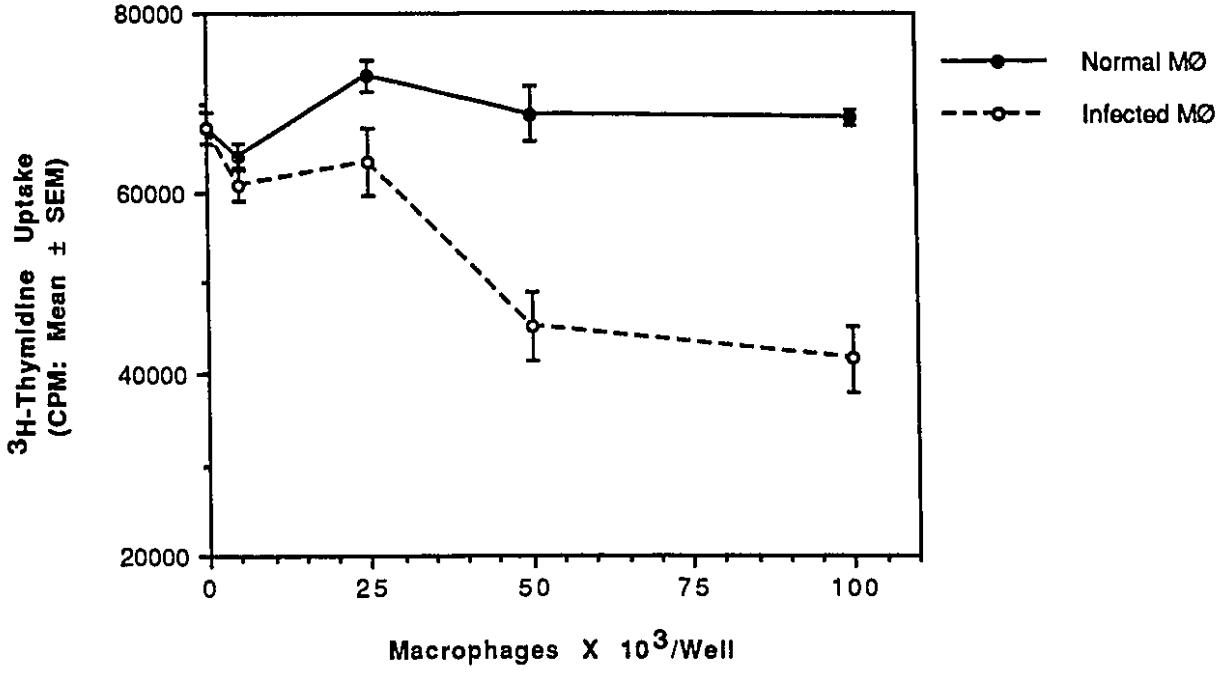
1
Sin-1 (mM)

Figure 4. Effect of macrophages from normal or infected mice on Con A-stimulated proliferation of spleen cells from normal mice. Spleen cells from normal mice were cultured alone or with various numbers of resident peritoneal macrophages either from normal mice (closed symbols) or day 7 infected mice (open symbols) and were stimulated with Con A. At the end of 72 hrs culture period, incorporation of [^{3}H]-thymidine was determined. Data represent the Mean cpm ± Standard Error of the Mean (SEM) of triplicate samples from 3-4 individual mice.


80000
60000
40000
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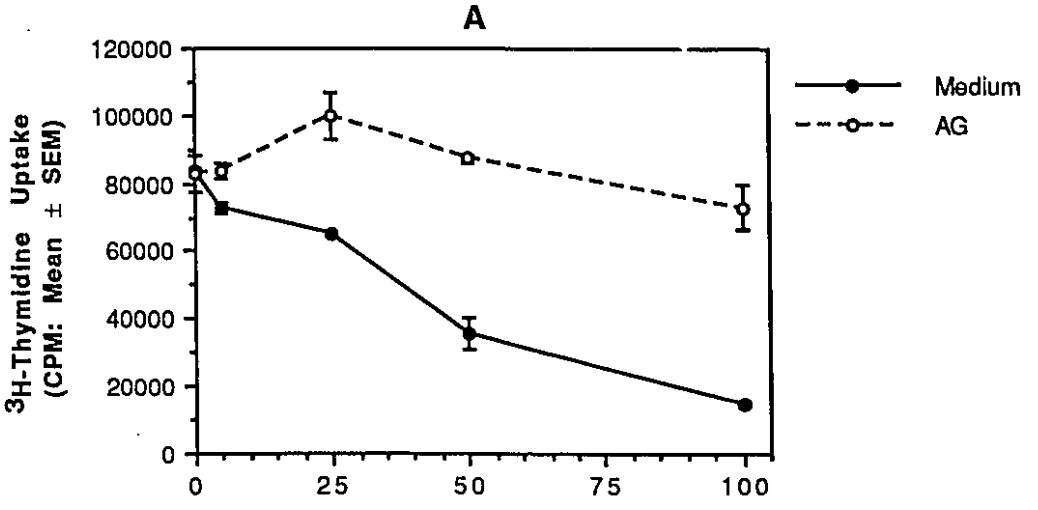
25
50
75
100
^{3}H-Thymidine Uptake
(CPM: Mean ± SEM)
Macrophages X 10^3/Well
Normal MØ
Infected MØ

