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ROLE OF THE SPLEEN IN ERYTHROPOIETIC AND ACQUIRED IMMUNE RESPONSES DURING MURINE BLOOD STAGE MALARIA

by

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A Thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Medicine, Division of Experimental Medicine
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Memo 2. THESIS TITLE For George So Yap

(short form, 52 characters including spaces):

Role of the Spleen in host Response to murine Malaria

To my parents, Leonardo and Mary
brothers, Sheldon and Kirk
and
sisters, Hazel and Iryn

ABSTRACT

Plasmodium chabaudi AS infections in mice result in lethal and non-lethal infections in a strain-dependent fashion. Resistant C57BL/6 mice exhibit moderate levels of peak parasitemia and anemia and survive the infection. Susceptible A/J mice develop significantly higher parasitemias, severe anemia and succumb to infection. Resistance is controlled by a single or tightly linked, dominant, autosomal, non-H-2 linked locus(i), and is associated with the development of enhanced splenomegaly during infection and enhanced reticulocytic response to phenylhydrazine-induced hemolytic anemia. In this thesis, the relationship between the splenic erythropoietic response and resistance to infection is examined. Impairment of splenic erythropoiesis is demonstrated in susceptible A/J mice. This impairment could not be explained by a deficiency in erythropoietin production or in the mobilization of erythroid progenitor cells from the marrow to the spleen. The kinetics and mechanism of the inhibition of erythropoiesis was examined using an in vitro erythroid proliferation assay. Increased inhibition was associated with the rise in parasitemia and was found to be invariant in resistant and susceptible mice. Evidence is presented to suggest that the inhibitor is erythroid-specific and is distinct from IL-1, TNF and IFN-gamma. In transfusion experiments, evidence was obtained for the importance of anemia in the mortality of susceptible A/J mice and in the induction of parasitologic crisis. In splenectomy and immune cell transfer experiments, evidence is presented for the conclusion that an intact spleen is required for the induction but not the expression of B cell-dependent immunity to *Plasmodium chabaudi* AS.

RESUMÈ

La létalité de l'infection à *Plasmodium chabaudi* AS varie selon les lignées de souris cosanguines utilisées. La souris résistante C57BL/6 qui survie a l'infection, montre des niveaux modérés de parasitémie et d'anémie. La souris sensible A/J développe, par contre, de plus hauts niveaux de parasitémie, une sévère anémie pour finalement succombe à l'infection. La résistance à cette infection est contrôlée par un seul locus ou quelques loci étroitement liés, dominant, autosomal et independent du complexe majeur d'histocompatibilité H-2. La resistance est associée au développement d'une splénomégalie accrue pendant l'infection et d'une réponse réticulocytaire accrue à l'anémie hémolytique provoquèe par la phénylhydrazine. Cette thèse a pour but d'étudier la relation existant entre la réponse érythropoïétique splénique et la résistance à l'infection à *P. chabaudi* AS. L'érythropoïèse splénique est réduite chez la souris sensible A/J. Cette diminution ne peut s'expliquer par une production déficitaire d'érythropoïétine, ni par une migration réduite des cellules souches de la moëlle osseuse vers la rate. La cinétique et le mécanisme d'inhibition de l'érythropoïèse ont été étudiés par un essai de prolifération des cellules érythroides in vitro. L'inhibition de l'érythropoïèse, qui est similaire autant chez la souris sensible, que chez la résistante, est associée à l'augmentation de la parasitémie. Certaines évidences nous portent a croire que le facteur responsable de l'inhibition est spécifique aux cellules érythroides mais est toutefois distinct de l'IL-1, duTNF et de l'IFN-γ. Les expériences de transfusion sanguine démontrent l'importance de l'anémie dans la mortalité des souris sensible A/J, ainsi que dans l'induction de la crise parasitologique observée chez les lignées sensibles et résistantes. Finalement, l'utilisation de souris splénectomisées et les expériences de transfert cellulaire permettent de conclure que la rate doit être intacte pour le developpement mais non pour l'expression d'une immunité, dépendante des cellules B, à *P. chabaudi* AS.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
BFU-E	burst forming unit-erythroid
CFU-E	colony forming unit-erythroid
CMI	cell mediated immunity
ELISA	enzyme linked immunosorbent assay
Epo	erythropoietin
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
MAb	monoclonal antibody
NK	natural killer
PBMC	peripheral blood mononuclear cells
PHZ-SP	phenylhydrazine-induced spleen cells
PRBC	parasitized red blood cells
PWM	pokeweed mitogen
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
SPCM	spleen cell conditioned medium
SPLX	splenectomized
TGF	transforming growth factor
TNF	tumor necrosis factor

PREFACE

In accordance with the "Guidelines concerning thesis preparation" of the Faculty of Graduate Studies and Research, manuscripts of papers which have been published or which have been submitted for publication haven been incorporated in the thesis. This format for the thesis preparation has been approved by the Division of Experimental Medicine, Department of Medicine. The following is quoted directly from the Guidelines:

> "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 a 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 texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collections 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 objectives of the study, (4) a comprehensive general review of the background literature to the subject of the study, when this review is appropriate, and (5) a final overall conclusion and/or summary."
>
> "Additional material (procedural and design data as well as desciptions of equipment) must be provided in sufficient detail (eg., in appendices)

to allow clear and precise judgement to be made of the importance and originality of the research reported in the thesis."

Each of the manuscripts included in this thesis (chapters 2, 3, 4, and 5) has its own Summary, Introduction, Materials and Methods, Results and Discussion. The general abstract, general introduction (chapter 1), and general discussion (chapter 6) relate to the combined work presented in this thesis. References appear at the end of each chapter.

The work described 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. The author received technical guidance and assistance provided by Miss MiFong Tam, senior technician in Dr. Stevenson's laboratory. The author and Dr. Stevenson are coauthors of all the manuscripts presented. The papers appearing in chapters 2 and 3 have been published*. Papers in chapter 4 and 5 have been submitted for publication.

---

*Yap, G. S. and M. M. Stevenson. 1992. *Plasmodium chabaudi* AS: erythropoietic responses in resistant and susceptible mice. Exp. Parasitol. 75:340-352.

Yap, G. S. and M. M. Stevenson. 1991. Production of soluble inhibitor(s) of erythropoiesis in resistant and susceptible mice, Ann. N. Y. Acad. Sci. 628:279-281.

Yap, G. S. and M. M. Stevenson. 1994. Inhibition of in vitro erythropoiesis by soluble mediators in murine malaria: lack of a major role for IL-1, TNF or INF-gamma. Infect. Immun. 62:357-362.

CHAPTER 1
GENERAL INTRODUCTION

MALARIA: OF MICE and MEN

Life Cycle of Plasmodia

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The life cycle of the parasite is complex, involving a vertebrate host, in which the asexual stages develop, and a mosquito vector in which the sexual phase of the cycle occurs. More than a hundred species of plasmodia exist, infecting reptiles, birds, rodents and mammals.

Natural infection in the vertebrate host is initiated by the bite of a female anopheline mosquito that injects sporozoites during a blood feed. Blood borne sporozoites reach the liver and invade hepatocytes. The sporozoite then divides asexually to form merozoites. Rupture of hepatocytes releases numerous exoerythrocytic merozoites into the blood. The merozoites invade red cells where they first appear as ring stages, later enlarging to become ameboid trophozoites. Inside red cells, the parasite undergoes asexual reproduction forming schizonts with a variable number of merozoites every 24-72 hours, depending upon the species. Each intraerythrocytic cycle ends with the rupture of the infected red cell, releasing merozoites which reinvade new red cells. Episodes of fever are associated with the synchronous rupture of red cells and subsequent release of merozoites. A small proportion of asexual parasites differentiate into male and female gametocytes. When a mosquito feeds on the vertebrate host, fertilization occurs in the mosquito gut. This process proceeds with the formation of ookinetes that encyst on the outer surface of the stomach and later release thousands of sporozoites that migrate to the salivary glands.

Clinico-Pathological Aspects of Malaria

Plasmodium falciparum , *P. vivax*, *P. malariae* and *P. ovale* are the species of *Plasmodium* that infect man. *P. falciparum* is the most virulent species and accounts for most of the mortality. The clinical presentation of malaria is known to vary from mild flu-like symptoms to deep coma. The clinical course is influenced by parasite factors, such as, the species and virulence of plasmodia, and host factors, such as, the immune, endocrine, and nutritional status (Greenwood et al., 1991). In uncomplicated attacks, fever, body pain, vomiting and nausea are common symptoms. Anemia is also common in *P. falciparum.* and in hyperparasitemic infections. In severe attacks of *P. falciparum* malaria, life-threatening pathologic sequelae include cerebral malaria, severe anemia, blackwater fever, renal failure, pulmonary insufficiency and bleeding disorders (Phillips and Warrel, 1986; White and Ho, 1992).

Rodent Malaria Infections

The host specificity of human malaria parasites represents a major obstacle for the study of malaria because the parasites cannot be maintained and studied in small laboratory animals. Thus, various avian, simian and rodent malaria models have been developed throughout the years (Mons and Sinden, 1990). The advantages of using rodent malarias include the easy maintainance of parasites in relatively inexpensive, well characterized small animals. Such models also allow for the study of host and parasite genetic factors in the susceptibility to and virulence of infections (Stevenson, 1989). Detailed studies to define the importance of various mechanisms in pathogenesis and immunity have been performed using mice with defined genetic or induced defects. However, the variability of the mechanisms elucidated with different host-parasite combinations has

highlighted the need for utmost caution in extrapolating these results to other rodent, and especially to human infections (Cox, 1988; Sayles and Wassom, 1993).

Four species of *Plasmodium*, namely *P. berghei*, *P yoelii*, *P. vinckei* and *P. chabaudi* are commonly used in rodent malaria models (Landau and Boulard, 1978). Infections with *P. berghei* and *P. vinckei* are invariably fatal. Infections with *P. yoelii* and *P. chabaudi* are generally self-resolving, although fatal infections are produced by certain parasite strains and in certain mouse strains. The types of red cell invaded by rodent plasmodia are different. *P. berghei* and *P. yoelii* parasites tend to invade reticulocytes whereas *P. vinckei* and *P. chabaudi* have an apparent preference for normal red cells. The red cell tropisms have profound effects on the evolution of the infections and on the development of immunologic and pathophysiologic responses (Ott, 1968; Jayawardena et al., 1983; Cox, 1988).

Blood stage infections are initiated by direct inoculation of infected red cells into laboratory mice, thereby bypassing the exoerythrocytic stage in the liver. The course of infection is monitored by quantitating the percentage of parasitized red cells in approriately stained blood smears taken at different times after infection. Typically, few parasites are seen in the prepatent period following inoculation. As the infection becomes patent, a rapid rise in parasitemia is observed. Fatal infections tend to be characterized by high peak parasitemias with the subsequent death of the infected mouse. Self-resolving infections tend to have more moderate levels of peak parasitemia. Parasitemia is rapidly controlled and suppressed and the infection is subpatent. In *P. chabaudi* infections, recrudescent parasitemia are often observed several weeks after infection.

These recrudescences presumably arise from antigenic variants of the original inoculum (Mc Lean et al., 1986).

Genetic Control of Resistance to Human and Murine Malaria

The molecular basis for genetic resistance to a major polygenic human disease is perhaps best known for malaria (Allison, 1954; Hill, 1991). Resistance in human populations is manifested either as a decrease in the intensity of infection or a decrease in the severity of disease. Most human malaria resistance genes are expressed at the level of the host red cell (Nagel and Roth, 1989). These resistance genes represent mutations in genes encoding red cell hemoglobin (e.g., HbAS), enzymes (e.g., glucose 6-phosphate dehydrogenase), cytoskeletal (e.g., spectrin) and membrane (Band 3) proteins. They are thought to act by interfering with the processes of invasion, growth, metabolism and release of intraerythrocytic parasites. Furthermore, they may render the infected red cells more sensitive to oxidative stress or to clearance by reticuloendothelial cells (Yuthavong and Wilairat, 1993). Recently, human HLA class I and II genes have been associated with protection from severe disease. The protective class I molecule, HLA Bw53, has been shown to bind to a highly conserved T cell epitope of liver stage specific antigen 1 (LSA-1) (Hill et al., 1993). Presumably, the 40% protection from cerebral malaria and severe anemia in carriers of this haplotype is mediated by cytotoxic T lymphocyte (CTL) responses to the peptide bound to HLA Bw53 on hepatocytes.

Among experimental animals, genetic differences in the levels of resistance to malaria have also been observed. Different patterns of resistance are exhibited by strains of inbred mice infected with different murine *Plasmodium* species (Stevenson, 1989). In stark constrast to the

situation in human malaria, the identity of the resistance genes in mouse malaria is virtually unknown. In almost all cases, however, they do not appear to involve structural or metabolic alterations in the mature red cell.

Early studies on the infections with the uniformly lethal *Plasmodium berghei* K173 showed a variability in the survival times of mice (reviewed by Stevenson, 1989). Resistance, as defined by a longer survival time appeared to be controlled by a single or closely linked dominant autosomal locus. A subsequent study documented the course of infection, the biochemical and histopathological changes in spleen, liver and thymic tissues in different strains of mice infected with *P. berghei* K173 (Contreras, 1980; Mackey, 1980). None of the pathological parameters studied was predictive of, or correlated with the survival time of the strains of mice.

A wide spectrum of parasitemia courses and outcomes have been observed in different strains of mice infected with *P. yoelii* XNL (Hoffman et al., 1984; Sayles and Wassom, 1988). The general conclusion from these studies is that several genes probably interact to control resistance. Both H-2 and non-H-2 encoded genes are involved. Resistance has been associated with the development of earlier and enhanced anti-*P. yoelii* IgG2 and IgG3 responses (Taylor et al., 1988). Susceptibility has been associated with accelerated production of erythrocytophilic antibodies (Sayles and Wassom, 1991). An entirely different pattern of resistance has been reported for the virulent strain of *P. yoelii* YM.

Strain differences in susceptibility to yet another rodent malarial parasite, *P. chabaudi* AS, have also been reported. Previous studies from this laboratory and others have characterized the strain distribution patterns of resistance to this species (Eugui and Allison, 1980; Stevenson et

al., 1982). Susceptible mouse strains develop high levels of parasitemia, severe anemia and succumb to infection regardless of the infective dose. On the other hand, resistant mouse strains develop moderate levels of parasitemia, and survive and clear the infection. During peak parasitemia, resistant mice have significantly higher hematocrits and spleen indices compared to susceptible mice. Resistant mouse strains also exhibit an enhanced reticulocytic response to chemically-induced hemolytic anemia. Genetic analysis showed that a dominant, single, major, non-H-2 linked gene controlled resistance. Analysis of resistance patterns in two separate series of recombinant inbred mice indicated the presence of the major controlling element on mouse chromosome 1 (Borwell et al., 1983; Stevenson and Skamene, 1985; Stevenson et al., 1988). The existence of other genes located on separate chromosomes was also indicated. When spleen indices were analyzed in the AXB series of RI mice, there was a high concordance between the trait of a high spleen index and resistance. These observations suggested that genetically determined resistance may be related to some aspect of splenic function, possibly an enhanced erythropoietic response in the spleens of resistant mice. The relationship between ability of the murine host to respond in terms of erythropoiesis and its survival characteristics is explored in this thesis. Since the spleen plays a dominant role during stress erythropoiesis in rodents, we postulated that differential expansion of the splenic erythroid compartment may explain, at least in part, the difference in the severity of anemia and the outcome of infection between resistant and susceptible. Thus, the kinetics and regulation of erythropoiesis in resistant and susceptible mice during *P. chabaudi* AS infection is presented in detail in chapters 2 and 3 (Yap and Stevenson, 1992; Yap and Stevenson, 1994). Our postulate presupposes

that severe anemia is the main cause of death of susceptible mice. Evidence is presented in chapter 4 to support this supposition

RED CELL HOMEOSTASIS AND THE ANEMIA OF MALARIA

Red cell production

The physiological function of red cells is to transport oxygen from the lungs to the body tissues. This critical function necessitates that a relatively constant amount of red cells be maintained in the circulation. Since red cells have a finite life span, senescent cells are constantly replaced by newly formed cells released from hemopoietic organs. A balance between the amount of red cell destruction and efflux from hemopoietic organs maintains the equilibrium. This equilibrium is maintained by signals mediated by the glycoprotein hormone erythropoietin (Epo) produced in kidney cells, which act on erythroid progenitors in hemopoietic organs (Erslev, 1991; Krantz, 1991). Red cells, as are all blood cells, are continuously generated from proliferative precursors in the hemopoietic organs. A hierarchy of developmental stages in the sequence of cell proliferation, differentiation and functional maturation can be delineated (Dexter and Spooncer, 1987). First, self renewing pluripotent stem cells are capable of producing progenitors committed to differentiate into various cell lineages. Under the influence of factors such as Epo, committed progenitors give rise to mature blood cells by proliferative and differentiative events.

The development of clonal assay systems in the 1970's has led to the concept that Epo acts mainly on committed erythroid progenitors and early stages of morphologically recognizable erythroblasts. The earliest lineage restricted erythroid progenitor cell is the burst forming unit-erythroid

(BFU-E), which gives rise to large multiple clusters of hemoglobinized cells after 7-10 days in culture (Axelrad, 1973; Gregory, 1976). The in vitro growth and development of BFU-E requires burst promoting activities (later identified as IL-3 and GM-CSF) and high levels of Epo. By proliferation and differentiation, BFU-E's give rise to late erythroid progenitors called colony-forming units erythroid (CFU-E), which give rise to small clusters of hemoglobinized cells after 2 days in culture (Iscove et al., 1974). The CFU-E's are very sensitive to Epo, and require 10 times less Epo as BFU-E. Moreover, Epo is absolutely required for the survival, proliferation and maturation of CFU-E. CFU-E probably correspond with proerythroblasts or their immediate precursors. Proerythroblasts undergo two proliferative cycles, giving rise to basophilic and polychromatic erythroblasts. Extensive hemoglobinization results in orthochromatic erythroblasts and subsequent enucleation gives rise to reticulocytes (Koury and Bondurant, 1988). Reticulocytes are released and mature into normocytes in the periphery.

Studying how the committed progenitor compartments behave under conditions of excess or deficiency in the red cell mass has defined the concepts of how homeostasis in the erythropoietic system is maintained (Iscove, 1977; Hara, 1976, 1977). In normal steady state conditions, approximately 30% and 75% of BFU-E and CFU-E are in cycle. In anemic states, Epo titers increase, the numbers of marrow CFU-E's increase and marrow BFU-E's decrease but the percentages of cycling cells in either progenitor compartment are not altered. Upon hypertransfusion, BFU-E's increase and CFU-E's decrease, again without much alteration in percentage of cells in cycle. Thus, erythropoietic stimulation occurs via amplication of the CFU-E pool. It has also been proposed that the

expansion in the CFU-E compartment results from increased influx from the BFU-E pool (Umemura et al., 1989; Jelkman, 1992).

An important level of regulation of erythropoiesis in vivo is in the hemopoietic inductive microenvironment (Trentin, 1970). During states of profound erythropoietic demand, erythropoiesis is shifted to extramedullary sites. It has been demonstrated that there is mobilization of BFU-E and other early progenitors from marrow to the blood and presumably homing to the splenic and hepatic sites (Hara and Ogawa, 1976, 1977; Goris et al., 1990; Rencricca et al., 1970). In addition, the efficiency of generation of CFU-E from BFU-E and erythroblasts from CFU-E has been determined to be 8 times and 5 times, respectively, more efficient in the spleen compared to the bone marrow (Pantel et al., 1989). Up to 70% of the total erythroid cell mass could be localized in the spleen in anemic mice. In contrast, the granulocyte-macrophage precursors in the spleen account for only 10% of the total pool. Thus, during erythropoietic stimulation, the splenic microenvironment is especially suited for the recruitment and amplification of erythroid precursors (Nijhof et al., 1993).

Red Cell Destruction

Destruction of aged and damaged red cells occurs in the spleen by a combination of rheological and immunological mechanisms (Drenckhahn, 1988). During the circulation of blood cells through the splenic sinusoids, red cells are forced to deform in order to traverse the interendothelial slits (Weiss and Tavassoli, 1970). Thus, erythrocytes with rigid membranes and cytoskeletons and those with hemoglobin precipitates are retarded and destroyed (Chen and Weiss, 1973). More subtle alterations in cell surface charge, membrane phospholipid asymmetry, membrane sialylation and opsonization and clustering of red cell neoantigens (e.g., band 3) allow

recognition and phagocytosis by macrophages in the splenic cords (Drenckhahn, 1988; Lutz, 1990). Similar mechanisms have been suggested to operate in the recognition of parasitized red cells by non-immune mechanisms (Turrini et al., 1992).

The Pathogenesis of the Anemia of Malaria

Anemia is an important cause of morbidity and mortality in patients with acute and chronic malarial infections. Despite several studies addressing the pathogenesis of anemia in human malaria, the mechanisms remain unclear (Abdalla et al., 1980; Pasvol, 1986; Woodruff et al., 1979). Aside from the variability in responses to parasitemia, human patients may have complications, such as, iron or folate deficiency, other parasitic infections, or liver and kidney dysfunction, factors which may exacerbate anemia. Under normal steady state conditions the numbers of circulating red cells are maintained by a balance between the numbers of senescent red cells removed and the number of reticulocytes released from marrow stores. In theory, anemia occurs as a result of increased red cell destruction or a decrease in the production and release of new red cells or a combination of both factors.

Since malarial parasites reside and mature within the red cell, hemolysis inevitably occurs as a result of schizogony and release of merozoites. Acute red cell destruction as it occurs in hyper-parasitemic states is manifestated by hyperbilirubinemia, hemoglobinuria, hemoglobinemia and a dramatic fall in red cell counts which could only result from acute parasite induced lysis.

Aside from direct effects of parasitization, evidence has been obtained from animal and human studies suggesting that normal unparasitized red cells are also lysed or cleared in an accelerated fashion,

thereby, compounding the effects induced by parasitization. For instance, the survival of autologous or heterologous radiolabelled red cells was significantly shortened several days after patients have cleared the parasitemia (Looaresuwan et al., 1987). The extrinsic nature of the hemolytic mechanism has led to the suggestion of an autoimmune basis. Facer (1980 a,b) found a higher incidence of a positive direct antiglobulin test (Coomb's test) among malarious children in Africa. Analysis of the antigen-antibody specificities of the red cell bound complexes indicates the presence of both parasite and autoimmune specificities. Later studies have not confirmed a correlation between the Coomb's positivity and anemia (Merry, 1986; Phillips, 1986). Another possible explanation (that is, extrinsic to the red cell) for the decreased life span of red cells is in the generalized hyperactivity of reticuloendothelial organs. This is perhaps best exemplified by the hypersplenism syndrome, which is characterized by, among other things, chronic anemia (Lewis, 1982).

Under normal conditions, a rapid loss of blood cells induces an increase of Epo levels which induces increased erythropoiesis in the bone marrow and the spleen. It has been described that the reticulocytic response in malaria is inadequate (Abdalla et al., 1980). In theory, this could be explained by decreased or ineffective erythropoiesis or an inhibition in the release of newly formed red cells. Earlier studies in human bone marrow smears have described the abnormal morphological features of erythroid precursors, generally known as dyserythropoiesis (Srichaikul et al., 1967). These changes include erythroblast multinuclearity, nuclear clefts and blebbing and cytoplasmic blebbing. Using autoradiographic studies, Wickramasinghe and colleagues (1982) have described an increase in precursors at G2 and a generalized arrest of

progress through S phase. A specific loss of cells in the polychromatic erythroblast has been described in the bone marrow of patients with acute *P. falciparum* malaria (Dormer, et al., 1983). Whether these dyserythropoietic changes are caused by parasites or host factors is unclear, but these changes are known to disappear as the parasites are cleared. Recently, Clark and Chaudri (1988) have been able to reproduce some of these dyserythropoietic changes upon injection of TNF into mice infected with *P. vinckei.* The negative regulation of erythropoiesis during *P. chabaudi* AS infections and the role of immune cytokines in this process is the main theme of chapter 3.

PROTECTIVE IMMUNITY TO BLOOD STAGE MALARIA: THE ROLE of ANTIBODIES, CELL-MEDIATED IMMUNITY AND THE SPLEEN

Antibody Mediated Immunity

An extensive body of literature on the protective role of antibodies in malaria exists. Evidence for the role of antibodies in the immune response to malaria comes mainly from passive transfer studies and studies in immunodeficient mice. Cure of acute malaria was achieved by passive transfer of purified immunoglobulins from adult immune plasma to children in the Gambia (Cohen, 1961; Bouharoun-Tayoun, 1990). Passive transfer experiments have also demonstrated the ability of immune serum or monoclonal antibodies to confer protection in rats and mice infected with *P. berghei, P. yoelii and P. chabaudi adami.* (Boyle et al., 1982; Diggs and Oster, 1975; Majarian et al, 1983; McDonald and Phillips, 1978) Passive transfer necessitates the transfer of large amounts of hyperimmune serum prior to infection. Protection was often manifested as a delay in the

onset of patent parasitemia and a decrease in the intensity of patent parasitemia. Passively transferred animals are generally not immune to rechallenge, reflecting the short lived protection conferred by passively transferred antibodies. Definitive evidence for the role of antibodies came from the demonstration that B cell deficient mice were unable to clear infections with *P. yoelii* (Grun and Weidanz, 1981). In contrast, B cell deficient mice were able to clear infection with *P. chabaudi adami*. This indicates that immunity to *P. yoelii* is antibody dependent whereas immunity to *P. chabaudi adami* is antibody independent. Infections of T cell deficient mice with *P. yoelii* were also non-resolving, indicating that the generation of the protective antibody response was T cell dependent (Weinbaum et al., 1976). However, passive transfer of serum was effective in T cell deficient recipients indicating that T cells were not required for the action of antibodies.

Several recent studies have addressed the qualitative aspects of the antibody response associated with protective immunity. White and colleagues (1991) have fractionated hyperimmune sera from *P. yoelii* infected mice according to isotypes and showed that the protective fraction resided in the cytophilic IgG2a fraction. The antigens recognized by the protective and non-protective antibody fractions were similar, if not identical. In vaccination studies using *P. yoelii* antigens in combination with various adjuvants, protective responses were again correlated with the presence of high titers of IgG2a (ten Hagen et al., 1993). Furthermore, higher recognition of membrane surface determinants as opposed to cytoplasmic determinants was associated with protection.

The mechanism by which antibodies exert protection is obviously dependent upon the stage specificity of the antigen recognized. Antibodies

to the major merozoite surface antigen are thought to act by agglutination and inhibition of red cell invasion (Burns et al., 1989; Epstein et al., 1981; Lew et al., 1989). Antibodies to trophozoite and schizont stage infected red cells act by opsonization and subsequent monocyte-macrophage antibody dependent cell-mediated inhibition or cytotoxity (Coleman et al., 1975; Bouharoun-Tayoun et al., 1990; Lunel and Druilhe, 1989; Shear, 1988).

Cell Mediated Immunity

As stated above, mice rendered B cell deficient by anti-IgM treatment while incapable of resolving acute *P. yoelii*, are capable of resolving acute parasitemias of *P. chabaudi adami* (Grun and Weidanz, 1981). In contrast, athymic mice were unable to resolve infection with *P. chabaudi adami*. This indicated that resolution of acute *P. chabaudi adami* parasitemia could occur via cell mediated, antibody independent mechanism(s). This phenomenon has been extended to a variety of other plasmodia, including *P. chabaudi chabaudi , P. vinckei peteri and Babesia microti* (Cavacini et al., 1990; Stevenson et al., 1990b) Subsequently, the ability of normal T cells, immune T cells, parasite-specific T cell lines and clones to confer protection to nude mice has been demonstrated (Brake et al., 1986, 1988; Cavacini et al., 1986). The transferred T cells did not suppress the timing and extent of patency but allowed T cell deficient mice to resolve the acute parasitemia. This suggested that the T cells did not act directly, but most likely induced the development and expression of an effector mechanism. The final clearance of the parasites, however, required antibody, as B cell deficient mice were unable to sterilize *P. chabaudi adami* infections (Grun and Weidanz, 1981).

The phenotypic and functional characteristics of T cells capable of conferring protective immunity has been the subject of recent intensive studies. T cells are generally subdivided by the expression of accessory molecules CD4 and CD8. Results of several studies indicate that $CD4^+$ T cells play a major role while $CD8^+$ T cells play a negligible or minor role in the development and expression of cell mediated immunity (CMI) to blood stage malaria. $CD4^+$ T cell lines and T cell clones are capable of conferring protection in nude mice (Brake et al., 1986, 1988). More importantly, depletion/deletion of $CD4^+$ T cells by antibody treatment or by homologous recombination and transgenic technology abrogated the ability to resolve *P. chabaudi adami* and *P. chabaudi* AS infections (Podoba and Stevenson, 1991; Suss et al., 1988; van den Heyde, 1993). In contrast, mice depleted of $CD8^+$ cells were capable of resolving infection, albeit with a slight delay (Podoba and Stevenson, 1991; Van den Heyde, 1993). These studies clearly established the primacy of $CD4^+$ T cells in host immunity to this group of plasmodia.

Antigen primed $CD4^+$ T cells have recently been differentiated on the basis of distinct profiles of lymphokine production and, thus, functional capacities (Street and Mossman, 1991). Th1 type cells secrete IFN-γ and IL-2 and little IL-4, IL-5 and IL-10 whereas Th2 type cells secrete predominantly, IL-4, IL-5 and IL-10. Th1 cells are effective in mediating delayed type hypersensitivity, by virtue of the potent macrophage activating properties of secreted IFN-γ. Th2 cells are especially suited for the induction of B cell differentiation and maturation.

Studies on the relative roles of Th1 and Th2 cytokines in immunity to blood-stage malaria were initiated by characterizing the lymphokine profiles of T cells from *P. chabaudi chabaudi* infected mice in bulk cultures

and in limiting dilution cultures (Langhorne et al., 1989; Stevenson and Tam, 1993; Taylor-Robinson and Phillips, 1992, 1993). It was found that T cells harvested from mice during the acute phase consisted predominantly of the Th1 type whereas T cells taken from mice after resolution of acute parasitemia were predominantly Th2 type cells. This has led to the proposition that Th1 cells activate effector macrophages, which mediate the resolution of acute parasitemia while Th2 cells provide help for B cell antibody synthesis (Langhorne, 1989). Antibodies were presumably responsible for keeping the residual parasitemia from becoming patent.

A plethora of effector mechanisms has been proposed for the expression of Th1 dependent cell mediated immunity during the acute phase of infection (Melancon-Kaplan and Weidanz, 1989). These putative mechanisms are reviewed here briefly.

Th1 type T cells are thought to effect resolution of acute parasitemia by activating macrophages and other non-antigen specific effector cells to produce reactive oxygen (ROI) and reactive nitrogen intermediates (RNI) which kill or inactivate parasites. The evidence for the role of ROI are threefold. First, in vivo administration of hydrogen peroxide or drugs which generate ROI reduced parasitemia of *P. vinckei* and *P. yoelii*-infected mice (Clark et al., 1983, 1987; Dockrell and Playfair, 1983) and in vitro addition of hydrogen peroxide inhibited multiplication of *P. falciparum* (Ockenhouse and Shear, 1984b). Second, scavengers of ROI inhibited in vitro and in vivo killing of parasites (Clark et al., 1987; Ockenhouse and Shear, 1984a and b). Third, splenic macrophages have been shown to have an enhanced capacity for ROI generation during infection with *P. berghei*, *P. yoelii* and *P. chabaudi* (Brinkman et al., 1984;

Lee et al., 1986; Stevenson et al., 1992). However, mutant P/J mice which are unable to generate ROI resolve *P. chabaudi adami* infections as efficiently as non-mutant mice, suggesting an ROI-independent mechanism (Cavacini et al., 1989). RNI have been shown to kill *P. falciparum* in culture (Rockett et al., 1991). Recently, nitric oxide (NO) has been suggested to be the mediator responsible for the suppression of acute parasitemia exerted by a protective Th1 type T cell clone (Taylor-Robinson et al., 1993). However, upon closer examination of the data presented, the mice treated with (nitrogen oxide synthase) NOS inhibitor exhibited only an unremarkable increase in peak parasitemia, but nonetheless suppressed acute parasitemia. These seemingly contradictory observations either indicate that macrophage-derived ROI and RNI play no role in the resolution of acute parasitemia or, more likely, that multiple mechanisms operate during this process.

A multiplicity of putative non-antigen specific effector cells and molecules have been implicated in the killing of intra-erythrocytic parasites. In addition to macrophages, granulocytes, NK cells and gamma delta TCR-bearing T cells have been suggested to act as effector cells against blood stage malaria (Taverne, et al., 1989; Langhorne, et al.,1992). Aside from ROI and RNI, lipid peroxides, various cytokines and a protein factor called crisis form factor have been shown to induce parasite death in vitro and in vivo (Carlin et al., 1985; Haidaris et al., 1983; Jensen, 1989; Orago and Facer, 1993; Rockett et al., 1988; Taverne et al., 1987). Episodes of fever and systemic shock have also been suggested to limit rapid parasite multiplication (Kwiatkowski and Novak, 1991; chapter 4).

The current paradigm for the development and expression of immunity to both murine and human plasmodia incorporates both non-

antigen-specific, cell mediated immunity and antibody dependent mechanisms (Kwiatkowski, 1992; Long, 1993). Non-specific mechanisms, whether they be T-cell dependent or independent, are induced rapidly and act to control and suppress acute parasitemia. A more delayed onset of antibody synthesis confers protection from patency and clinical attacks. With respect to murine malaria models, immunity to *P. yoelii* and *P. chabaudi adami* appears to represent polar extremes, requiring antibody and cell mediated mechanisms, respectively. Immunity to *P. chabaudi* AS appears to require sequential action of cell-mediated and antibody-mediated mechanisms (Taylor-Robinson, 1993).

Role of the Spleen in Protective Immunity

The importance of the spleen in immunity to plasmodia is well established (Taliafero and Cannon, 1936; Wyler, 1983). Splenic functions in malaria are incompletely understood, but are, however, likely to be complex. During the course of malaria infection, parasite-specific antibody forming cells increase in the spleen (Sayles and Wassom, 1991). Increased activation and homing of T cells to the spleen has been described (Langhorne and Simon, 1991). Chemotactic factors are secreted which recruit monocytes and other effector cells (Wyler and Gallin, 1977). Dynamic changes occur in the microcirculation and filtration capacity of the spleen (Wyler et al., 1981). Splenic macrophages develop an enhanced capacity for monokine and ROI and RNI release (Brinkman, et al., 1984; Stevenson, et al., 1992). The spleen is, thus, likely to be the site of cellular activation and interactions, and trapping and destruction of rheologically or immunologically modified parasitized red cells. The role of the spleen in the induction and expression of immunity to *Plasmodium chabaudi* AS is the subject presented in chapter 5.

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CHAPTER 2

The paper presented in this chapter describes and compares the development of anemia and the kinetics of erythropoietic responses in the bone marrow, spleen and blood of resistant C57BL/6 and susceptible A/J mice during infection with *P. chabaudi* AS. The role of erythropoietin and progenitor cell migration is examined.

Plasmodium chabaudi AS: ERYTHROPOIETIC RESPONSES DURING INFECTION IN RESISTANT AND SUSCEPTIBLE MICE

ABSTRACT

The course of anemia and the erythropoietic response in the bone marrow, spleen and blood were studied during *Plasmodium chabaudi* AS infection in resistant C57BL/6 (B6) and susceptible A/J (A) mice. Infections in B6 mice were characterized by moderate levels of both parasitemia and anemia and survival. In contrast, A mice experienced high parasitemia, severe anemia and high mortality. During the period of anemia, erythropoiesis, as measured by in-vivo ^{59}Fe incorporation, was significantly more depressed in bone marrow and more increased in the spleen in resistant B6 mice. The increase in splenic ^{59}Fe incorporation was a function of the size of the spleen. Bone marrow CFU-E were decreased to 50% of control in both strains, while splenic CFU-E were increased 2-fold greater in B6 mice in comparison to A mice. However, the absolute numbers of CFU-E per spleen in the two strains were not significantly different during peak parasitemia. Bone marrow BFU-E were transiently increased before peak parasitemia whereas splenic BFU-E peaked during peak parasitemia. A mice had significantly lower numbers of BFU-E per spleen on all days except at peak parasitemia. The frequency of blood-borne BFU-E as well as plasma erythropoietin titers were increased earlier and to a greater extent in A mice. These results suggest that an impaired amplification of late stage splenic erythropoiesis may be an important determinant in the severity of anemia and lethality of infection with *P. chabaudi* AS in A mice. Moreover, these results demonstrate that the defective amplification of splenic erythropoiesis in A mice is neither caused by a defect in the mobilization of BFU-E from the bone marrow to the spleen nor by a defect in erythropoietin production.

INTRODUCTION

Anemia represents a major cause of morbidity and mortality in severe human malaria (Greenwood *et al.* 1991; Phillips and Warrell 1986). It has been recognized that the etiology of the anemia of malaria is both complex and multifactorial (Pasvol 1986). Parasite mediated hemolysis (Phillips *et al.* 1986), decreased and ineffective red cell production by the bone marrow (Dormer et al. 1983) and accelerated clearance of unparasitized red cells by reticuloendothelial organs (Looaresuwan *et al.* 1987) have been implicated as major factors in the pathogenesis of malarial anemia in man.

In an effort to understand the contribution of impaired red cell genesis to the anemia of malaria, numerous investigators have studied erythropoietic responses in experimental murine malarias (Maggio-Price *et al.* 1985; Silverman *et al.* 1987; Villeval *et al.* 1990; Weiss *et al.* 1989). These studies demonstrate that, in general, bone marrow erythropoietic activity is depressed while splenic erythropoiesis is dramatically amplified. It has also become apparent that the quantitative and qualitative aspects of the observed erythropoietic changes are dependent upon the species, strain, virulence and cellular tropism (ie., normocyte versus reticulocyte preference) of the parasite as well as the genetic background of the host .

Our laboratory has previously shown that inbred mouse strains differ in their resistance to infection with the murine malaria parasite *Plasmodium chabaudi* AS (Stevenson *et al* 1982). Upon infection with a uniform dose of 10^6 parasitized red blood cells (PRBC), C57BL/6 (B6) mice and other resistant strains experience moderate levels of parasitemia and anemia, marked splenomegaly and survive the infection. In contrast, parasitemia and anemia are more severe in A/J (A) mice. In these hosts,

which were demonstrated to be the most susceptible inbred mouse strain, infection is invariably fatal and splenomegaly minimal. Genetic analyses of hybrid and backcross progeny and recombinant inbred strains derived from susceptible A and resistant B6 progenitor mice showed that resistance is controlled by a single, autosomal, non-H-2 linked, dominant locus, designated as *Pchr*. Furthermore, the phenotype of marked splenomegaly was found to be genetically linked to resistance (Stevenson and Skamene 1985; Stevenson *et al.* 1988). Since the spleen plays a dominant role during stress erythropoiesis in rodents, we postulated that differential expansion of the splenic erythroid compartment may explain, at least in part, the difference in the severity of anemia and the outcome of infection between resistant and susceptible mice.

In the present study, we have compared the temporal, topologic and quantitative changes in the levels of early and late erythroid progenitors during the entire course of *Plasmodium chabaudi* AS infection in resistant B6 and susceptible A mice. Our results suggest that splenic amplification of the later stages of erythropoiesis is impaired during lethal infection in A mice. Furthermore, we provide evidence that this is caused neither by a defect in the mobilization of early erythroid progenitors (BFU-E) from the bone marrow to the spleen nor by a defect in erythropoietin production.

MATERIALS AND METHODS

Experimental Animals Six to eight week old female B/6 mice and male A mice were purchased from Charles River (Quebec) and the Jackson Laboratory (Bar Harbor, ME) respectively. Animals were maintained ad libitum on standard mouse chow and water under a standard 12:12 light dark cycle.

Parasite and Infection Protocol An LDH virus free isolate of *Plasmodium chabaudi* AS, originally obtained from Prof. David Walliker (University of Edinburgh, Edinburgh, Scotland), was maintained as frozen stocks in the vapor phase of liquid nitrogen. Experimental infections were performed as previously described (Podoba and Stevenson 1991). Briefly, the parasite was maintained by weekly passage in B6 mice by infection with 10^7 parasitized red blood cells (PRBC). Experimental mice were infected intraperitoneally (IP) with 10^6 PRBC suspended in 1 ml of pyrogen-free saline.

Determination of Parasitemia and Hematologic Parameters Parasitemia was determined by Diff-Quik (Baxter Corp, IL) staining of thin blood smears prepared from tail vein blood. Reticulocytes were visualized on thin blood smears stained supravitally with New Methylene Blue (AJP Scientific, Clifton,NJ). The percentage of PRBC and reticulocytes were determined by counting at least 200 cells. Hematocrits were determined by spinning blood in microhematocrit tubes for 4 minutes in a microcapillary centrifuge (International Equipment Co, Boston, MA).

In vivo ^{59}Fe Incorporation Assay 0.5 µCi of $^{59}FeCl_3$ (ICN Biochemicals, Inc., Mississauga, Ontario) in 0.2 ml pyrogen free saline was injected into each mouse subcutaneously at designated days after infection. Blood samples (100 µl aliquots), spleens and femoral pairs were obtained 6 hours later and the radioactivity determined using a gamma counter (5500B, Beckman Instruments, Palo Alto, CA). Radioactive iron incorporation for each sample was calculated as a percentage of counts in tissue over total counts of radioactivity injected.

Preparation of Cell Suspensions Spleens and femora were excised aseptically from mice at indicated times after infection. Heparinized blood was obtained by cardiac puncture from previously anesthetized mice. Cell suspensions were prepared by passing minced spleens through a sterile, fine wire mesh. Bone marrow cells were flushed with 1 ml of cold Iscove's Modified Dulbecco Medium (IMDM, Gibco, Grand Island, N.Y.) supplemented with 5% fetal calf serum (FCS, Hyclone, UT), 0.12% gentamycin (Schering Canada Inc., Montreal, Quebec) and 2 mM glutamine (Gibco), using a syringe attached to a 25-gauge needle. Total nucleated peripheral blood cells were obtained by density gradient centrifugation on Ficoll-Hypaque media (Lympholyte M, Cedar Lane, Ontario). Low density mononuclear cells were obtained after 20 minutes of centrifugation at 2000 rpm at 25°C. Cell suspensions were washed 2-3 times in IMDM and total viable nucleated cells determined using a hemacytometer after appropriate dilution in trypan blue.