Figure 5. Macrophages from infected mice inhibit Con A-stimulated proliferation of spleen cells from normal mice through NO production. Spleen cells from normal mice were cultured alone or with various numbers of resident peritoneal macrophages from day 7 infected mice and were stimulated with Con A in the absence or presence of 0.5 mM AG. At the end of 72 hrs culture period, incorporation of [^{3}H]-thymidine (A) and accumulation of NO (B) were determined. Data represent the Mean ± Standard Error of the Mean (SEM) pooled from triplicate samples from 3-4 individual mice. N.D., not detectable.


A
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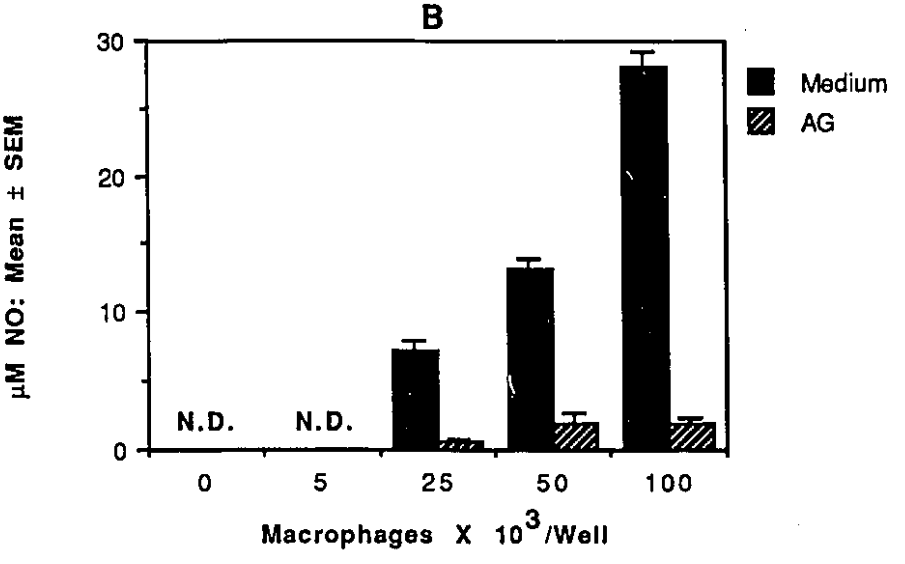
^{3}H-Thymidine Uptake
(CPM: Mean ± SEM)
Medium
AG


B
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N.D.
N.D.
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5
25
50
100
µM NO: Mean ± SEM
Macrophages X 10^{3}/Well
Medium
AG

Table 1

Effect of Aminoguanidine on Con A or PHA Induced Proliferation of Spleen Cells from *P. chabaudi* AS Infected Mice

Spleen Cells From[a]:	[^{3}H]-Thymidine Incorporation: CPM (Mean ± SEM)			
	Con A		PHA	
	-AG	+AG	-AG	+AG
Normal	76,202 ± 9985[b,c]	58,965 ± 2532[c]	4222 ± 125[b,c]	3785 ± 184[c]
Infected				
Mouse #1	2605 ± 286[b,d]	11,231 ± 315[d]	285 ± 51[b,d]	2858 ± 68[d]
Mouse #2	2927 ± 207[b,d]	8761 ± 444[d]	105 ± 9[b,d]	1437 ± 58[d]
Mouse #3	3540 ± 431[b,d]	9316 ± 397[d]	276 ± 25[b,d]	2076 ± 48[d]

[a]Spleen cells were harvested from 2 normal C57BL/6 mice and pooled, or from 3 individual *P. chabaudi* AS infected (day 7) mice. Cells were cultured with Con A or PHA in the presence or absence of 0.5 mM AG.