Erythropoietic Progenitor Assays The CFU-E assay was performed according to Iscove and coworkers (Iscove *et al.* 1974). The CFU-E medium contained 0.8% methylcellulose (Terry Fox Laboratory, Vancouver, BC), 30% FCS, 200 mU/ml recombinant human erythropoietin rhEpo (a kind gift from Genetics Institute, Cambridge, MA), 2 mM glutamine and 5 X 10^{-5} M 2-mercaptoethanol (Sigma, MO) in IMDM. Bone marrow and spleen cells were plated in 35 mm dishes (Sarstedt, Montreal, Canada) at a density of 2 X 10^5 cells/ml and 4 X 10^5 cells/ml, respectively. Benzidine positive clusters of 8 or more cells were scored after 2 days of incubation in a 5% CO_2 humidified incubator kept at 37°C. The BFU-E assay was performed according to Monette and coworkers (Monette *et al.* 1987). The BFU-E medium consisted of 0.8%

methylcellulose, 30% FCS, 10% pokeweed mitogen spleen cell conditioned medium (as a source of burst promoting activity), 2000 mU/ml rhEpo, 0.1 mM hemin (Eastman Kodak, USA), 2mM glutamine and 5 $X10^{-5}$ M 2-mercaptoethanol in IMDM. Cells were plated at the same density as in CFU-E cultures. Hemoglobinized colonies consisting of at least 50 cells were scored unstained after 7 days of culture.

Erythropoietin Bioassay Erythropoietin titers in serum samples were estimated using the method of Krystal (Krystal 1983). Spleen cells, which were obtained from B6 mice made anemic by two daily consecutive phenylhydrazine (Fischer Scientific Canada, Montreal Quebec) injections (60 mg/kg/day), were used as responder cells. Responder cells were plated in 96 well plates (Linbro, ICN) at a density of 5 X 10^{6} cells per ml in 20% FCS, 5 X 10^{-5} M 2-mercaptoethanol and IMDM in a total volume of 100 µl. Dilutions of sera samples were assayed in triplicate. A standard curve using rhEpo was established for each assay. Cultures were incubated for 24 hours. In the last 2 hours, 20 µl of IMDM containing 1µCi of ^{3}H-thymidine (ICN) were added to each well. Two hours later, cultures were harvested onto glass fiber filters using a Skatron automatic cell harvester (Skatron, Inc., Sterling VA). DNA synthesis was measured by liquid scintillation counting of air dried disks suspended in 2.5 ml of Ecolume scintillation fluid (ICN) using a Beckman beta counter.

Statistical Analysis Values are expressed as mean ± standard error of the mean. Differences in the mean values between B6 and A mice were analyzed by Student's t-test. A probability of less than or equal to 0.05 was considered significant.

RESULTS

Course of Infection and Anemia During *P. chabaudi* A S Infection

Following infection with 10^6 *P. chabaudi* AS PRBC, both susceptible A and resistant B6 mice experienced a prepatent phase until day 6, during which time very few parasites could be detected in the peripheral blood smears. Infections became patent thereafter and the peak parasitemia levels occurred on day 10. The percentages of parasitemia were significantly higher on days 6, 8, and 10 in A mice compared to B6 mice although, as we have previously demonstrated (Stevenson et al. 1982), there was no difference in the absolute number of PRBC on the day of peak parasitemia. Approximately 75% of A mice succumbed to the infection between days 10-13. No deaths were recorded among B6 mice and these mice cleared the infection by day 28 (Figures 1a and 1b).

The development of anemia during the course of infection was monitored by determining changes in the hematocrit of peripheral blood taken from mice at various days post infection. As shown in Figure 1c, hematocrits of A and B6 mice were similar at the start of the experiment. As parasite levels increased, anemia developed progressively as evidenced by the precipitous fall in hematocrit values. On the day of peak parasitemia, the time at which deaths among A mice started to be observed, these hosts had significantly lower hematocrits (9.9 ±.005%) compared to B6 mice (23.24 ± .01%) ($P < 0.01$).

The erythropoietic response to anemia was monitored by reticulocyte release into the peripheral blood. Reticulocyte levels remained within the normal range of less than 5% in both strains of mice until day 8. On day 10, A mice had higher percent reticulocytes (% reticulocytes= 16.1) than

B6 mice (5.8%, P<0.05). Upon correction for the difference in hematocrits, A mice actually had equivalent levels of reticulocytes compared to B6 mice (corrected % reticulocytes A=3.2%, B6=2.7%). Therefore, the increase in % reticulocytes in A mice was artificially caused by lysis of normocytes rather than representing a true release of reticulocytes from the hemopoietic organs. Reticulocytes were released into the blood starting on day 12 in B6 mice. Two peaks of reticulocytosis on days 14 and 20 were observed separated by a minor decrease on day 17. Reticulocyte levels returned to normal values on day 28, when the parasites had been cleared and hematocrits had returned to normal suggesting a complete regeneration of the peripheral red cell pool.

In Vivo Ferrokinetic Analysis

In order to estimate the in vivo erythropoietic activity in the bone marrow and spleen during the course of infection, six-hour ^{59}Fe incorporation studies were conducted on groups of 3-4 mice on different days following infection with 10^6 PRBC. ^{59}Fe incorporation was determined on tissue and blood samples.

Values, expressed as percentage of total radioactivity injected, for femoral ^{59}Fe incorporation were significantly higher in normal, uninfected B6 mice (2.90 ± 0.31% of total radioactivity injected) compared to A mice (1.74 ± 0.21%) (P=0.05). The results of a representative experiment are presented in Figure 2; data presented in a, b and d are expressed as per cent ^{59}Fe incorporation of normal, control animals. In both strains of mice, femoral ^{59}Fe incorporation (expressed as percent control) increased transiently and then decreased below normal values on day 3 and day 6, respectively. Thereafter, femoral ^{59}Fe incorporation returned to near normal values in A mice, whereas B6 femoral ^{59}Fe

incorporation remained low (approximately 50% of control values) for most of the rest of the experiment. On day 8 and 10, femoral ^{59}Fe incorporation was significantly greater in A mice compared to B6 mice (values expressed as percentage of control; day 8: A=99.4% vs. B6=48.3%, $P<0.05$; day 10: A/J=93.7% vs. B6=52.7 % , $P<0.01$) suggesting less depression in bone marrow erythropoiesis in A mice compared to B6 mice.

A completely different picture emerged from determination of the splenic ^{59}Fe incorporation data (Figure 2b). In contrast to the femoral ^{59}Fe incorporation, normal values for splenic ^{59}Fe incorporation were significantly higher in A mice (3.60 ± 0.31%) than in B6 mice (2.34 ± 0.31%), $P<0.05$. No significant change in splenic ^{59}Fe incorporation was observed until day 6 in either strain. On day 8, ^{59}Fe incorporation in B6 spleens increased about 4.5 fold whereas no significant change was observed in A mice. At the time of peak anemia on day 10, the mean value for splenic uptake was still higher in B6 mice, although this difference was not statistically significant. Splenic ^{59}Fe incorporation increased progressively in B6 mice reaching peak levels (up to 10-fold) on day 14. Subsequently, values decreased but did not return to normal levels even by day 28.

In order to normalize for the significant changes in spleen size observed during the course of infection, splenic ^{59}Fe incorporation values were divided by the weights of individual spleens. This index (% ^{59}Fe incorporation/mg spleen) gives an estimate of erythropoietic activity per unit weight of tissue. As shown in Figure 2c, this index was higher in spleens of normal, uninfected A mice. A drop in the index was observed in both mouse strains between days 6 and 8 post infection. On day 10, the

index increased reaching normal values in B6 but not in A mice. A second decrease occurred between days 17 and 20 in B6 mice.

Blood ^{59}Fe incorporation exhibited an increase in parallel with the appearance of reticulocytes in the peripheral blood. No statistically significant differences in ^{59}Fe incorporation in the blood were observed between A and B6 hosts during the entire course of the experiment (Figure 2d).

These results suggested to us that the relative distribution of ^{59}Fe between the bone marrow and the spleen and, thus, the erythropoietic activities during steady state and during anemia in these two hemopoietic organs differs significantly between the two mouse strains studied. The shunting of erythropoiesis from the bone marrow to the spleen was more pronounced in the non-lethal *P. chabaudi* AS infection characteristic of B6 mice. In order to investigate the putative cellular and systemic mechanisms involved in this observed difference, we determined the numbers of the late, sessile erythropoietic progenitor CFU-E in the bone marrow and spleen as well as the earlier, mobile erythropoietic progenitor BFU-E in the bone marrow, spleen, and blood of both B6 and A hosts during the entire course of *P. chabaudi* AS infection.

Bone Marrow Cellularity and Splenic Size and Cellularity

As shown in Figure 3a, normal uninfected B6 mice had a higher bone marrow cellularity ($1.92 \pm 0.15 \times 10^7$ cells per femur) compared to A mice ($1.46 \pm 0.20 \times 10^7$ cells per femur). In both strains of mice, bone marrow cellularity was consistently observed to be decreased during infection as the anemia progressed, approaching 50% of control values between day 9 and day 12.

Spleen cellularity was similar in uninfected A/J and B6 mice (Figure 3b). As early as day 7, spleen cellularity was increased above normal levels in both mouse strains. At the time of peak parasitemia, spleen cellularity was approximately 2 fold greater in B6 mice. The spleens of B6 mice continued to enlarge reaching peak levels on day 12 and day 18. B6 spleen cellularity remained significantly higher than normal through 40 days post infection (data not shown).

Bone Marrow and Splenic CFU-E Compartments

Figure 4a depicts the changes in the numbers of CFU-E in the femur during the course of infection. In the experiment shown here, normal uninfected A mice had a higher number of CFU-E per femur compared to B6 mice. This observation was, however, not consistently observed. CFU-E numbers increased transiently on day 1 and 3 in B6 and A femurs, respectively. Thereafter, there was a decrease in CFU-E numbers per femur between days 3 and 7 for B6 mice and between days 5 and 7 for A mice. On the day of peak parasitemia, the numbers of CFU-E increased in A mice, such that there were twice as many as in B6 mice. An increase in B6 femoral CFU-E was observed much later on day 17 and day 21.

The normal splenic CFU-E pool was consistently observed to be twice as large in A mice compared to B6 mice (Figure 4b). Significant increases in the splenic CFU-E pool were observed starting day 7 in both strains. During peak parasitemia, there was no significant difference in the absolute numbers of CFU-E in the spleens of A and B6 mice. It is interesting, however, to note that at this time, the splenic CFU-E pool was amplified approximately eight times normal in B6 mice compared to only four times in A mice. Also, the hematocrit of A mice (approximately 10%) was less than half that of B6 mice (approximately 25%). Thereafter,

the B6 splenic CFU-E pool was further amplified reaching maximal levels (representing an increase of 156 times over normal levels) on day 12. Splenic CFU-E amplification exhibited a bimodal response in parallel with changes in splenic cellularity. Considering the more severe anemia which occurred in A compared to B6 mice, it is, thus, apparent that the degree of amplification in the splenic CFU-E pool was inappropriate in A mice.

Blood, Bone Marrow and Splenic BFU-E Compartments

The BFU-E levels per femur were similar in uninfected animals of both strains (Figure 4c). Furthermore, the changes observed during *P. chabaudi* AS in the bone marrow BFU-E compartment were strikingly similar in A and B6 mice. Significant increases in the number of BFU-E per femur were observed between days 7 and 8, the time at which femoral CFU-E and cellularity were low. In each case, the numbers of BFU-E returned to normal levels and in resistant B6 hosts remained within the normal range throughout the experiment. In contrast to the bone marrow, the numbers of BFU-E in the spleen were two-fold greater in normal B6 mice compared to normal A mice (P=0.01). In both strains, the numbers of splenic BFU-E started to increase around day 6, reaching peak levels on day 10. This peak preceded the peak levels of the more mature CFU-E compartment by approximately 2 days. B6 mice had significantly higher numbers of splenic BFU-E except on day 10. Splenic BFU-E numbers decreased subsequently remaining slightly above normal for the rest of the experiment in B6 mice (Figure 4d).

Table 1 shows the frequencies of BFU-E per 5 x 10^6 peripheral blood mononuclear cells (PBMC) in A and B6 mice as a function of time after infection. Results from one of two experiments are shown. Normal blood from either strain contained relatively low frequencies of BFU-E (5-

25 per 5 x 10^6 PBMC). As the infection progressed and anemia ensued, a dramatic increase in the frequencies of blood BFU-E (50-104 BFU-E/ 5 x 10^6 PBMC) was observed. There was an earlier increase and a higher peak frequency of blood BFU-E in A hosts. Frequencies of blood BFU-E decreased as B6 hosts recovered from anemia. Based on these data, it is obvious that A mice are able to mobilize BFU-E from the bone marrow to the blood.

Erythropoietin Production

To determine if differences in the production of Epo occurred in resistant and susceptible mice, groups of A and B6 mice were bled at various days post infection and plasma Epo titers were determined by in vitro bioassay. In both strains, Epo titers remained low until hematocrits dropped to 33% on day 8. The plasma of A mice were found to have significantly higher Epo titers on days 8-10, reflecting the severity of anemia in these mice (Table 2). Thus, A mice are capable of secreting biologically active Epo into the bloodstream in response to anemia .

DISCUSSION

The present report compares the erythropoietic responses of resistant B6 and susceptible A mice during infection with the rodent malaria *P. chabaudi* AS. We provide a detailed description of the changes in the peripheral red cell pool and the terminally differentiating, hemoglobin-synthesizing cells as well as in the early and late compartments of the committed erythroid progenitors, BFU-E and CFU-E. We have attempted to define the interaction between the erythropoietic compartments of the bone marrow and the spleen and to correlate the hemopoietic changes with the course and outcome of malaria infection.

Significant changes in the bone marrow parameters were observed as early as day 3 of infection. As the anemia developed, bone marrow cellularity, ^{59}Fe incorporation and CFU-E numbers decreased progressively. Similar changes have been observed in mice infected with *P. berghei*, *P. yoelii* or *P. chabaudi adami* (Maggio-Price *et al.* 1985; Silverman *et al.* 1987; Weiss *et al.* 1989). In spite of the decrease in bone marrow cellularity, the numbers of marrow BFU-E were elevated preceeding the peak of parasitemia and anemia. This illustrates that the changes in bone marrow parameters often correlate with but do not necessarily follow the changes in cellularity. It is interesting to note (especially in light of the splenic ^{59}Fe incorporation data) that resistant B6 mice had significantly depressed marrow ^{59}Fe incorporation for a sustained period of time compared to susceptible A/J mice.

A completely contrasting situation occurred in the spleen. No significant changes in splenic parameters were observed early in the infection. As soon as anemia developed, splenic cellularity, CFU-E, BFU-E and ^{59}Fe incorporation increased progressively. Of note, we observed significant differences in the cellularity and ^{59}Fe uptake in the spleens of resistant versus susceptible mice before and during peak parasitemia. In contrast, splenic CFU-E and BFU-E numbers were not significantly different between the two strains at the time of peak parasitemia. It must be noted that in spite of a more severe anemia, A mice had amplified the splenic CFU-E compartment 2-fold less than the B6 mice. We, therefore, consider the erythropoietic response in A mice to be inappropriate even at the level of splenic CFU-E. More importantly, ^{59}Fe incorporation per spleen which provides a direct measure of hemoglobin synthesizing activity was significantly less in A mice compared to B6 mice. These observations

have led us to conclude that 1) splenic amplification of erythropoiesis is impaired in A mice during infection with *P. chabaudi* AS and 2) the defect most likely occurs in the late (hemoglobin synthesizing-erythroblast) rather than early (BFU-E and CFU-E) stages of erythropoiesis.

The basis of the defect in splenic amplification in susceptible mice may be due to a number of mechanisms. In the next few paragraphs, we discuss the possible contribution of different hemopoietic regulatory mechanisms to the observed defect in susceptible hosts.

The differential behaviour of hemopoietic stem cells and the hemopoietic inductive microenvironment in bone marrow and spleen has long been recognized (Trentin 1970). Recently, Pantel et al. (Pantel *et al.* 1990) presented a mathematical model of erythropoiesis based on theoretical calculations and experimental data, which illustrate the differential roles of the bone marrow and spleen in erythroid homeostasis. The most salient features of this model are: 1) that during normal steady state, one extra erythropoietic cell division takes place in the spleen compared to the bone marrow and 2) that during states of erythropoietic demand, for example, bleeding or hypoxia, erythroid precursors can undergo 5-6 additional cell divisions in the spleen but only 2 additional divisions in the bone marrow. This allows the splenic contribution to total erythropoiesis to rise from less than 10% to greater than 40% during stimulation. The factors which regulate the number of extra cell divisions as well as the presence of any differences in the number of extra divisions between mouse strains are presently not known. Furthermore, Pantel et al. (Pantel *et al.* 1990) demonstrated that the dose response curves between erythropoietic stimulation (low hematocrit or hypoxia) and erythropoietic activity were steeper in the spleen compared to the bone marrow. To

determine if resistant and susceptible mice differed in this respect, we performed linear regression analyses between the degree of erythropoietic stimulation (hematocrit) and the erythropoietic activity (^{59}Fe incorporation) in the bone marrow and spleen of the two mouse strains (not shown). As expected from the data presented in Figure 2, the erythropoietic activity in bone marrow was positively correlated with the hematocrit whereas the erythropoietic activity in the spleen was negatively correlated with the hematocrit in both strains of mice. More importantly, the absolute values of the slopes were higher in B6 mice compared to A mice for both bone marrow and spleen. The theoretical y-intercept values for the spleen and the bone marrow were higher and lower respectively in B6 mice. We interpret this to mean that the two strains of mice differed with respect to the rate of erythropoietic response to a constant amount of erythropoietic demand as well as to the relative distribution of erythropoietic activity between the spleen and bone marrow.

The differences in erythropoietic activity between the spleen and bone marrow may be under microenvironmental regulation. Although we observed that the egress of BFU-E as well as CFU-GM and CFU-Mix (data not shown) from the bone marrow to the blood occurred to a similar extent in B6 and A mice (Table 1), it is possible that the degree of homing to the spleen, mediated by unique lectin pairs expressed on hemopoietic and stromal cells (Konno and Tavasolli 1990), is more efficient in B6 mice. Alternatively, A mice may have a defect in the development of erythroid-supportive stromal cells. Yanai et al. (Yanai *et al.* 1989), have recently established spleen stromal cells which show an enhanced support capacity for CFU-E and their subsequent maturation into enucleated red cells in vitro. A defect in the development of similar stromal cells in vivo may

impede full erythroid amplication even if sufficient numbers of BFU-E and CFU-E are present.

Defective amplification of erythropoiesis may also be caused by inappropriate production of positive and negative growth regulatory factors. In vivo, erythropoietin is acknowledged to be the primary humoral regulator of erythropoiesis (Krantz 1991). Our results (Table 2) as well as previous studies (Maggio-Price *et al.* 1985; Rencricca *et al.* 1974; Silverman *et al.* 1987; Villeval *et al.* 1990; Weiss *et al.* 1989) demonstrate that appropriate and sufficient quantities of bioactive erythropoietin are produced during both lethal and non-lethal malaria. Based on our determination of Epo levels in A and B6 mice, it is, therefore, unlikely that a deficiency in erythropoietin caused the impaired splenic erythropoiesis in A mice. It has been proposed that splenic erythropoiesis might be regulated by serum factors distinct from erythropoietin (Weiss *et al.* 1989). The molecular nature of these factors is presently unknown but they are thought to be similar to endotoxin-induced serum factors which mediate shunting of erythropoiesis from the bone marrow to the spleen (Fruhman 1967; Staber and Metcalfe 1980; Vos *et al.* 1972). It is known that a cascade of cytokines, including IL-1, TNF, IL-6 and myeloid colony stimulating factors, are synthesized shortly after endotoxin injection (Broudy *et al.* 1990; Ulich *et al.* 1990). We have not ascertained whether there is a difference in the production of these factors in the two strains of mice. Given that A mice seem to release stem cells from the bone marrow quite efficiently, it is unlikely that they are deficient in the production of these factors. Another possible level of erythroid regulation is in the production of inhibitors of erythroid cell proliferation and differentiation. Miller and colleagues (Miller *et al.* 1989) demonstrated that both bone

marrow and spleen adherent cells from *P. berghei* and *P. vinckei*-infected mice secreted soluble factor(s) which inhibited erythroid cell proliferation in vitro. We have confirmed that such soluble inhibitor(s) are also secreted by bone marrow and spleen cells from *P chabaudi* AS infected mice (Yap and Stevenson 1991). We believe, however, that the production of these factors may not be crucial in determining the extent of erythroid amplification as equivalent levels of inhibitory activity are produced by cells from both strains.

While the experiments described herein were in progress, Villeval published results of similar experiments with resistant B6 and susceptible C3H mice infected with *P chabaudi* DS and DK strain (Villeval *et al.* 1990). Similar to our observations, they noted an impairment in marrow erythropoiesis and an increase in splenic erythropoiesis. Furthermore, they observed that splenic amplification of the erythroblast, CFU-E and BFU-E compartments was 2.5 -fold lower in susceptible C3H mice compared to resistant B6 mice. They concluded that a defect in amplification of splenic erythropoiesis is a crucial determinant of the fatal outcome of malaria infection. They also suggested that a defective migration or multiplication of early stem cells may account for this defect. Our results confirm their observation that indeed a defect in amplification of late erythropoiesis occurs in the spleens of susceptible mice. However, we were unable to demonstrate a disparity in the absolute numbers of splenic CFU-E between the two mouse strains we studied. Differences in the strain of susceptible mice (A/J versus C3H) and parasites (AS versus DS and DK) used as well as tissue culture conditions may have contributed to the divergent results. Furthermore, our results extend the observations of Villeval *et al.* (1990)by demonstrating that the migration of early stem cells

from the bone marrow to the spleen is not defective in susceptible mice. Their results and ours , thus, emphasize the prime importance of splenic erythropoiesis in determining the severity of anemia and the outcome of malaria infection .

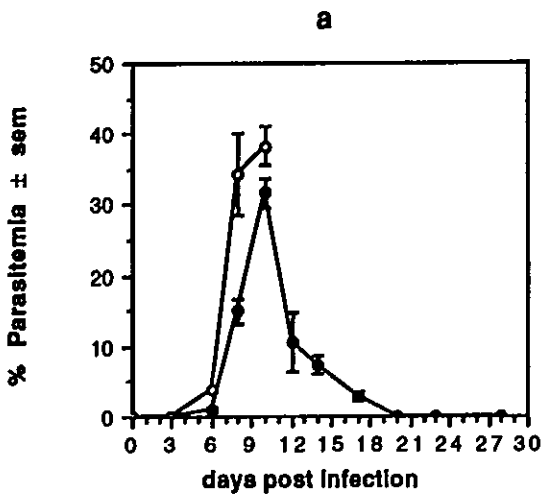
While it is not possible to establish a direct causal relationship between the impairment in splenic erythropoietic response and host susceptibility to *P. chabaudi* AS infection, several lines of evidence suggest that such a relationship may exist. First, splenectomy of resistant B6 mice produces a more severe anemia and increases the mortality rate from 0 to 30-50% (Rae 1987; Stevenson *et al.* 1990). Splenectomy in A mice is invariably 100% fatal. Second, blood transfusion, which alleviates the anemia and presumably compensates for the deficit in erythropoietic response, has been shown to have a rescuing effect in both lethal *P. berghei* infection (Hejna *et al.* 1974) and *P. chabaudi* AS infection (Yap, unpublished observations). Thirdly, the strong genetic linkage between splenomegaly and host resistance to *P. chabaudi* AS (Stevenson *et al.* 1988) as well as the concordance between the results of our studies with A mice and of Villeval's studies with C3H mice (Villeval *et al.* 1990) provide further support for this linkage. It is, of course, entirely possible that the defect in splenic amplication may have been an effect rather than the cause of the lethal course of infection. However, we believe that the impaired erythropoietic response may contribute to the development of severe anemia in A mice. Severe anemia compounded by metabolic dysregulation (e.g., lactic acidosis, hypoglycemia and hepatic injury) may eventually lead to death of susceptible mice (Holloway *et al.* 1991).

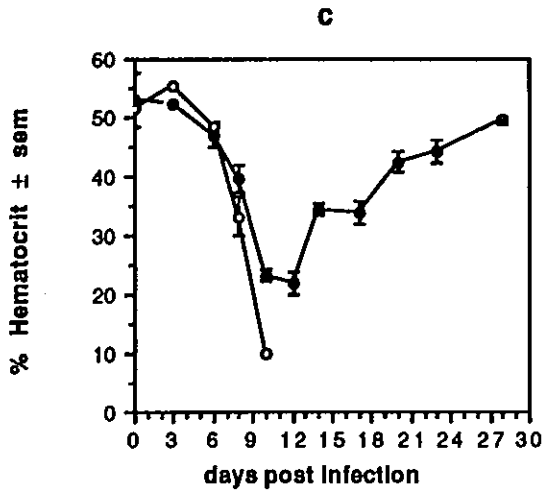
In conclusion, we have demonstrated that splenic amplification of late erythropoiesis is impaired during the lethal course of *P chabaudi* AS

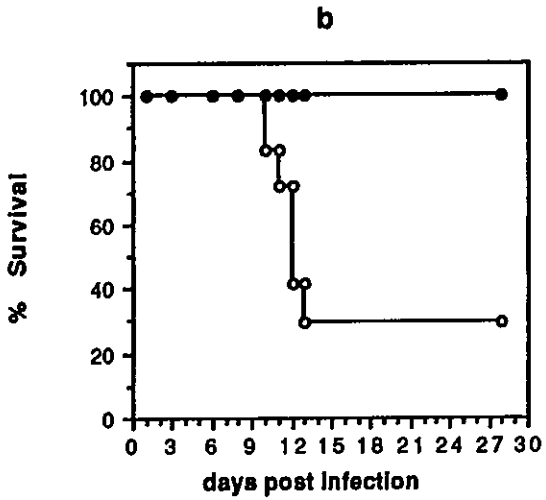
infection in susceptible A mice. We provide evidence that this is neither caused by a deficiency in erythropoietin production nor by ineffective mobilization of stem cells and progenitors from the bone marrow to the spleen. Further studies are required in order to define the exact pathophysiologic mechanisms involved.

Figure 1.

Course of infection and changes in hematologic parameters during the course of *Plasmodium chabaudi* AS infections in B6 (closed symbol) and A (open symbol) mice. a) course of parasitemia, b) percentage mortality, c) changes in hematocrit, and d) reticulocytosis. Results are expressed as mean ± SEM of 4 mice per time point.


a
% Parasitemia ± sem
0
10
20
30
40
50
0 3 6 9 12 15 18 21 24 27 30
days post infection


c
% Hematocrit ± sem
0
10
20
30
40
50
60
0 3 6 9 12 15 18 21 24 27 30
days post infection


b
% Survival
0
20
40
60
80
100
0 3 6 9 12 15 18 21 24 27 30
days post infection

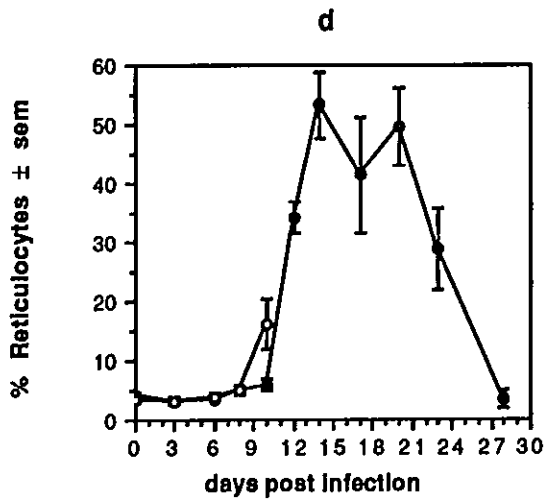
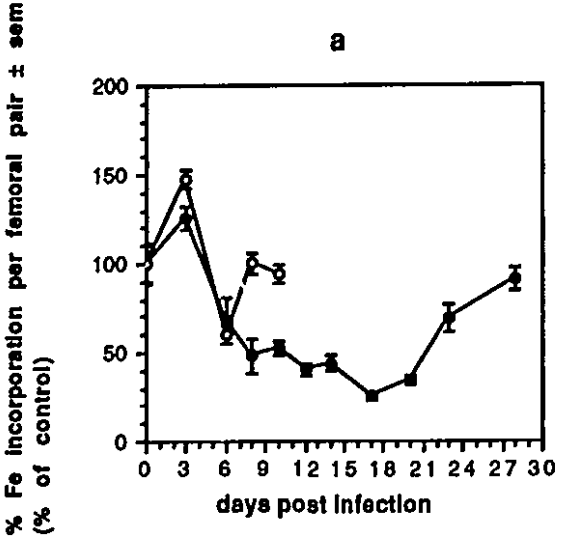
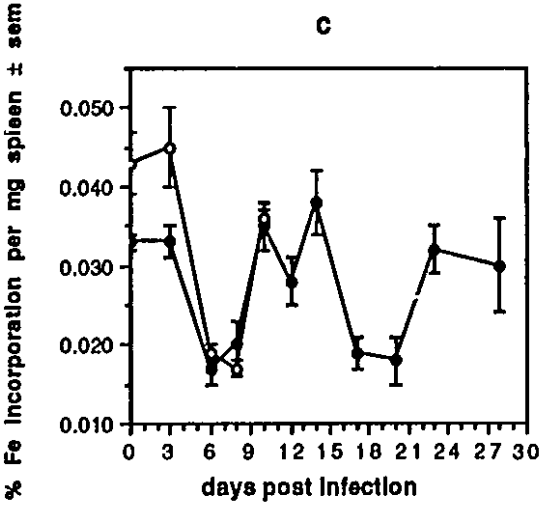
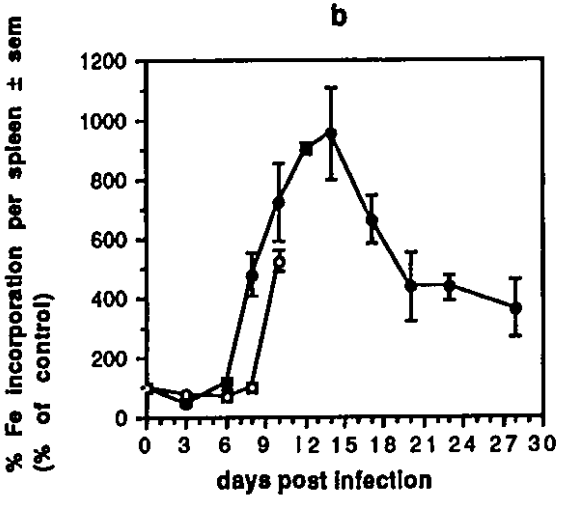

d
% Reticulocytes ± sem
0
10
20
30
40
50
60
0 3 6 9 12 15 18 21 24 27 30
days post infection

Figure 2.

Changes in ^{59}Fe incorporation in femora (a), spleens (b) and blood (d) and in ^{59}Fe incorporation per mg spleen (c) during the course of *Plasmodium chabaudi* AS infections in B6 (closed symbol) and A (open symbol) mice. ^{59}Fe incorporation is expressed as percentage incorporation of total radioactivity injected into experimental mice divided by percentage incorporation in normal mice X 100%. Results are expressed as mean ± SEM of 4 mice per time point.


a
% Fe incorporation per femoral pair ± sem
(% of control)
200
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30
days post infection


c
% Fe incorporation per mg spleen ± sem
0.050
0.040
0.030
0.020
0.010
0 3 6 9 12 15 18 21 24 27 30
days post infection


b
% Fe incorporation per spleen ± sem
(% of control)
1200
1000
800
600
400
200
0
0 3 6 9 12 15 18 21 24 27 30
days post infection

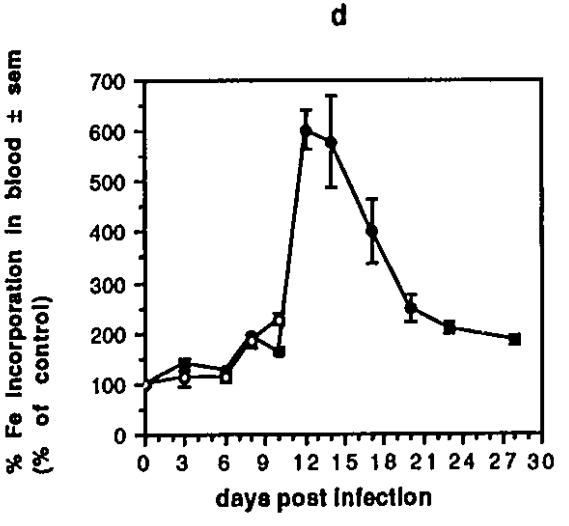
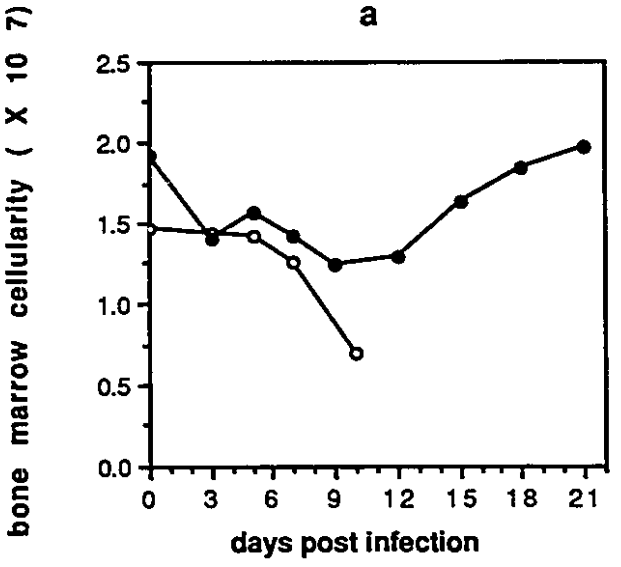

d
% Fe incorporation in blood ± sem
(% of control)
700
600
500
400
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30
days post infection

Figure 3.

Changes in bone marrow (a) and spleen (b) cellularity during the course of *Plasmodium chabaudi* AS infections in B6 (closed symbol) and A (open symbol) mice. Results are expressed as mean of 3-4 mice per time point. Standard errors (not shown) were within 10-20 % of the means.


a
bone marrow cellularity (X 10 7)
2.5
2.0
1.5
1.0
0.5
0.0
0 3 6 9 12 15 18 21
days post infection

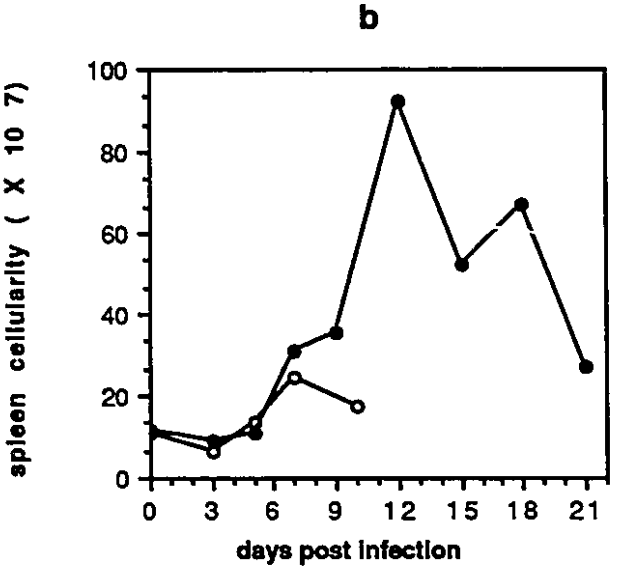
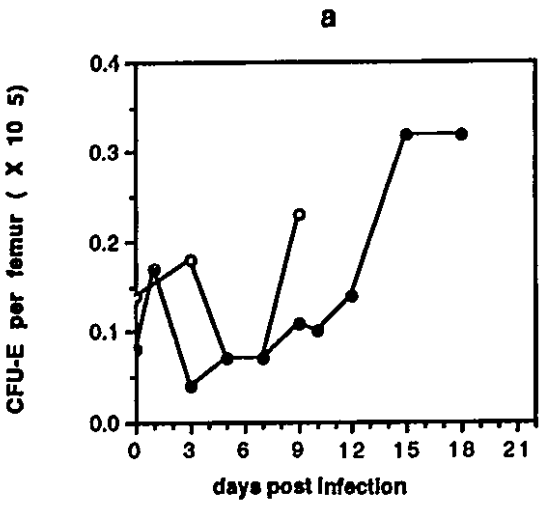
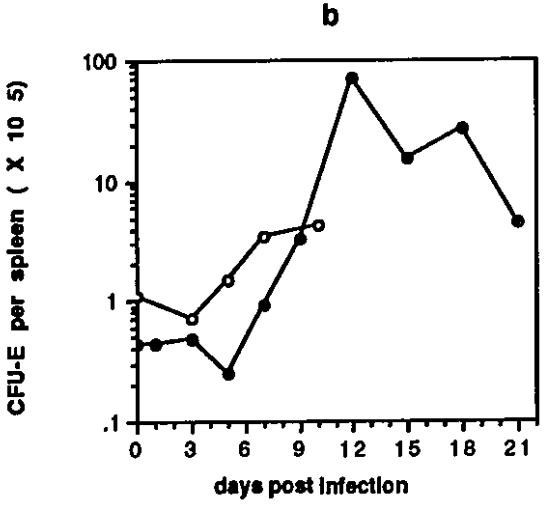
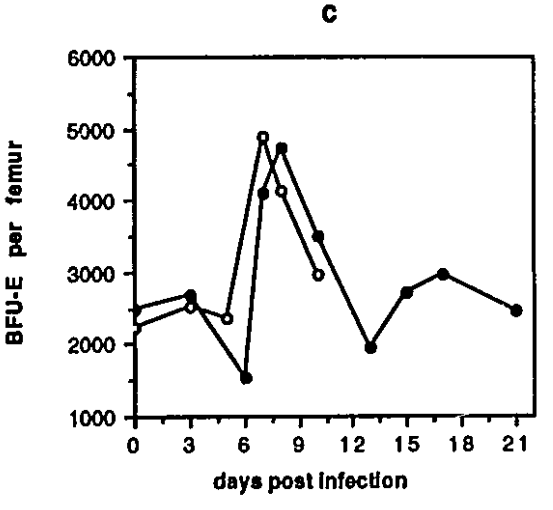

b
spleen cellularity (X 10 7)
100
80
60
40
20
0
0 3 6 9 12 15 18 21
days post infection

Figure 4.

Changes in the numbers of CFU-E (a,b) and BFU-E (c,d) in femora (a,c) and spleens (b,d) of B6 (closed symbol) and A (open symbol) mice during the course of *Plasmodium chabaudi* AS infections. Results are expressed as means of 3-4 mice per time point. Standard errors (not shown) were within 10-20% of the means.


a
CFU-E per femur (X 10 5)
0.4
0.3
0.2
0.1
0.0
0 3 6 9 12 15 18 21
days post infection


b
CFU-E per spleen (X 10 5)
100
10
1
.1
0 3 6 9 12 15 18 21
days post infection


c
BFU-E per femur
6000
5000
4000
3000
2000
1000
0 3 6 9 12 15 18 21
days post infection

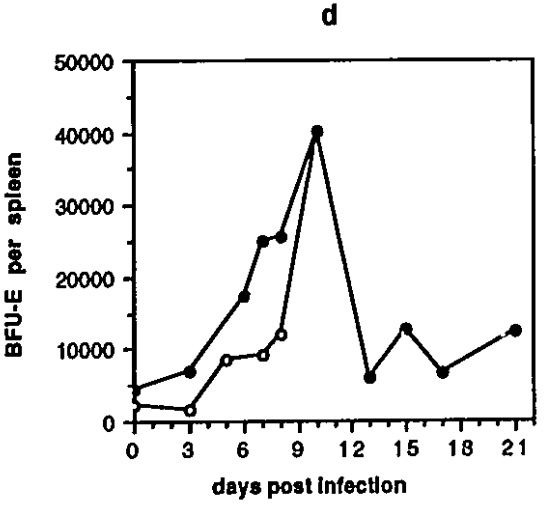

d
BFU-E per spleen
50000
40000
30000
20000
10000
0
0 3 6 9 12 15 18 21
days post infection

TABLE 1

Changes in BFU-E frequencies in PBMC during *Plasmodium chabaudi* AS infection in C57BL/6 and A/J mice.[a]

Day	C57BL/6	A/J
0	7,6,8,6,3,12,6,18,4	11,18,18,10,
3	8,3	9,21
6	4,8	12,30
7	10,10	90,50
8	52,20	102,104
10	70,66	
14	78,80	
17	50,48	
21	8.14	

[a]PBMC pooled from 2-3 mice were obtained and cultured for BFU-E as described in Materials and Methods. Results are expressed as frequency of BFU-E per five million PBMC plated.

TABLE 2

Plasma Erythropoietin (Epo) Titers during *Plasmodium chabaudi* AS infection in C57BL/6 and A/J Mice.[a]

	C57BL/6		A/J	
Day	% Hct	Epo[b]	%Hct	Epo[b]
0	48.1 ± 0.5	43 ± 15	48.4 ± 0.4	49.± 14
3	48.2 ± 0.1	32 ± 0	48.2 ± 0.3	41 ± 15
6	46.9 ± 0.7	16 ± 4	45.9 ± 0	30 ± 6
8	32.6 ± 1.4	610 ± 146[a]	32.4 ± 0.6	993 ± 245[a]
10	21.0 ± 0.6	2510 ± 74[a]	14.5 ± 1.5	6264 ± 933[a]
11	19.0 ± 1.6	2890 ± 580	10.1 ± 0.1	8095 ± 2318

[a]Plasma samples were obtained from groups of 3-4 uninfected and infected mice and individually assayed for Epo titers as described in Materials and Methods.

[b]Results are expressed in mU/ml ± sem.

[c]$P < 0.05$.