[b]$P<0.001$, normal versus infected

[c]Not significant, -AG versus +AG

[d]$P<0.001$, -AG versus +AG

Table 2

Effect of L-NMMA or AG on Spleen Cell Proliferation to Malaria Antigen

Spleen Cells From[a]:	[^{3}H]-Thymidine Incorporation: CPM (Mean ± SEM)	
Experiment 1		
	-NMMA	+NMMA
Normal		
Medium	1196 ± 311[b]	1111 ± 175[b]
PRBC	752 ± 77[b]	868 ± 52[b]
Infected Mice		
Medium	1775 ± 211[b]	1114 ± 98[b]
PRBC	2456 ± 224[c]	3611 ± 153[c]
Experiment 2		
	-AG	+AG
Normal Mice		
Medium	271 ± 30[b]	326 ± 42[b]
PRBC	490 ± 87[b]	354 ± 46[b]
Infected Mice		
Medium	1260 ± 72[d]	3396 ± 330[d]
PRBC	3706 ± 146[c]	6311 ± 633[c]

[a]Spleen cells were harvested from individual normal or *P. chabaudi* AS infected (day 7) mice and cultured with 10^6 PRBC/ml in the presence or absence of 0.5 mM NMMA(Experiment 1) or 0.1 mM AG (Experiment 2).

[b]Not significant, -NMMA versus +NMMA or -AG versus +AG.

[c]$P<0.005$, -NMMA versus +NMMA or -AG versus +AG.

[d]$P<0.001$, -AG versus +AG

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CHAPTER V

GENERAL DISCUSSION

The murine model of infection with the rodent malaria parasite *Plasmodium chabaudi* AS has been established in our laboratory (Stevenson et al, 1982). The level of host resistance to infection in inbred mice with *P. chabaudi* AS is genetically determined by a major, dominant, autosomal, non H-2 linked gene. Intraperitoneal infection of resistant C57BL/6 mice with 10^6 PRBC results in a moderate level of peak parasitemia, enhanced splenomegaly and acquired immunity resulting in the control and elimination of the parasite. Previous observations by Stevenson and her colleagues have suggested that the development of protective immunity to blood-stage infection with *P. chabaudi* AS requires $CD4^+$ T cells, macrophages and an intact spleen (see review in Chapter II). Since the spleen and its cellular components play a critical role in host defence and immunoregulation, the purpose of this study was: first, to characterize the profile of *in vitro* production of the Th1-derived cytokine, IFN-γ, versus the Th2-derived cytokines, IL-4, IL-5 and IL-10, by spleen cells from infected mice; second, to quantitate the production of polyclonal and malaria-specific antibodies; and, third to define the role of nitric oxide and prostaglandins in suppression of lymphocyte proliferation during blood-stage infection with *P. chabaudi* AS. Since each experimental Chapter contains its own detailed discussion, the principal findings of this thesis are summarized below.

The studies presented in Chapter III provide evidence that *in vitro* stimulation of spleen cells with parasite antigen results in production of the Th1-derived cytokine, IFN-γ, followed by the Th2-derived cytokines, IL-4, IL-5 and

IL-10, during the course of blood-stage *P. chabaudi* AS infection. As previously observed (Stevenson et al, 1990), it was found that high levels of IFN-γ are produced a few days prior to peak parasitemia. It is likely that IFN-γ production activates macrophages during the course of acute *P. chabaudi* AS infection, resulting in the control of peak parasitemia. In this respect, recent studies by Stevenson et al (1992) demonstrated that splenic macrophages are activated and produce a variety of soluble mediators, including reactive oxygen intermediates and TNF-α, during parasite multiplication and just prior to the peak parasitemia. Furthermore, our results show that spleen cells produce high levels of IL-10 at the time of peak parasitemia followed by IL-4 and IL-5 production during the chronic phase of infection. Although numerous studies have documented that both Th1 and Th2 cells are involved in immunity to *P. chabaudi* (see review in Chapter II), none of these studies have demonstrated by which mechanism(s) cell-mediated immune responses are switched toward humoral responses. Our results suggest that IL-10 production by PRBC-stimulated spleen cells at the time of peak parasitemia may be a candidate to initiate a Th2 response. Moreover, IL-10, which is known as cytokine synthesis inhibitory factor and is produced by a variety of immune cells, has been shown to inhibit cytokine production by Th1 cells indirectly through down-regulation of macrophage antigen presenting function (Moore et al, 1993). Since IFN-γ is critical for activation of macrophages to kill plasmodia (Ockenhouse and Shear, 1984), the diminution of IFN-γ production and expansion of Th2 subset inhibitory activities underscores the profound dysfunction of immune responses that underlies susceptibility to this organism (Stevenson et al, 1993).