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CHAPTER 3*

In principle, a number of regulatory mechanisms can explain the differential expansion of erythropoiesis in the bone marrow and spleen of resistant and susceptible mice. Using an in vitro erythroid cell proliferation assay, the papers presented in this chapter describe the detection and characterization of an inhibitory activity produced by bone marrow and spleen cells from *P. chabaudi* AS infected mice.

* This chapter consists of an extended abstract and a full length manuscript.

I. PRODUCTION OF SOLUBLE INHIBITOR OF ERYTHROPOIESIS DURING *Plasmodium chabaudi* AS INFECTION IN RESISTANT AND SUSCEPTIBLE MICE

Anemia is a major cause of morbidity and mortality in malaria. Parasite mediated hemolysis, bone marrow dyserythropoiesis and increased splenic clearance of non-infected red cells may contribute to the development of anemia (1-2). The cellular mechanism(s) for a depressed erythropoietic response is not well understood. Recently, Miller and coworkers (3) reported that a soluble factor released from bone marrow and spleen cells of mice infected with *P. berghei* and *P. vinckei* was able to depress erythropoietin induced proliferation of enriched splenic erythroid precursors.

We investigated the production of this soluble inhibitor of erythropoiesis during infection with *P. chabaudi* AS using a previously described ^{3}H-thymidine assay for the determination of erythropoietin (4). In order to determine its importance in the development of anemia and host survival, we used a genetic model of resistance/susceptibility to *P. chabaudi.* AS (5). In this system, resistant C57BL/6 strain of mice survive the infection while susceptible A/J mice succumb to infection within 10-14 days. Death is associated with a profound anemia and high parasitemia.

Table 1 shows that conditioned media from spleen and bone marrow cells of both C57BL/6 and A/J mice infected with *P. chabaudi* AS inhibited the proliferative response of C57BL/6 splenic erythroid cells to varying degrees during the period of increased parasitemia. Maximal inhibition was consistently observed during the peak of parasitemia, suggesting that the increased parasite load is the stimulus for the production of the inhibitory factor. However, at peak parasitemia, spleen conditioned media from A/J mice exhibited an apparently higher inhibition than C57BL/6 (not statistically significant, P=0.388), most likely due to a higher parasitemia.

Using splenic erythroid cells from both strains of mice, we have found equivalent levels of sensitivity to the factor.

We have further characterized some properties of this inhibitory factor. Dose response studies indicate that the inhibitory factor is present in low concentrations in conditioned media. Inhibitory activity can be enriched by membrane filtration (molecular weight cut off = 10,000 daltons), ammonium sulfate precipitation (50-70% saturation), and elutes in the void volume of a Sephadex G-25 column. These observations indicate a macromolecular nature of this factor. Neutralization experiments with antibodies to tumor necrosis factor and interferon-gamma have failed to reverse inhibition. Heat treatment at 95 ^{o}C partially inactivates inhibitory activity. While it inhibits erythropoietin-induced cell proliferation and colony formation of the erythroid lineage, macrophage cell proliferation and colony formation in response to CSF-1 (LCM) are not affected (Table 2).

We conclude that cells in the spleen and bone marrow of mice infected with *P. chabaudi* AS secrete soluble factor(s) which inhibits erythropoietin-induced proliferation of splenic erythroid precursors in vitro. The molecular nature of this factor is presently unknown. The in-vivo contribution of this factor to the development of anemia during malaria remains to be determined. Our results indicate that neither the sensitivity to nor the production of this factor is a determinant in the expression of genetically determined resistance to murine malaria infection.

Table1. Course of parasitemia and production of soluble inhibitor in bone marrow (BMCM) and spleen cell conditioned media (SPCM) in resistant C57BL/6 and susceptible A/J mice[a]

	C57BL/6 (B)			A/J(A)[b]		
day	parasitemia	BMCM[c]	SPCM[c]	parasitemia	BMCM[c]	SPCM[c]
1	0	104.3	100.1	0	102.3	82.9
3	0	119.7	94.5	2.5	110.8	109.6
5	0.6	113.3	77.5	8.8	133.1	56.8
7	8.1	94.2	100.6	23.5	133.5	58.0
9(B)/10(A)	28.7	65.0	62.2	47.0	68.1	53.6
11	10.7	70.0	98.6			
14	2.0	81.8	95.7			

[a] Data obtained from groups of 2-4 mice sacrificed at indicated days during infection.

[b] 100 % mortality was observed at day 10.

[c] Single cell suspensions of bone marrow and spleens were cultured at 5 X 10^6/ml for 2 days. Supernatants were tested at 20 % v/v using 25 mU/ml erythropoietin. Values are expressed as percentage fraction of proliferative response with test supernatant/ proliferative response with supernatant prepared from uninfected mice.

Table 2. Lineage specificity of inhibitory activity of spleen cell conditioned media.

	Epo + medium	Epo+ SPCM[a]
CFU-E/ 2X10^5 cells [bc]	550 ± 45	220 ± 32
^{3}H-thymidine incorporation[bd] 24-hr assay	36,888 ± 2814	7101 ± 1420
	LCM + medium	LCM+SPCM[a]
CFU-C/ 5 X 10^5 cells	141 ± 5	130 ± 5
^{3}H-Thymidine Incorporation 96-hr assay	2011 ± 462	3862 ± 201

[a]Spleen cell conditioned medium from C57BL/6 mice infected with 1 x 10 7 parasitized red blood cells (PRBC) were prepared at peak of parasitemia. SPCM was used at 10% and 20% v/v in colony and proliferation assays respectively.

bSpleen cells were obtained from C57BL/6 mice injected with phenylhydrazine on two consecutive days and sacrificed 3 days later.

[c]P=.035
[d]P=.004

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II. INHIBITION OF IN VITRO ERYTHROPOIESIS BY SOLUBLE MEDIATORS IN *Plasmodium chabaudi* AS MALARIA: LACK OF A MAJOR ROLE FOR IL-1, TNF-α and IFN-γ

ABSTRACT

Using erythropoietin (Epo)-dependent proliferation of splenic erythroid cells as an in-vitro erythropoiesis model system, we demonstrate that spleen cells from *Plasmodium chabaudi* AS-infected C57BL/6 mice potently inhibited erythroid cell proliferation. Inhibitory activity was detected in spleen cell conditioned media (SPCM) prepared from infected but not from uninfected mice. The inhibitory activity in SPCM was characterized as being heat-sensitive, macromolecular and host-derived. The inhibitory activity was not reversed by increasing Epo concentration and was found to be specific for the late erythroid lineage. Mouse strains, which differ in their resistance to *Plasmodium chabaudi* AS infection, produced and responded to the inhibitory activity to a similar extent. Putative immune mediators, IL-1α, IL-β and IFN-γ, were found to be potent inhibitors of erythroid cell proliferation. However, antibody neutralization experiments failed to demonstrate a major role for these cytokines in the inhibitory activity of SPCM. Our results suggest that the elaboration of inhibitor(s) of erythropoiesis in hemopoietic organs of *Plasmodium* infected mice may impair erythroid regeneration. The identity of the inhibitory mediator(s) is presently unknown, but is distinct from IL-1, TNF-α and IFN-γ.

INTRODUCTION

Anemia together with cerebral malaria account for the highest proportion of deaths due to severe malaria in Africa (5). The pathophysiologic mechanisms of the anemia of malaria are complex and multifactorial (13,15). Since *Plasmodium* parasites reside within host erythrocytes, schizogony and the subsequest release of merozoites inevitably lead to intravascular hemolysis. In addition, it is thought that erythrophagocytosis of non-infected red cells and a depressed erythropoietic response contribute to the development of anemia (8,14,15). Morphologic studies of bone marrow smears obtained from patients as well as mice with malaria have provided cytologic evidence for dyserythropoiesis (2,4,14). Detailed analysis of erythroblast cell kinetics using quantitative ^{14}C autoradiography in bone marrow samples of patients with acute *P. falciparum* malaria revealed a reduction in proliferative rates in all erythroblast stages and a significant intramedullary deletion in the polychromatophilic erythroblast compartment (4).

The mechanisms which account for these changes are not well understood. It has been postulated that host-derived factors, such as tumor necrosis factor, produced in response to parasites may cause the depression in erythropoiesis (2). Recently, Miller and colleagues (11) reported that a soluble factor released from cultured bone marrow and spleen cells of mice infected with *P. berghei* or *P. vinckei* was able to depress the in vitro erythropoietin-induced proliferation of enriched splenic erythroid precursors. The identity of this soluble mediator and its possible contribution to the development of anemia in vivo is unknown.

In a preliminary report, we demonstrated that a soluble inhibitory activity is also produced by bone marrow and spleen cells derived from *P. chabaudi* AS-infected mice (22). In the present investigation, we have characterized the biological and physicochemical properties of the inhibitory activity present in conditioned media of spleen cells from infected C57BL/6 mice. Furthermore, we demonstrate that inhibition of erythropoietin-induced proliferation of splenic precursors by conditioned media from *P. chabaudi* AS infected mice is not mediated by either IL-1, TNF-α or IFN-γ, cytokines which have previously been suggested to play major roles in the suppression of erythropoiesis during malaria (2, 11).

MATERIALS AND METHODS

Animals and Experimental Infection: C57BL/6, BALB/C, AKR, and DBA/2 mice were purchased from Charles River (St. Constant, Quebec) and A/J and C3H/HeJ mice from the Jackson Laboratory (Bar Harbor, ME). Six to eight week old, age and sex-matched mice were routinely used for infection and for phenylhydrazine injection. Experimental mice were infected intraperitoneally with 10^6 *Plasmodium chabaudi* AS parasitized red cells (PRBC) as previously described (23).

Preparation of Spleen Cell Conditioned Media (SPCM): Spleens were obtained aseptically from normal and infected mice at peak parasitemia (typically day 7 post infection). Cell suspensions were prepared by passing minced spleens through a sterile wire mesh and were washed three times with complete medium consisting of Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (Hyclone, UT), 0.12% gentamycin (Schering Canada, Montreal, Quebec), 2 mM glutamine and 5×10^{-5} M 2-mercaptoethanol.

Total viable cell counts were determined by hemacytometer after appropriate dilution with trypan blue. SPCM were prepared by culturing the spleen cells at a density of 5×10^6 cells per ml for 2 days in a well humidified CO_2 incubator. Supernatants were harvested and stored at -20°C until use.

Inhibition Assay for Erythropoietin-Induced Proliferation: This assay was performed essentially according to Krystal (7). Spleen cells were obtained from mice made anemic by two consecutive daily phenylhydrazine injections (60 mg/kg/day). Unless otherwise indicated, spleen cells were prepared from phenylhydrazine-treated C57BL/6 mice. Aliquots of 100 ml of spleen cells at 5×10^6 cells per ml in complete IMDM medium with 20% FCS and 100 mU/ml recombinant human erythropoietin (Epo; Genetics Institute, Cambridge, MA) were distributed into 96 well plates. SPCM samples were added at 20% v/v and each sample was assayed in triplicate. Cultures were incubated for 24 hours. Two hours before harvesting, 20 ml of IMDM containing 1 μCi of ^3H-thymidine (ICN, Montreal, Quebec) were added to each well. DNA synthesis was measured by liquid scintillation counting of cells harvested on glass fiber discs using a Skatron automatic cell harvester (Skatron, Inc., Sterling, VA). Inhibition of cell proliferation was assessed by dividing the mean counts of cultures with SPCM by the mean counts of cultures with medium alone. In some cases, LPS (*E. coli* 055:B5, Difco, Detroit, MI), polymyxin B (25 mg/ml), washed normal red cells (NRBC) or parasitized red cells (PRBC) or soluble extracts of NRBC or PRBC were added directly to the splenic erythroid cultures. Soluble extracts were prepared by incubating NRBC or PRBC at 10^8 cells/ml in HBSS at 37°C in roller tubes for 24 hrs (1). Supernatants were cleared by boiling for 5 min,

centrifuged again and passed through a 0.2 μm filter (Gelman, Ann Arbor, MI) and stored at 4°C.

Cytokines and Antibodies: Recombinant human IL-1α, IL-1β and murine IL-4 were kindly provided by S. Gillis (Immunex Corp, Seattle, WA). rhIL-6(2×10^8 U/mg), rmTNF-α (4×10^7 U/mg) and rmIFN-γ (4.5×10^6 U/mg) were purchased from Boehringer Mannheim (Montreal) and Genzyme (Cambridge, MA), respectively. Cytokine preparations were diluted in IMDM and added to cultures as detailed above. DB-1, a murine anti-rat IFN-γ MAb was generously given by P. van der Meide (TNO Primate Centre, The Netherlands). Polyclonal sheep anti-mouse IL-1α and goat anti-mouse IL-1β antisera were kindly provided by H. Ziltener (The Biomedical Research Centre, Vancouver, B.C.). Antibodies were used at concentrations which completely inhibited 10 U/ml IFN-γ and 10 pg/ml IL-1. Higher concentrations of antibodies gave similar results.

Hemopoietic Colony Assay: Bone marrow cells obtained from femora of C57BL/6 mice were plated at a density of 5×10^5 cells per ml in a semisolid medium as previously described (12). This medium consists of 0.8% methylcellulose, 30% FCS, 10% WEHI-3 CM (as source of IL-3), 2 U/ml Epo, 0.1 mM hemin (Eastman Kodak, Rochester, NY), 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol in IMDM. Test supernatants or medium was added at 20% (v/v). Colonies were scored in situ after 6 days. The CFU-E assay was done as previously described (22 23).

Statistics: Differences between groups of cultures assayed at the same time were analyzed by Student's t-test. A probability of less than 0.05 is reported as significant. Data shown are from representative experiments which have been repeated at least twice.

RESULTS

In order to evaluate the inhibitory activity of spleen cells from *P. chabaudi* AS-infected mice on erythropoiesis in vitro, we utilized a previously described proliferation assay using splenic erythroid cells derived from mice rendered anemic by phenylhydrazine injection as responder cells (PHZ-SP) and recombinant human Epo as mitogen (7, 11). This cell population has recently been shown to contain greater than 90% erythroid precursor cells as judged by morphology and high level expression of transferrin and Epo receptors (21).

The effect of spleen cells from uninfected and infected C57BL/6 mice at peak parasitemia on the proliferation of splenic erythroid precursor cells was determined using this in vitro assay. Figure 1a illustrates that spleen cells from *P. chabaudi* AS infected mice added at different concentrations (to give a range of spleen cell: responder cell ratios of 1:10 to 2:1) almost completely abrogated the proliferative responses of PHZ-spleen cells to Epo ($P<0.0001$). In contrast, normal spleen cells were not inhibitory at a 1:5 ratio but at higher concentrations (1:1 to 2:1) modestly but significantly inhibited proliferative responses ($P<0.001$). However, at cell ratios higher than 1:50 spleen cells from infected mice significantly inhibited PHZ-spleen proliferation in comparison to spleen cells from normal mice ($P\leq0.001$). We next determined whether soluble mediators released by spleen cells from *P. chabaudi* AS infected mice are responsible for the inhibition. CM prepared from *P. chabaudi* AS infected spleen cells were found to significantly inhibit the proliferative response of PHZ-spleen cells ($P<0.001$) while CM from normal, uninfected spleen cells failed to significantly inhibit the response relative to medium control (Fig 1b). It is notable that addition of SPCM from infected mice inhibited proliferative

responses by approximately 10-fold less than the addition of spleen cells from infected mice.

Characterization of the physicochemical properties of the inhibitory activity in SPCM from infected C57BL/6 mice showed that following treatment at 56°C for 30 minutes, there was no significant loss of inhibition. Inhibitory activity was completely lost after boiling for 1 hr. Ultrafiltration of SPCM using a 10 kD molecular mass cut-off membrane resulted in the enrichment of inhibitory activity in the concentrate of SPCM prepared from infected but not from control mice (data not shown). Passage of the concentrate on a Sephadex G-25 column and subsequent assay of the fractions showed that most of the inhibitory activity was in the void volume (data not shown). These results, thus, demonstrate that the inhibitory activity is heat-sensitive and macromolecular. Addition of polymyxin B (25 μg/ml) had no effect on the inhibitory activity (25% and 32% of control response with and without polymyxin B, respectively). This observation together with the heat sensitivity of the activity demonstrate that inhibition is not caused by LPS contamination. Direct addition of parasitized red cells or soluble parasite extracts did not suppress erythroid cell proliferation compared with normal red cells or extracts. Therefore, the inhibitory activity is host and not parasite-derived.

In order to estimate the relative concentration of the inhibitory activity, SPCM from infected C57BL/6 mice were serially diluted in complete medium and assayed for inhibitory activity. There was a dose dependent inhibition with only concentrations of 20% and 10% (v/v) resulting in significant inhibition (Figure 2a) ($P=0.0004$ and 0.0007, respectively). The effect of SPCM added at a constant concentration (20% v/v) with increasing concentrations of Epo on the proliferative response of

splenic erythroid cells was also investigated. Figure 2b illustrates that proliferative responses of erythroid progenitors were significantly inhibited even at high Epo concentrations. Proliferative responses in cultures containing SPCM were 20-30% of control responses at all Epo concentrations tested. These results suggest that inhibitory activity in SPCM from infected C57BL/6 mice is present in low concentrations and that high concentrations of Epo fail to reverse the inhibition.

Our previous results demonstrated that the inhibitory activity in SPCM was erythroid-specific, that is, it inhibited CFU-E formation and erythroblast proliferation but not CFU-C and monocyte-macrophage proliferation (22). We extended this observation by examining the effects of SPCM on interleukin-3 and Epo driven colony formation of bone marrow cells of the myelo-erythroid lineage. Table 1 shows that in comparison to control cultures incubated with medium alone or cultures incubated with SPCM from uninfected mice, SPCM from infected mice did not inhibit BFU-E, CFU-GM or multipotential CFU-GEMM colony formation by bone marrow precursor cells. However, addition of SPCM from infected mice significantly inhibited CFU-E colony formation ($P<0.05$). These observations suggest that the inhibitory activity is effective primarily in the late erythroid compartment.

Our previous studies have shown an impairment of splenic erythropoiesis in genetically susceptible A/J mice (23). We reasoned that an increased sensitivity to and/or production of the inhibitory activity may explain this observation. We tested a standard SPCM preparation from infected C57BL/6 mice on splenic erythroid cells from different inbred mouse strains. Inbred mouse strains which have been previously typed as resistant (DBA/2 and C57BL/6) or susceptible (BALB/C and A/J) to *P.*

chabaudi AS were used (18). The responsiveness to the inhibitory activity did not differ among the strains studied (49, 60, 61 and 60% of control, respectively). We also prepared SPCM from these 4 inbred strains at peak parasitemia following infection with 10^6 *Plasmodium chabaudi* AS PRBC and tested for inhibitory activity on splenic erythroid precursor cells from C57BL/6 mice. There was no striking difference in production of inhibitory activity among the strains examined (26, 31, 17 and 23 % of control, respectively).

In order to identify candidate cytokines which may mediate the inhibitory activity in SPCM, several recombinant cytokines were added directly into the cultures. As shown in Figure 3, IFN-γ, IL-1α, IL-1 β and IL-4 inhibited proliferative responses maximally at concentrations of 10 U/ml , 10 pg/ml, 10^3 pg/ml and 10^4 pg/ml, respectively. TNF-α, IL-6 and TGF-β (not shown) were not found to be inhibitory over a wide range of concentrations tested. Next, antibody neutralization experiments were carried out in order to ascertain the contribution of IL-1 and IFN-γ to the inhibitory activity of SPCM from infected mice (Table 2). DB-1, a mouse anti-rat IFN-γ, slightly increased the proliferative response in 3 of 3 SPCM preparations ($P<0.05$). These SPCM preparations were found to contain low but detectable levels of IFN-γ (2-5 U/ml). Nonetheless, these cultures were significantly inhibited compared with cultures containing control SPCM and DB-1 ($P<0.001$). Polyclonal antisera to mouse IL-lα and IL-1β had no significant effect on inhibitory activity. Addition of anti-IL-1 and DB-1 together did not increase thymidine labelling above that in DB-1 containing cultures. It, thus, appears that neither IL-1 nor IFN-γ, alone or in combination, could account for a majority of the inhibitory activity of SPCM.

DISCUSSION

In the present report, we demonstrate that spleen cells recovered from mice infected with *P. chabaudi* AS at peak parasitemia completely suppressed the Epo-induced proliferative response of enriched erythroid precursors. This suppression appears to be due to the presence of an inhibitory activity in SPCM prepared from *P. chabaudi* AS-infected mice. Our results confirm and extend earlier work from Miller and colleagues (11) which demonstrated the presence of soluble inhibitor(s) of erythropoiesis in conditioned media from spleen cells of mice infected with the more virulent *P. berghei* and *P. vinckei*. Their results and ours suggest that, at least in mice, the production of this soluble inhibitory activity may be a universal feature of malaria infection. However, the relative potency of spleen cells over SPCM in inhibiting proliferative responses argues for additional mechanisms which may require cell contact or operate within short range.

We performed a series of experiments in order to characterize the inhibitory activity in SPCM from *P. chabaudi* AS-infected mice. The possibility that LPS contamination contributed to the inhibition was excluded by demonstrating the heat sensitivity of SPCM and the lack of effect of polymyxin B. Another concern which has been addressed in our study is the possibility of non-specific cytotoxicity causing the decreased thymidine incorporation in SPCM containing cultures; this concern has also been excluded. Examination of the inhibitory activity of SPCM on bone marrow precursor cells of various lineages indicates that it is specific to cells of late erythroid lineage. As we have previously shown, SPCM inhibited proliferation of spleen cells from phenylhydrazine-treated mice

and CFU-E colony formation but did not inhibit M-CSF driven bone marrow cell proliferation and CFU-C colony formation (22). As demonstrated here, SPCM also failed to inhibit the early erythroid progenitor, BFU-E, and the multipotential progenitor, CFU-GEMM (Table 1). This apparent lineage specificity of inhibition by SPCM from *P. chabaudi* AS infected mice, thus, excludes the possibility of a non-specific cytotoxic or cytostatic effect. Furthermore, direct observation of splenic erythroid cells incubated with SPCM over a 24-hour period failed to demonstrate significant loss of viability as judged by trypan blue exclusion (data not shown).

The identity of the factor(s) which mediates the inhibitory activity of SPCM is not yet known. It has been postulated that a Mac 1^+, adherent cell, presumably a monocyte-macrophage, secretes cytokines, such as IL-1 and TNF, which inhibit erythropoiesis (11). Both IL-1α and IL-1β have been shown to inhibit Epo-induced proliferation of anemic mouse spleen cells (17, Fig.3). Alternatively, IFN-γ production by activated T cells and/or NK cells may be responsible for the inhibitory activity. Indeed, IFN-γ has been implicated as a major mediator of erythroid suppression in patients with aplastic anemia and rheumatoid arthritis (9, 24). We have previously demonstrated that spleen cells from *P. chabaudi* AS infected C57BL/6 mice secrete high levels of IFN-γ in response to antigenic or Con A stimulation (19). However, neutralizing antibodies to IL-1 and IFN-γ are ineffective in reversing inhibition by SPCM. Furthermore, antibody and complement depletion of Thy-1^+ cells from spleen cell suspensions, which almost totally eliminated IFN-γ in SPCM, does not significantly affect inhibitory activity (unpublished observations). These results indicate that IL-1 and IFN-γ do not play an essential role in the inhibitory activity of

SPCM. We, however, cannot discount the possibility that the small quantities of these cytokines may cooperate with other as yet unidentified cytokines to mediate the inhibitory activity. It is, therefore, necessary to consider other cytokines as candidate inhibitory factors. TNF-α, which inhibits erythropoiesis in vivo (6,20), has no inhibitory activity in the in vitro assay used in this study (17 and Figure 3). Combined treatment with IFN-γ and TNF-α did not significantly reduce proliferative responses compared with IFN-γ alone. Furthermore neutralizing antibodies to TNF-α added together with anti-IFN-γ (at concentrations which neutralized 10 U/ml of each cytokine added together) had no significant effect on SPCM activity (Unpublished results). Recently, it has been demonstrated that TNF-α exerts a negative effect on human CFU-E growth indirectly by inducing IFN-β production by stromal fibroblastic cells (10). In addition, IFN-β has been implicated in natural suppressor activity and is spontaneously produced by spleen and bone marrow cells from mice undergoing graft versus host disease, post-irradiation and neonatal immunosuppressive states (3). Indeed, in our hands, murine IFN-α/β was found to be inhibitory at a concentration range of 100-1000 U/ml. However, a polyclonal antiserum against IFN-α/β (at concentrations which neutralized 500 U/ml) was unable to reverse the effects of SPCM (Unpublished observation).

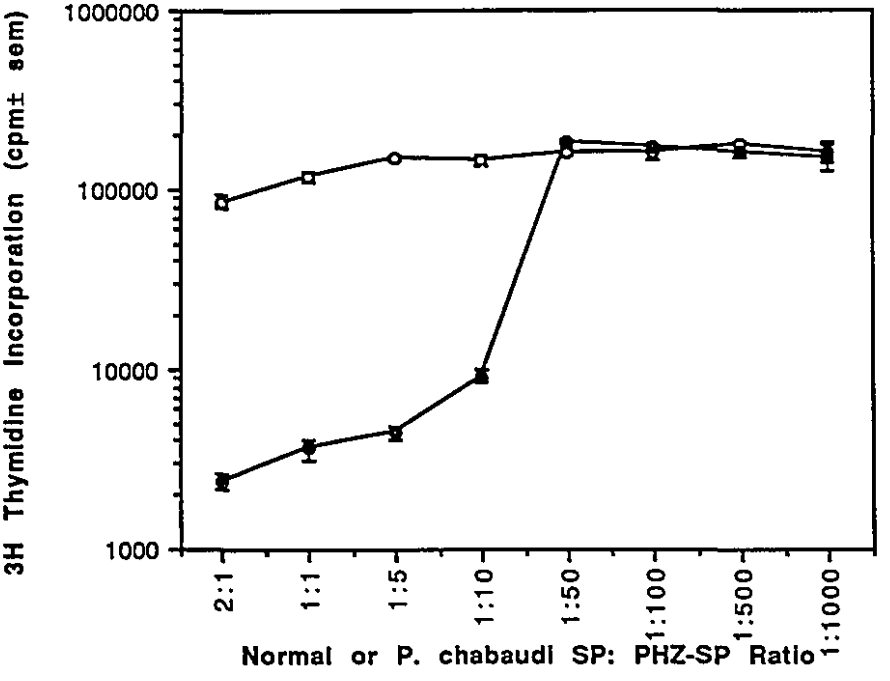
How relevant is the inhibitory activity in vivo? Histological examination of spleen tissue from malaria infected mice revealed macrophages engorged with malarial pigment closely apposed to developing erythroblasts in the red pulp (our unpublished observations). It is, therefore, likely that sufficiently high concentrations of the inhibitor can be achieved in the immediate microenvironment of the developing

erythroblasts. Previous studies have shown that spleen cells produce the inhibitor transiently at the time of peak parasitemia (22). Inhibitory cytokines produced during this critical period may delay or decrease the reticulocytic response which follows within 2-4 days. Comparison of the production and responsiveness to the inhibitory activity among resistant and susceptible inbred mouse strains, however, failed to demonstrate any significant strain differences. This observation implies that neither the production nor responsiveness to the inhibitory activity can be directly correlated with the survival or death of the infected mice. Our previous in vivo studies demonstrated a concurrent decrease in erythropoietic activity in the bone marrow and increase in the spleen at the time of peak parasitemia (23). However, maximal inhibitory activity occurred in both bone marrow and spleen cell conditioned media at the time of peak parasitemia (22). Thus, this apparent paradox implies that additional mechanisms (e.g., preferential migration and homing to the spleen and differences in hemopoietic stromal support) regulate the erythropoietic activity in the two organs. Identification of the cytokine and the cellular requirements for inhibition of erythropoiesis represent initial steps to further our understanding of the complex pathophysiology of anemia which occurs during malaria and other infections and inflammatory states.

Figure 1.

a) Inhibition of Epo-induced proliferation of splenic erythroid cells (5 x 10^5 per well) by spleen cells from normal mice (open symbol) and *Plasmodium chabaudi* AS-infected mice (closed symbol). Thymidine incorporation of PHZ-SP alone with Epo or without Epo (100 mU/ml): 169,227 ± 6876 and 1672 ± 231 CPM, respectively. b) Inhibition of Epo-induced proliferation of splenic erythroid cells by spleen cell conditioned medium (SPCM used at 20% v/v) from *Plasmodium chabaudi* AS-infected mice. sem, standard error of the mean.

a


3H Thymidine Incorporation (cpm± sem)
1000000
100000
10000
1000
2:1
1:1
1:5
1:10
1:50
1:100
1:500
1:1000
Normal or P. chabaudi SP: PHZ-SP Ratio

b

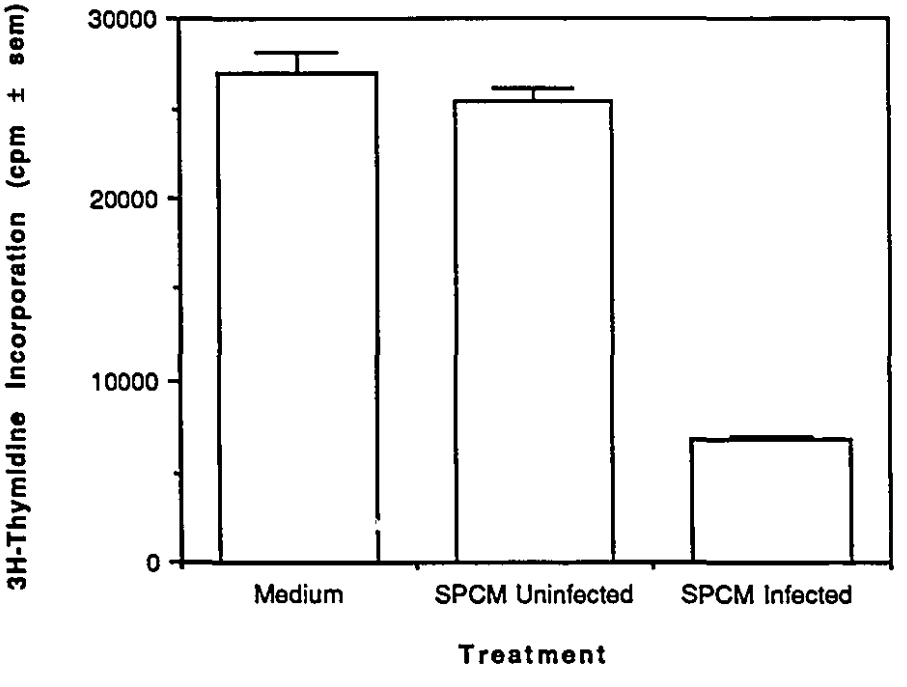

3H-Thymidine Incorporation (cpm ± sem)
30000
20000
10000
0
Medium
SPCM Uninfected
SPCM Infected
Treatment

Figure 2.

Dose response studies of SPCM inhibitory activity: a) Dose response of SPCM inhibition at 10 mU/ml Epo, b) Effect of increasing Epo concentration on inhibition with SPCM (20 % vol/vol).

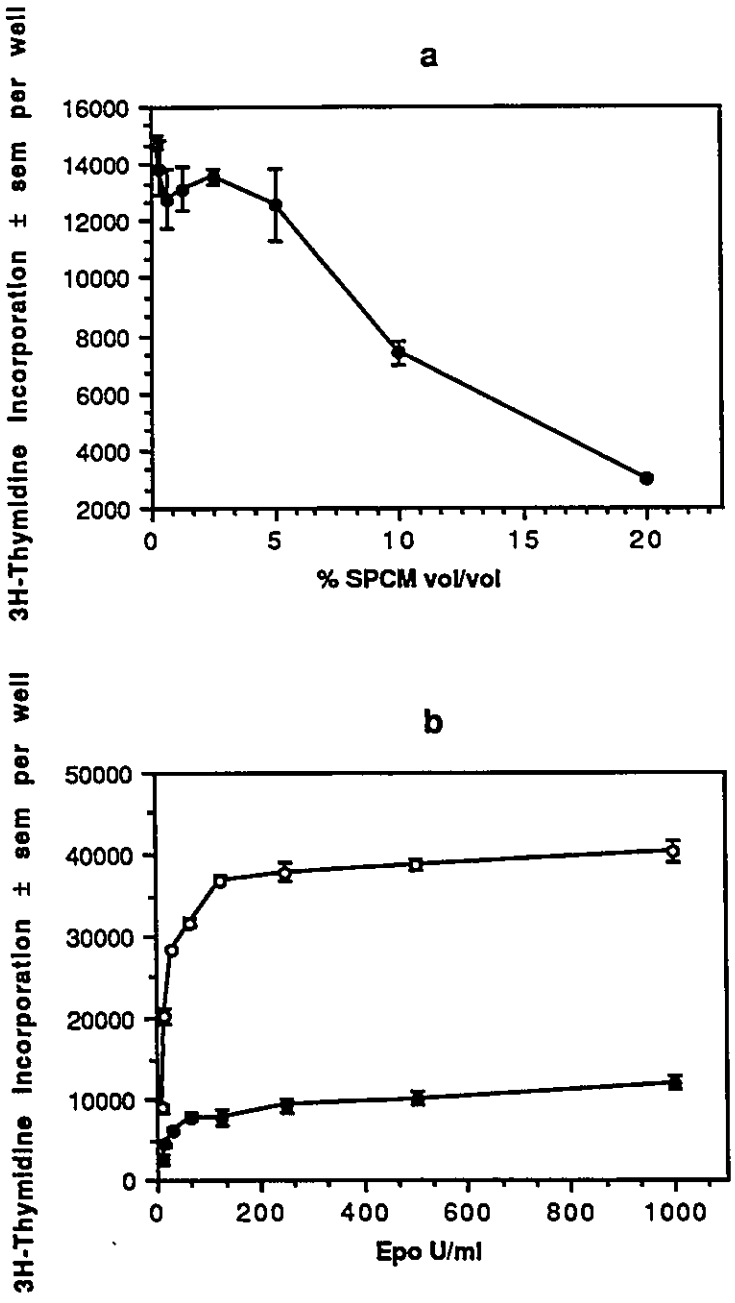

a
3H-Thymidine Incorporation ± sem per well
16000
14000
12000
10000
8000
6000
4000
2000
0
5
10
15
20
% SPCM vol/vol
b
3H-Thymidine Incorporation ± sem per well
50000
40000
30000
20000
10000
0
0
200
400
600
800
1000
Epo U/ml

Figure 3.

Inhibitory activity of recombinant cytokines on Epo-induced proliferation of PHZ-SP cells. Dose range used for IL-1α, IL-1β, IL-4 (10^{-2} to 10^{4} pg/ml) and for IFN-γ, TNF-α , IL-6 (10^{-3} to 10^{3} U/ml). Epo was added at 100 mU/ml.

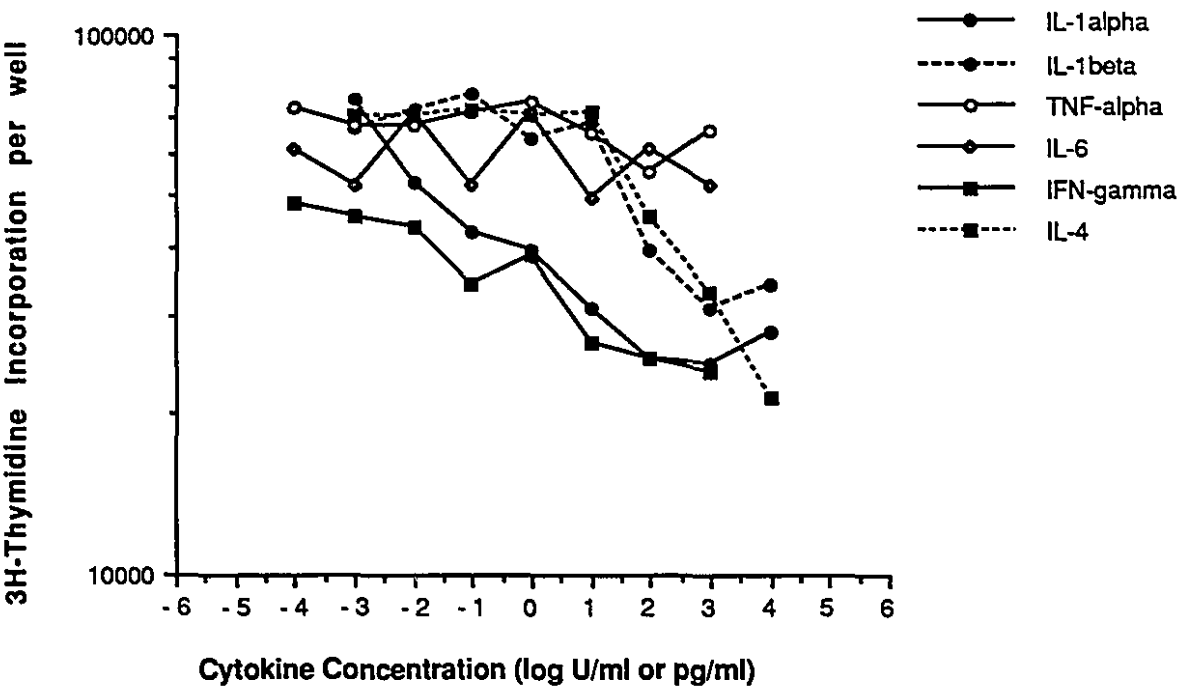

100000
10000
3H-Thymidine Incorporation per well
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
Cytokine Concentration (log U/ml or pg/ml)
IL-1alpha
IL-1beta
TNF-alpha
IL-6
IFN-gamma
IL-4

Table 1

Effect of SPCM from *P. chabaudi* AS Infected C57BL/6 Mice on IL-3 and Epo Driven In Vitro Colony Formation by Bone Marrow and Spleen Cells

	Mean Colonies ± SEM[b]			
	BFU-E	CFU-GM	CFU-GEMM	CFU-E
Medium	31 ± 3	292 ± 6	7 ± 1	805 ± 33
SPCM[a] Uninfected	29 ± 1	302 ± 7	8 ± 1	795 ± 39
Infected 1	30 ± 1	297 ± 5	12 ± 1	403 ± 72[c]
Infected 2	35 ± 3	309 ± 8	12 ± 2	407 ± 57[c]
Infected 3	33 ± 2	278 ± 18	12 ± 1	487 ± 95[c]

[a]SPCM was prepared from 1 uninfected and 3 individual *P chabaudi* AS infected C57BL/6 mice as described in Materials and Methods.Infected animals are designated numerically. SPCM from infected mice exhibited significant inhibitory activity on splenic erythroid precursor cells incubated in the presence of 100 mU/ml Epo.

[b]Colony counts per 2×10^5 bone marrow or spleen cells cells from C57BL/6 mice plated 1 ml per tissue culture dish in the presence of 20% v/v of SPCM from uninfected or infected C57BL/6 mice. Results are presented as mean colonies ± SEM for triplicate cultures.

[c]$P < 0.05$

Table 2
Effects of Neutralizing Antibodies to IFN-γ and IL-1 on the Inhibitory Activity of SPCM

	Antibody Treatment					
	medium	anti-IL-1α	anti-IL-1β	anti-IL-1α + anti-IFN-γ	anti-IL-1β + anti-IFN-γ	anti-IFN-γ
medium	48,816 ±3,368	48,371± 828	48,216 ± 555	47,510 ±1,096	47,191 ±1,199	46159 ±1328
SPCM						
uninfected[e]	38,809 ±2,147	38,880 ± 439	43,463 ±1,934	44,334 ±1,108[d]	37,186 ± 361[d]	39115 ±1469[b]
infected 1[e]	13,635 ±1,910[a]	11,675 ± 182	12,960 ±402	21,244 ±2,336[c,d]	17,999 ±1,079[c,d]	19673 ± 308[a,b,c]
infected 2[e]	16,950 ± 519[a]	16,307 ±1,824	18,891 ± 800	21,284 ±1,484[c,d]	18,856 ± 396[c,d]	20135 ±1230[a,b,c]
infected 3[e]	16,588 ± 673[a]	19,163 ±2,167	17,661 ±1,381	18,341 ±1,314[c,d]	17,449 ± 450[c,d]	19232 ± 457[a,b,c]

[a] $P < 0.05$ compared with medium control.

[b] $P < 0.001$ compared with control, uninfected cultures treated with anti-IFN-γ.

[c] $P > 0.1$ compared with anti-IFN-γ treated cultures within same row.

[d] $P < 0.001$ compared with uninfected SPCM within same column.

[e] IFN-γ titers by ELISA (19): uninfected (< 1U/ml), infected 1(5 U/ml), 2(2.2 U/ml), 3 (<1U/ml).

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CHAPTER 4

That severe anemia causes the mortality in susceptible mice infected with *P. chabaudi* AS is presumed but not proven. Therefore, the ability of blood transfusion to rescue susceptibie mice was examined. Blood transfusion rescued susceptible mice, but prolonged the patency of parasitemia in both resistant and susceptible mice. The implications of the latter observation for our understanding of the mechanisms which operate to induce parasitologic crisis is discussed.

BLOOD TRANSFUSION ALTERS THE COURSE AND OUTCOME OF *Plasmodium chabaudi* AS INFECTION IN MICE

ABSTRACT

The importance of severe anemia in the mortality of susceptible A/J strain mice during blood stage *Plasmodium chabaudi* AS infection was assessed. Blood transfusion during and 2-3 days after peak parasitemia rescued 90% of susceptible mice from severe anemia and death and allowed these mice to clear the infection and acquire immunity to reinfection. However, blood transfusion prolonged the patency of the infection for up to 5 days post peak parasitemia. Blood transfusion of resistant C57BL/6 strain mice produced an identical effect, that is, prolongation of the patency of parasitemia. In addition, blood transfusion increased the numbers of gametocytes. In both strains of mice, the rapid reduction in parasitemia, which occurs during crisis, was associated with the development of moderate levels of anemia. The possible mechanisms for the modulation of parasitemia by blood transfusion and the implications of the present observations for our understanding of the events which occur during crisis are discussed. It is proposed that parasitologic crisis is induced and/or maintained by physiological alterations associated with anemia.