The finding of enhanced Th1 and Th2 cytokine production was confirmed by increased polyclonal immunoglobulin levels as well as *P. chabaudi* AS-specific IgG2a and IgG1 immunoglobulin isotypes during infection. The Th2

cytokines IL-4 and IL-10 can induce B cell activation and differentiation. Dramatic polyclonal B cell activation has also been reported in other murine models of malaria infection, *P. chabaudi adami* and *P. yoelii* (Langhorne et al, 1985; Rosenberg, 1981). Indeed, our results demonstrate that poly-isotypic hypergammaglobulinemia occurs during *P. chabaudi* AS infection, a characteristic feature of humans chronically infected with *P. falciparum* (Ho and Webster, 1989). In addition, we also show that high titers of *P. chabaudi* AS-specific antibodies of IgG2a and IgG1 isotypes were present during primary infection on days 10 and 21 post infection, at the time of peak parasitemia and when animals had almost cleared the infection, respectively. Similarly high levels of malaria-specific IgG2a and IgG1 isotypes were detected in sera from hyperimmune mice, except that only 50% of the mice exhibited high titers of malaria-specific IgG1 isotype.

Studies in Chapter IV demonstrate that *in vitro* proliferation of spleen cells from *P. chabaudi* AS infected C57BL/6 mice in response to the mitogens Con A, PHA and LPS are suppressed, with the most severe suppression occurring between days 5 and 14 post infection. Our results demonstrate that in addition to PG, increased NO production by macrophages contributes to immunosuppression during the acute phase of infection. The evidence for the role of NO, in particular, and PG in suppression of proliferation to both T and B cell mitogens is threefold. First, addition of either L-NMMA or AG, specific inhibitors of NO synthase, or INDO, a prostaglandin inhibitor, partially but significantly abrogated the suppression of Con A- or PHA-stimulated proliferation of spleen cells from infected mice. Evidence is presented which demonstrates that only the addition of INDO, but not L-NMMA, abrogated the suppression of LPS-stimulated proliferation of spleen cell from infected mice. Addition of L-NMMA or AG in combination with INDO partially but significantly abrogated the suppression in

response to Con A and completely abrogated the suppression to PHA. Our results also demonstrate that addition of L-NMMA or AG significantly increased proliferation of spleen cells from infected mice to *P. chabaudi* AS antigen.

Second, our results confirm the role of NO in suppression of lymphocyte proliferation by demonstrating that the addition of exogenous NO-donor agents, such as SNP or Sin-1, to spleen cells from normal mice decreased mitogen-stimulated proliferation. SNP and SIN-1 were found to significantly suppress proliferation of spleen cells from normal mice in a dose dependent manner in response to either Con A or PHA.

Third, confirmation of the role of NO-dependent inhibition of lymphocyte proliferation was obtained in co-cultures of spleen cells from normal mice and high numbers of peritoneal macrophages from day 7 infected mice but not peritoneal macrophages from normal mice. The suppression of Con A- or PHA-stimulated proliferation of spleen cells from normal mice correlated with production of high levels of macrophage-derived NO. Moreover, addition of L-NMMA or AG to co-cultures completely restored the lymphoproliferative responses to normal levels, with a concurrent reduction in NO production.

Collectively, the results presented in this thesis illustrate that immune activation (as demonstrated by activation and production of Th1 and Th2 cytokines as well as polyclonal and malaria-specific antibodies), and immunosuppression (as shown by impaired proliferation of spleen cells in response to mitogens and specific antigen), occurs simultaneously during infection with blood-stage *P. chabaudi* AS in C57BL/6 mice. Although the mechanism(s) by which immune responses during malaria switch from a state of activation to a state of suppression are not yet fully understood, many hypotheses have been proposed (see review in Chapter II). The evidence from the studies presented in this thesis suggests that production of NO and PG by

activated macrophages play a role in suppression of cell-mediated immune responses, inasmuch as there is strong evidence for the role of Th1-derived IFN-γ in activation of macrophages to control the parasite multiplication and peak parasitemia during the acute phase of infection (Stevenson, 1990). Moreover, NO and PG have been shown to inhibit production of Th1-derived cytokines, IL-2 and IFN-γ, but not of Th2-derived cytokines (Taylor-Robinson et al, 1994; Betz and Fox, 1991). Consequently, it can be hypothesized that enhanced production of IL-10, presumably by macrophages and B cells or other cells yet to be identified, at the time of peak parasitemia, initiates a Th2 response with production of IL-4 and IL-5 during the chronic phase of infection. Finally, it is well documented that the course of malaria infection in human has certain features, in addition to immunosuppression, that parallel observations made in these studies, including hypergammaglobulinemia. A better understanding of the host immune response to *P. chabaudi* AS may, therefore, lead to the development of safe and effective vaccine strategies or alternatively, effective immunotherapy for use against blood-stage infection with plasmodia in man.

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