INTRODUCTION

Blood stage *Plasmodium chabaudi* AS infection in mice follows a lethal or non-lethal course in a host strain-dependent fashion. Our laboratory has previously characterized the strain distribution pattern of resistance and susceptibility to *P. chabaudi* AS infection (17). Resistant C57BL/6 mice develop moderate levels of peak parasitemia, clear the infection to subpatent levels and are subsequently immune to challenge infection. On the other hand, susceptible A/J mice experience higher levels of parasitemia and succumb to infection within ten to fourteen days post infection. Susceptible mice suffer from severe anemia and shock prior to death. Severe anemia has been presumed to be the cause of death in susceptible mice, although this has not been formally proven (23). The experiments reported herein were initiated to assess the contribution of severe anemia to the mortality of susceptible A/J strain mice as a result of *Plasmodium chabaudi* AS infection. Our finding that blood transfusion rescues susceptible mice but prolongs the period of patent parasitemia and retards the occurrence of crisis forms the basis of our present communication.

MATERIALS AND METHODS

Parasite and Experimental Infections: C57BL/6 and A/J strain mice bred at the animal facility of the Montreal General Hospital were used at age 8-10 weeks old. Mice were infected intraperitoneally (IP) with 10^6 parasitized red cells (PRBC) prepared as previously described (23). Blood smears and RBC counts were obtained using standard procedures as previously described (23).

Blood Transfusion: Blood was obtained from noninfected donors (same strain and sex as recipients) by cardiac puncture using heparinized

syringes. Pooled blood was washed twice and resuspended in saline. Blood transfusions were done by IP injection of washed blood cells (0.7 - 0.9 x 10^9 RBC in 1 ml saline per mouse). Unless otherwise stated, transfusions were given beginning on the day of peak parasitemia and 2-3 days subsequently.

Histological and Functional Clearance Studies: For histological studies, mice were injected with 0.2 ml of a dialyzed suspension of India ink and sacrificed 15 minutes later. Spleens and livers were weighed, fixed in buffered formalin and processed for paraffin embedding. Four micron sections were prepared and stained with hematoxylin and eosin. For clearance studies, parasitized red cells from mice at peak parasitemia were harvested and labelled with ^{51} Cr as previously described (16). Mice were injected intravenously with 10^8 ^{51}Cr-labelled PRBC in 0.2 ml and bled at indicated time points from the retro-orbital plexus using a calibrated 25 *u*l micropipette. Spleens and livers were dissected out at the end of the observation period. Blood and tissue samples were counted in a Beckman gamma counter. Clearance curves were drawn using counts expressed as percent of the maximum counts obtained at either 2 or 5 minutes.

RESULTS AND DISCUSSION

In order to assess the importance of severe anemia in the lethality of *P. chabaudi* AS infections in susceptible mice, infected A/J mice were transfused with washed syngeneic blood cells at the time of peak parasitemia (day 7, in these experiments) and on 2 consecutive days thereafter. As shown in Figure 1, 10 of 10 (100 %) untransfused A/J mice succumbed to infection by day 10 whereas only 1 of 10 (10%) transfused mice died through day 21 when the experiment was terminated (chi-square test, P =0.0001). The kinetics of parasitemia and changes in red cell counts in control, untransfused and transfused A/J mice are shown in Figure 2. As previously shown by our laboratory (17), A/J mice developed high levels of peak parasitemia which occurred in this experiment on day 7. Parasitemia decreased between day 8 and day 9 when the nadir in red cell counts occurred ($1.52 \pm 0.2 \times 10^9$ RBC /ml). During this period of severe anemia, infected mice lost weight, became lethargic, hypothermic, and died. As expected, blood transfusion of infected A/J mice on days 7-9 prevented the development of severe anemia (Figure 2B). Unexpectedly, the parasitemia in transfused mice was maintained at patent levels (20 to 40 %) for a period of 5-6 days post peak parasitemia. Parasitemia decreased to subpatent levels on day 14, five days after the last blood transfusion. Interestingly, the decrease in parasitemia was coincident with the development of moderate levels of anemia (RBC count: $3.05 \pm 0.58 \times 10^9$ /ml). These mice developed symptoms similar to, albeit less severe than those of untransfused mice on days 8-9, but most of the animals survived and cleared the parasitemia to subpatent levels by day 15 post infection. A single mouse died during this period. Transfused A/J mice rechallenged

with 10^6 PRBC 30 days after primary infection did not develop patent parasitemia.

These observations, thus, indicate that anemia is a proximate cause of mortality in susceptible A/J mice and that susceptible mice are inherently capable of developing acquired immunity to *Plasmodium chabaudi* AS. The same conclusions were obtained in an earlier study with a different rodent *Plasmodium* species (4), whereby repeated blood transfusions rescued mice from an otherwise uniformly lethal *P. berghei* infection and allowed adequate time for the development of acquired immunity. However, unlike the present results, blood transfusion induced an early and steady decline in parasitemia in *P. berghei*-infected mice. Differences in red cell host preference between these two *Plasmodium* species may explain the divergence. Blood transfusion, consisting predominantly of normocytes, into *P. berghei*-infected mice may have inhibited endogenous erythropoiesis, thereby, decreasing the frequency of reticulocytes, which are the preferred host cell of this species. On the other hand, blood transfusion into *P. chabaudi*-infected mice could conceivably provide the parasites with more target cells, as this species has been regarded as being predominantly normocytophilic (10).

We sought to confirm the phenomenon of transfusion-induced prolongation of patent parasitemia in resistant C57BL/6 mice infected with *P. chabaudi* AS. Infection in this mouse strain follows a self-limiting, non-lethal course, characterized by a primary patent parasitemia, followed by a parasitologic crisis. One or more smaller recrudescences may occur before the infection is finally resolved by 4 weeks post infection. As shown in Figure 3, untransfused C57BL/6 mice experienced a progressive decrease in red cell counts reaching a nadir on days 9-10, at the time when

crisis was occurring. Daily blood transfusion of infected C57BL/6 mice starting at peak parasitemia (day 7) up to day 11 maintained the red cell counts at normal or slightly subnormal levels and the parasitemia at patent levels (between 10-20 %) for 6 days post peak parasitemia. As in the transfused A/J mice, resolution of patent parasitemia (day 14-15) was coincident with the development of moderate levels of anemia. Waves of reticulocytosis followed the episodes of anemia in both untransfused and transfused animals with peaks occurring on day 12 (57.1 ± 1.3% reticulocytes) and day 17 (42.2 ± 4.8% reticulocytes), respectively. Red cell counts had approached normal levels by the end of the experiment. It is noteworthy that the rebound in red cell counts was transiently delayed in untransfused mice as a result of a secondary parasitemia which peaked on day 14. Interestingly, no such secondary peak was observed in the transfused mice. Taken together, these observations demonstrate the modulation of parasitemia and timing of parasitologic crisis by infusion with syngeneic blood cells.

An obvious mechanism by which blood transfusion is able to modulate the course of infection is that red cell transfusion provides a pool of preferred target cells and prevents the release of reticulocytes which are thought not to be the preferred target cell of this parasite species (10). This simple explanation seems untenable for two reasons. First, blood transfusion using preparations enriched for reticulocytes produced the same effect as the normocyte preparations (not shown). Second, a recent report has indicated that the normocyte preference of *P. chabaudi* is more apparent than real (5).

In addition to its effects on course of infection, blood transfusion increased the numbers of sexual stage parasites (gametocytes) seen in the

blood smears. This was most obvious in the blood of mice transfused with reticulocyte rich preparations (Figure 4). This observation is consistent with the results of recent in vitro studies with *P. falciparum* which demonstrate that inclusion of either reticulocytes or hemolysates of infected red cells in the cultures increased the propensity of the parasites to differentiate into gametocytes (15,20). Both factors are likely to occur in the transfused mice and possibly favor sexual differentiation.

Experimental infection in animals with *Plasmodium* species is characterized by a prepatent phase of varying duration, a patent phase, when parasite growth rates are maximal and a period of crisis, defined as a period of spontaneous attrition of the parasite population. During crisis, developmental degeneration and cytostasis of intraerythrocytic parasites occur. The process of crisis has been largely attributed to cell-mediated immune mechanisms (8). The cellular mechanisms which destroy intraerythrocytic parasites are thought to involve macrophages, lymphocytes, granulocytes and factors derived from them (11). It is believed that activated T cells secrete lymphokines which activate effector cells to kill parasites by oxidative and non-oxidative mechanisms. These events are thought to occur in the microvascular space of the spleen, the liver and the bone marrow. Based on ultrastructural studies of splenic tissue from *P. yoelii*-infected mice (21) and blood clearance and trafficking studies of physically damaged red cells in *P. berghei* -infected rats (22), a model of how parasite destruction may occur in situ has been suggested (21). It has been proposed by Weiss and his colleagues (21) that patent infection is associated with the development of a splenic barrier system which effectively shunts blood circulation into the splenic sinuses. This results in a decreased capacity of the spleen to filter rheologically altered

red cells. During crisis, the splenic barrier system is "opened" with two interrelated consequences: entry of parasitized red cells into the cordal circulation, allowing contact with activated effector cells, and the release of newly formed reticulocytes, which replenishes the peripheral red cell pool. Whether or not this paradigm applies universally to all *Plasmodium* species has not been determined. We reasoned that if this same scenario occurred in *P. chabaudi*-infected mice, then blood transfusion would eliminate the physiologic trigger for the "opening" of the barrier systems required for the release of new reticulocytes, thereby preventing the close contact of PRBC with activated effector cells in the splenic cords. In order to provide evidence for or against our hypothesis, we performed histological and functional clearance experiments in normal mice, mice at peak parasitemia, mice undergoing crisis and transfused mice. Unlike the results with *P. berghei* and *P. yoelii*, we did not find evidence for an "opening" of splenic barriers at the time of crisis. In fact, infection was associated with a progressive decrease in clearance rate and splenic uptake of ^{51}Cr-labelled PRBC which was not reversed at the time of crisis (not shown). In normal spleen (Figure 5A), there is a sharp delineation of splenic red pulp and white pulp areas by a zone of carbon uptake by marginal zone macrophages. Carbon uptake in the marginal zone is diminished but still distinct in mice at peak parasitemia (Figure 5B). In contrast, spleens of mice undergoing crisis revealed congestion with erythroblastic islands and lack of carbon particle uptake in the marginal zone (Figure 5C). These results suggest that crisis in murine *P. chabaudi* infections is not associated with the "opening" of barrier cell complexes. Furthermore, these findings, although by no means definitive, would appear to be inconsistent with our hypothesis described above. However, direct ultrastructural analysis of

spleens and in situ distribution studies of microspheres would be required to resolve this question. It is interesting to note that the histologic features of spleens from transfused mice (Figure 5D) resemble those of the spleens from peak parasitemia rather than the spleens of mice at crisis. This probably reflects the similarity in parasitologic and hematologic features of the peripheral blood between transfused mice and mice at peak parasitemia.

The exact mechanism by which blood transfusion alters the course of parasitemia and the timing of parasite clearance is not known. We speculate that a shock-like syndrome associated with acute hemolysis and severe anemia initiates a cascade of systemic responses which may be detrimental to parasite growth and metabolism. Indeed, Clark and his colleagues (14) have previously shown that administration of endotoxin to mice infected with *P. vinckei* induced a cytostatic effect on the parasite (14). It has been proposed that mediators released during systemic shock have deleterious or inhibitory effects on parasites. Mediators which have been implicated in the process of crisis during malaria include tumor necrosis factor (9), immune interferon (9), lipid peroxides (13) and crisis form factor (6). It is noteworthy that host tissues also suffer from anoxic and ischemic injury during the period of crisis. Indeed, Jensen (6) has previously noted that crisis is usually associated with severe infections, either preceeding the death of the host or spontaneous resolution of parasitemia and survival of the host. Our suggestion that the mechanism(s) which operates during crisis is mainly a non-specific physiologic response, rather than a specific immune-mediated process is consistent with experiments in mice with genetic or experimentally-induced deficiencies in specific cell types or effector molecules. In murine hosts deficient in B cells and antibodies [anti-IgM treated mice (3)and scid mice (7)], T cells

[nude and scid mice (7); anti-CD4 and anti-CD 8 treated mice (12)], macrophages [silica-treated mice (18)], and in animals deficient in nitric oxide [NMMA-treated mice (19)] and in oxidative metabolites [P/J strain mice (2)], there is a strikingly consistent fall in parasitemia during the period immediately following peak parasitemia. This decrease in parasitemia does not differ either in timing or in rate from that in control, immunocompetent mice. The essential difference between immunocompetent and immunodeficient hosts lies mainly in the phase after crisis, whereby immunodeficient hosts experience a resurgence of patent parasitemia, which they are unable to suppress or sterilize. Thus, as previously suggested (1), different mechanisms likely operate to limit parasite multiplication, to induce and effect parasitologic crisis, to maintain the infection at subpatent levels and to sterilize the infection .

Regardless of the exact mechanisms, the present results demonstrate that crisis as it occurs in experimentally infected mice need not be constitutive and is influenced by the physiological status of the host. The blood transfusion model described here allows the experimentalist a measure of control over the timing of crisis and the opportunity to discern which mechanisms, whether they be immune or physiological, contribute to this process. Furthermore, this model allows for studies on the effects of prolonged exposure to high levels of parasites on the development of T-cell subsets and antibody responses, and on the development of acquired immune responses in genetically-susceptible mice.

In summary, our results clearly demonstrate that susceptible A/J mice succumb to infection as a result of severe anemia and other pathophysiologic alterations associated with it, and that these mice are inherently capable of mounting an effective acquired immune response to

P. chabaudi AS. Furthermore, we have presented novel observations which suggest that the process of crisis during blood-stage malaria may be regulated primarily by physiologic alterations associated with acute anemia. In addition, our observation of increased numbers of gametocytes in transfused mice suggest that the effect of blood transfusion on the transmission characteristics of the parasites (i.e., the infectivity to the mosquito vector) needs to be explored (9).

Figure 1.

Effect of blood transfusion on survival of susceptible A/J mice during *Plasmodium chabaudi* AS infection. Blood transfusions, performed as described in Materials and Methods, were given on days 7 to 9. Percentage survival of untransfused control mice (open symbols, N=10) and transfused mice (closed symbol, N=10) are shown. Chi-square test, $P < 0.0001$.

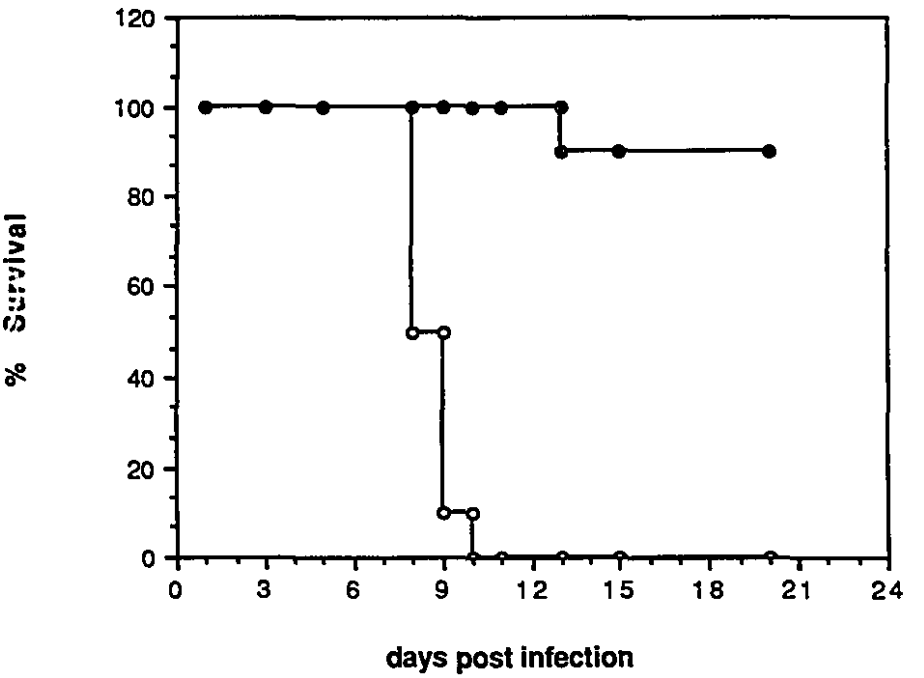
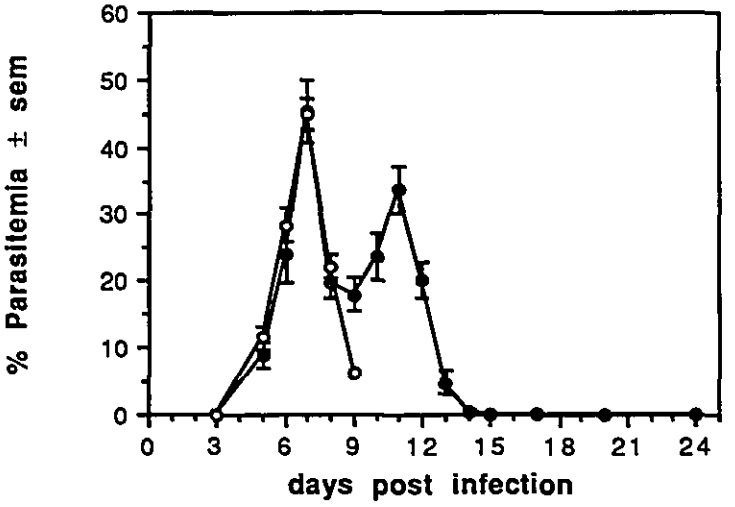

120
100
80
60
40
20
0
% Survival
0
3
6
9
12
15
18
21
24
days post infection

Figure 2.

Effect of blood transfusion on the course of *P. chabaudi* AS infection (A) and the course of anemia (B) in susceptible A/J mice. The parasitemia and red cell counts in untransfused control mice (open symbols) and in transfused mice (closed symbols) are shown. Values are mean ± sem of 5 mice per group. Data shown are representative of four independent experiments.

A


% Parasitemia ± sem
60
50
40
30
20
10
0
0 3 6 9 12 15 18 21 24
days post infection

B

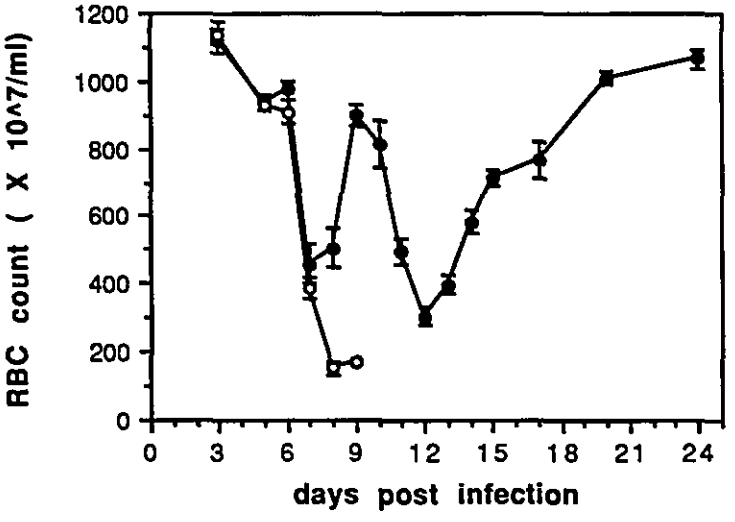
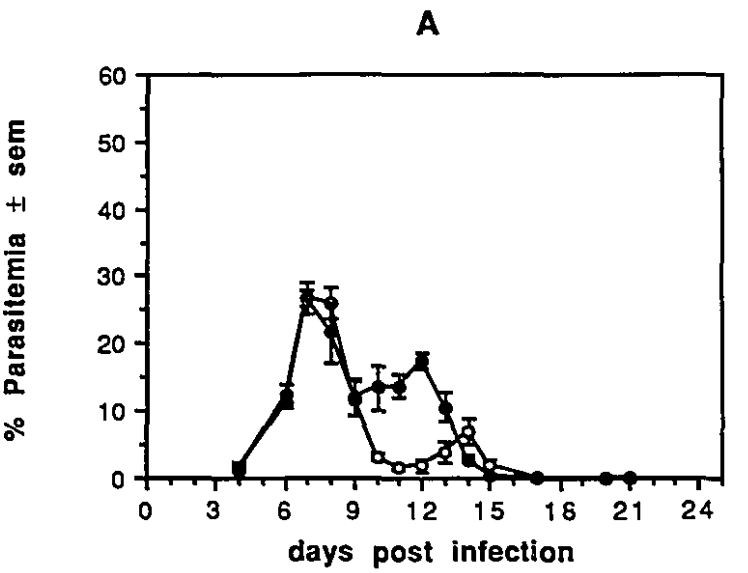

RBC count (X 10^7/ml)
1200
1000
800
600
400
200
0
0 3 6 9 12 15 18 21 24
days post infection

Figure 3.

Effect of blood transfusion on the course of *P. chabaudi* AS infection (A) and the course of anemia (B) in resistant C57BL/6 mice. The parasitemia and red cell counts in untransfused control mice (open symbols) and in transfused mice (closed symbols) are shown. Values are mean ± sem of 4 mice per group. This experiment was repeated twice with similar results.


A
% Parasitemia ± sem
60
50
40
30
20
10
0
0 3 6 9 12 15 16 21 24
days post infection

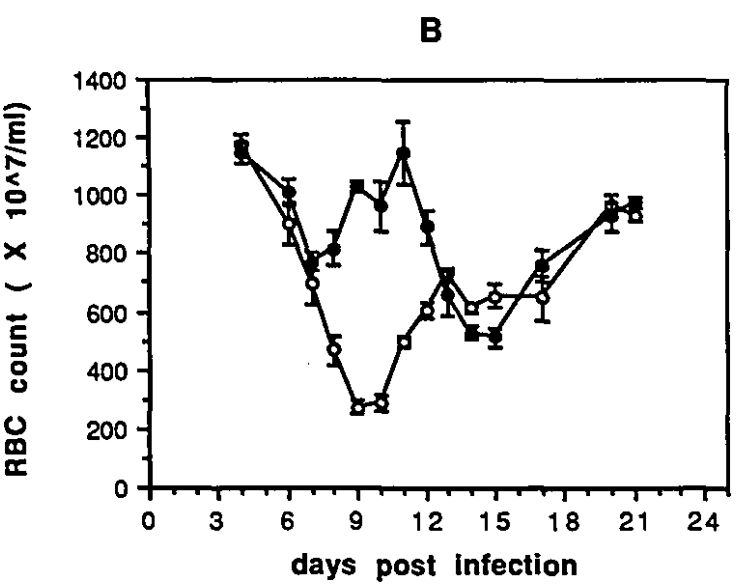

B
RBC count (X 10^7/ml)
1400
1200
1000
800
600
400
200
0
0 3 6 9 12 15 18 21 24
days post infection

Figure 4.

Blood smear from a representative, transfused C57BL/6 mouse, showing increased gametocyte differentiation. Three gametocytes are indicated with arrow heads. Magnification: 850 x

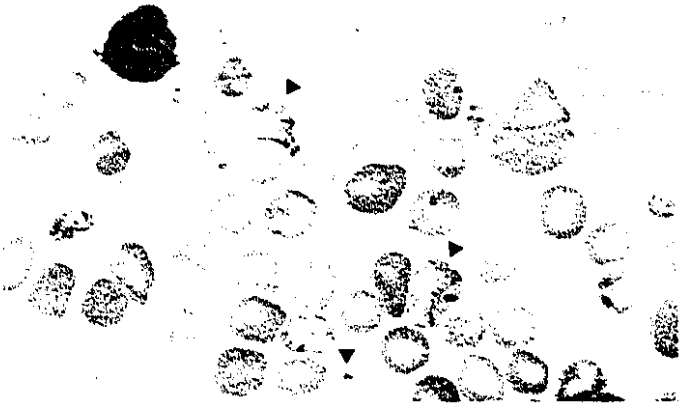
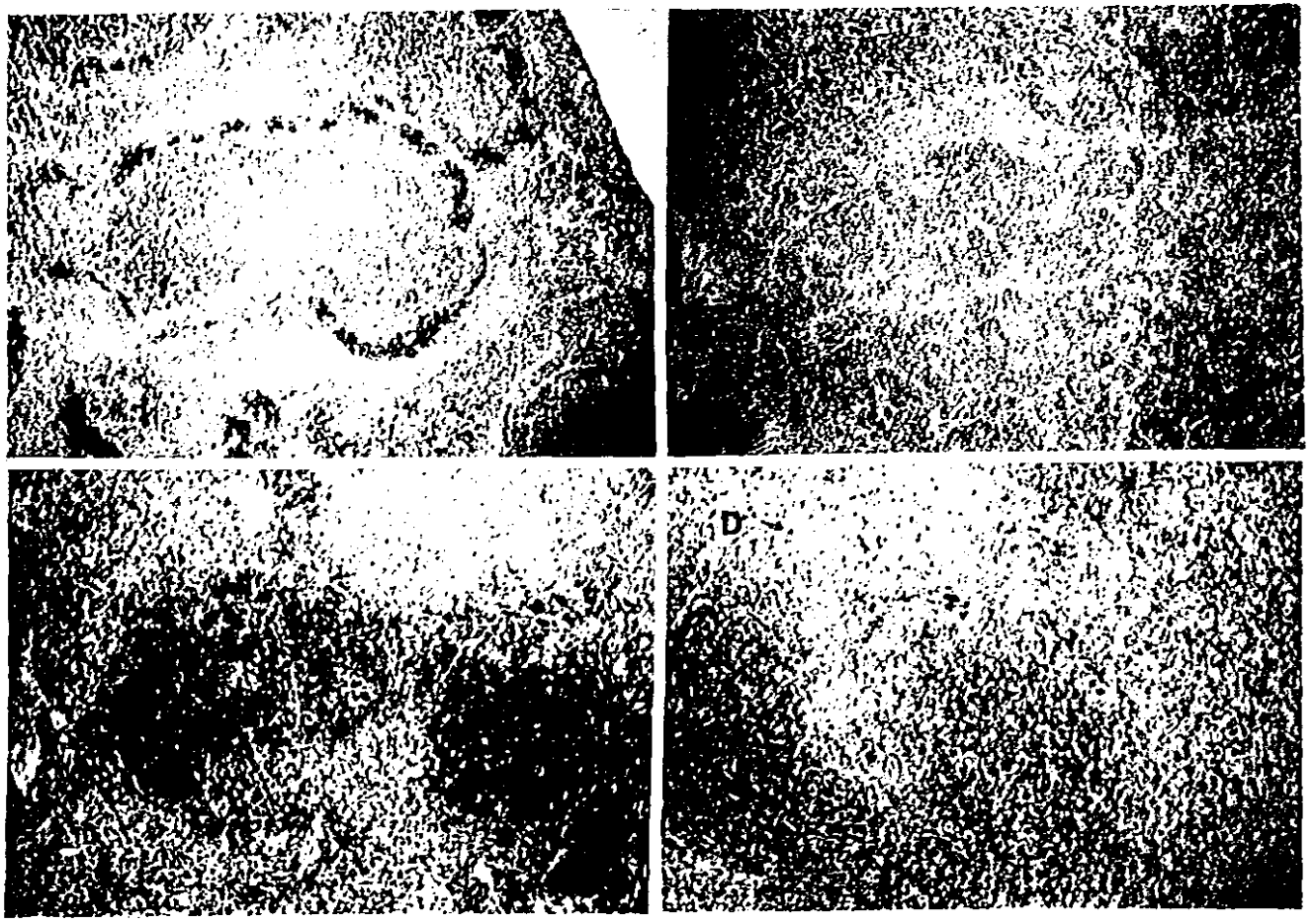


Figure 5.

Histology and carbon uptake in spleen sections obtained from a representative control C57BL/6 mouse (A), mouse at peak parasitemia, day 7 (B), mouse which was undergoing crisis, day 10 (C) and a transfused mouse also at day 10 (D). Magnification: 120x


A
D

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CHAPTER 5

If splenic erythropoiesis were crucial for the survival of C57BL/6 mice, as suggested in chapter 2, splenectomy would be expected to result in the development of more severe anemia and death. Splenectomized mice are unable to clear the parasites and suffer from severe anemia and many of them die. Whether anemia is caused by the absence of splenic erythropoiesis or the persistence of parasitemia is unclear. The paper presented in this chapter demonstrates that immune spleen cell transfer allows splenectomized mice to clear the parasitemia and recover from anemia and survive the infection. Therefore, splenic erythropoiesis *per se* is not essential for the expression of genetically-determined resistance in C57BL/6 mice. The manuscript dissects the cellular requirements for transfer of the capability to resolve and clear *P. chabaudi* AS in splenectomized C57BL/6 mice.

DIFFERENTIAL REQUIREMENTS FOR AN INTACT SPLEEN IN THE INDUCTION AND EXPRESSION OF B CELL DEPENDENT IMMUNITY TO *Plasmodium chabaudi* AS

ABSTRACT

The requirement for an architecturally intact spleen in the afferent and efferent arms of immunity to the murine malaria parasite, *Plasmodium chabaudi* AS, was analyzed. Spleen-intact C57BL/6 mice develop a single, patent parasitemia and resolve the infection. In contrast, surgically splenectomized mice experience persistent waves of patent parasitemia interrupted briefly by periods of parasitologic crises. Transfer of spleen cells from immune donors but not spleen cells from normal mice into splenectomized mice enabled the recipients to resolve the infection similar to spleen-intact mice. B-cell depletion but not T-cell depletion of spleen cells prior to transfer abrogated the ability of splenectomized recipients to resolve the infection. Compared to spleen-intact mice, splenectomized mice exhibited a delayed antibody response whereas all groups of immune cell recipients had an accelerated antibody response. Nevertheless, splenectomized mice and recipients of B-cell depleted cells failed to resolve infections, in spite of the development of high titer antibodies late during the course of infection. Analysis of IgG isotype responses showed a lower representation of IgG2a in mice which failed to resolve infections. The latter mice had characteristic histopathologic changes in the liver. These observations indicate a unique role of the splenic microenvironment for the induction and development of an effective B-cell dependent response against malarial parasites.

INTRODUCTION

That the spleen plays an important role in host defence against malarial infections is a well established fact [1]. This is perhaps best exemplified by the capacity of splenectomy to extend the host range of *Plasmodium* species. Furthermore, splenectomy of several rodent and primate hosts abrogates their capacity to control and clear the malarial parasites [2]. The precise mechanisms by which the spleen exerts its protective functions are less well understood. By virtue of its unique anatomical features and its cellular composition, three functions could be broadly ascribed to the spleen in the context of host responses to malarial infections. Firstly, the spleen serves as a mechanical filter which surveys and sequesters rheologically or immunologically modified red cells, including parasitized red cells [3]. Secondly, the spleen provides a microenvironment for the interaction of parasites, immune cells and their products during the generation and execution of immune effector functions [4]. Finally, the spleen participates in the generation of new blood cells, especially in the erythropoietic response to hemolytic stress caused by malaria infection [5].

The immune response to the intraerythrocytic stages of malarial parasites has been best characterized in the rodent models of malaria. Evidence has accumulated suggesting that humoral and cell mediated immunity may act in concert or sequentially to control and clear malaria parasites. The relative importance of either arm has been shown to depend upon the parasite species and specific phase of immunity in question. For instance, immunity to blood stage *P. yoelii* and *P. berghei* has been characterized as being antibody dependent whereas *P. chabaudi* and *P. vinckei* have been shown to be controlled primarily by cell mediated

immune mechanisms [7,8]. The cell mediated immune responses to *P. chabaudi* are thought to be mediated by a $CD4^+$ T cell dependent activation of an effector cell, generally regarded as the monocyte-macrophage, which mediates non-specific killing or inactivation of the parasites. More recently, Meding and Langhorne [8] have provided evidence for the involvement of antibody dependent mechanisms in the control and final clearance of the parasites. Thus, it is proposed that CD4+ T cells may activate effector cells via the elaboration of Th1 type cytokines in the acute phase, whereas Th2 type CD4+ T cells help B cells to produce antibodies which ultimately clear the infection [9, 10, 11]. The spleen is thought to be important both as a site of macrophage activation and non-specific parasite killing and a site for antibody synthesis, antigen focusing and phagocytosis. Indeed, recent studies with *P. chabaudi adami* and *P. vinckei* demonstrate an absolute requirement for the presence and modification of an architecturally intact spleen for the expression of T cell dependent cell mediated immunity [12, 13]. This requirement has been attributed to the activation and development of barrier cell complexes which increase the clearance function of the spleen. However, efforts to non-specifically induce barrier cell activation in the spleen by administering BCG, *Salmonella* or vaccinia virus have not resulted in protection against *P. vinckei* [14].

In the present study, we have investigated the requirements for an intact spleen in the development and expression of the immune response to *Plasmodium chabaudi* AS. Special attention was given to the early and late phases of the infection. Our results indicate that the splenic microenvironment is essential for the development but is not required for the expression of an effective immune response against this parasite.

Furthermore, our results suggest that the spleen provides a unique tissue microenvironment for the development of an effective B cell dependent immune response required to clear the parasites.

MATERIALS AND METHODS

Parasites and Experimental Infection of Mice

Plasmodium chabaudi AS originally obtained from Prof. D. Walliker, University of Edinburgh, was preserved and maintained by passage in C57BL/6 mice as previously described [16]. Experimental infection was initiated by intraperitoneal inoculation with 10^6 PRBC. Parasitemia was monitored by counting 200 to 10,000 RBC per slide on Diff-Quik stained blood smears.

Splenectomy

C57BL/6 mice obtained from our breeding colony at the Montreal General Hospital were used throughout. Splenectomy and sham splenectomy procedures were performed using standard techniques previously detailed [16]. Surgically manipulated mice were allowed to rest for at least two weeks before experimental infection.

In Vivo Cell Transfer

As a source of immune spleen cells for transfer, sex-matched C57BL/6 mice were infected with 10^6 PRBC and allowed to resolve the infection. A secondary challenge with the same dose of parasites was administered 30 days after the first infection. Routinely, the mice were smear negative at 7-10 days post challenge and were thence used as donors. Spleen cells from immune and control animals were prepared by perfusion in complete medium (RPMI 1640 with 2% BSA and antibiotics) and by washing twice. Erythrocytes were removed by hypotonic lysis. Spleen cells were either administered intravenously at a dose of 25 x 10^6 per

mouse or were subsequently depleted of T cells or B cells as outlined below.

T Cell and B Cell Depletion

T cells were depleted by two cycles of treatment with a cocktail of anti-T cell mAb (F5D5, GK 1.5 and 53-6.7.2 specific for Thy-1, CD4 and CD8 determinants, respectively) and rabbit C (Cedarlane, Hornby, Ontario, Canada) [17]. Viable cells were retrieved on a Lymphoprep gradient (Cedarlane). B cells were depleted by panning on petri dishes precoated with goat anti-mouse IgM+ anti-mouse IgG (Caltag, Cedarlane). The extent of depletion was monitored by cell surface staining with anti Thy-1 and anti-B220 as outlined below. Resulting cell suspensions were injected at the same dose as undepleted cells.

Cell Surface Staining and Flow Cytometry

Spleen or liver cell suspensions (10^6 cells per sample) were stained with the appropriate antibodies for 20 minutes at 4 C using standard procedures. For the T and B cell depletion experiments, the mAbs 30H12 (rat IgG 2b anti-Thy-1.2) and an anti-B220 antibody (Pharmingen, San Diego, CA) were used to monitor extent of T and B cell depletion.

***P. chabaudi* Specific Ab ELISA**

Plasmodium chabaudi Ag was prepared and solubilized from saponin-lysed parasitized red cells using a solution of 0.5% SDS, 1mM EDTA in 50 mMTris (pH 7) as previously described [18]. Immunolon II plates (Dynatech, Canada) were coated with Ag in bicarbonate buffer pH 9.6 overnight and subsequently blocked with 5% skim milk in PBS and washed with PBS-Tween 20. Serial ten fold dilutions of serum samples were incubated 2 hrs at room temperature. After extensive washing, the plates were developed with either HRP-conjugated goat anti-mouse IgG for

total parasite specific IgG or HRP conjugated anti-isotype antibodies (SBA, Birmingham, AL). Optimal dilutions of conjugated antibodies were determined in pilot experiments and were used at 1:3000 for total IgG and 1:250 for isotype reagents. Reactivity was developed using ABTS substrate kit and optical density read using an SLT Lab Instruments ELISA reader at 405 nm.

Histology

At the end of each experiment (day 60 or later), livers from splenectomized mice with or without various cells transferred were excised and processed routinely for paraffin embedding, sectioned and stained with haematoxylin and eosin. Two to four mice per group were examined.

RESULTS

To better define the role of the spleen in *Plasmodium chabaudi* AS infection, we initially compared the course of infection in surgically splenectomized mice with the course of infection in control, sham operated mice. Figure 1A shows the course of infection in control C57BL/6 mice. As previously reported [5, 15], infection in this strain of mice is self-healing and non-lethal. Mean peak parasitemia of 25% occurred on day 10-12, after which parasitemia was rapidly reduced to subpatent levels. A recrudescence followed on days 28-30, which had a lower peak of parasitemia of approximately 1% or less. In sharp contrast, splenectomized (SPLX) mice experienced a higher primary peak parasitemia of approximately 50% or higher and were chronically patent (Fig. 1B). The patent parasitemia was persistent, interrupted only by short episodes of parasitologic crises and severe anemia. These results indicate that splenectomized mice were able to decrease the level of patent parasitemia during the several parasitological crises observed, but were

unable to suppress the remaining parasitemia from resurgence into full-blown patency. The inability of the splenectomized mice to suppress infections from becoming patent may be due to either their inability to generate effector cells or the lack of a proper tissue microenvironment (that is, the splenic reticular network) required for the effector function had the relevant cells been present. In order to distinguish between these possibilities, we transferred normal and immune spleen cells into the splenectomized mice prior to infection. As previously reported for splenectomized mice infected with *P. chabaudi adami*, transfer of normal spleen cells did not enable the splenectomized recipients to clear the patent parasitemia as spleen intact animals would (not shown). However, transfer of immune cells totally suppressed the secondary and subsequent parasitemia, without affecting the timing and peak of the primary parasitemia. (Fig 1C) This indicates that the spleen is required for the development of the immune cells which directly or indirectly effect the suppression of secondary patent parasitemia. However, an architecturally intact spleen was not required for the effector function of the protective immune cells transferred.

In order to gain insight into the cellular requirement for the transfer of immunity to splenectomized recipients, the immune spleen cells were depleted of T or B cells prior to transfer. Figure 1D shows that the depletion of T cells (from 30% to 3% Thy 1^+ cells) did not result in the abrogation of the protective effect. In contrast, B cell depletion (from 44% to 3% $B220^+$ cells) of the cells transferred did not allow the mice to clear the infection. However, in recipients of B cell depleted immune cells, the T cells, null cells and possibly the remaining B cells were able to prevent the infections from becoming patent. Thus, recipients of B cell

depleted transfers experienced only low grade parasitemias not exceeding 1%. In a subsequent transfer of B depleted immune cells (0.5 % $B220^+$ cells), persistent, subpatent parasitemia was similarly observed. We, therefore, conclude that B cells but not T cells are required for the transfer of the capacity to resolve parasitemia. However, in the absence of transferred immune B cells, T cells and other cells from immune mice are able to confer the ability to control the primary parasitemia to subpatent levels. Nonetheless, immune B cell transfer was absolutely required to effect complete resolution of infections in splenectomized mice.

Since B cells function primarily as antibody producing cells, we compared the antibody response of splenectomized mice with or without various cell transfers at early and later time points during the infection. In order to compare the results of individual determinations done on separate days, normal mouse plasma and immune mouse plasma (obtained from immune spleen donors) were included as negative and positive reference controls, respectively. As shown in Figure 2, on day 17, parasite reactive IgG was undetectable in splenectomized mice without cell transfer. However, these mice developed higher levels of IgG later during the infection. Transfer of untreated or T cell depleted immune cells led to an accelerated IgG response relative to control, intact mice. Depletion of B cells prior to transfer did not result in the abrogation of the early antibody response when compared to B cell sufficient transfers. This indicates that the T cells transferred were able to cooperate with endogenous B cells to generate an accelerated antibody response. However, these antibodies were apparently not effective in the resolution of parasitemia (see Figure. 1D). Consistent with this notion is the observation that IgG levels continue to rise at later time points in B cell depleted transfer recipients whereas

antibody levels declined in the B cell replete transfer recipients. The antibodies produced in the splenectomized mice transferred B depleted cells or without any cell transfer were ineffective in resolving the parasitemia but may or may not have contributed to the control of patency. More importantly, these results strongly suggest that in the absence of an intact spleen, an effective B cell-dependent response fails to develop.

Since increased levels of antibodies are eventually produced in splenectomized mice with or without B cell transfer, it is reasonable to posit that a qualitative difference may exist in the antibody repertoire between these groups of mice. The difference may be related to either the antigen recognition or isotype distribution of the antibodies or both. In order to investigate potential differences in antigen recognition, Western blot analysis of *P. chabaudi* antigens were probed using sera from splenectomized mice with or without B cell transfer. The same banding patterns were obtained regardless of the serum used (data not shown). It appears that there is no gross difference in antigen recognition between the antibodies. We cannot discount the possibility that the antibodies may differentially recognize different epitopes on parasite molecules with similar mobilities.

A more likely difference may exist in the isotype distribution of antibodies in the different groups which may relate to the functional capacity of antibodies to effect resolution. Table 1 shows the relative amounts of parasite reactive IgG isotypes in the different groups of mice at early and later stages of the infection. Splenectomized mice exhibited a delayed antibody response with respect to all isotypes, when compared with control intact mice. Control mice had low levels of each isotype on day 17, but subsequently developed higher levels on day 43. In contrast,

splenectomized mice did not have detectable antibodies on day 17 and still had low levels of each isotype on day 43. Higher levels of IgG2b and IgG3 but not IgG2a and IgG1 were detected on day 56. As would be expected, splenectomized mice which received cell transfers had an accelerated response when compared with mice without cell transfer. On day 17, the isotype distribution among the three groups of cell recipients did not differ markedly. Subsequently, in splenectomized mice which received immune B cells and were able to clear the infection, the levels of IgG1 and IgG3 decreased progressively, while IgG2a and IgG2b remained elevated. In contrast, in recipients of B cell depleted transfers, which were unable to resolve subpatent parasitemia, IgG2b and to a lesser extent IgG3 were markedly increased on days 43 and 56. IgG2a levels did not increase substantially in the B cell depleted transfer recipients.

The liver has been shown to take over some of the splenic functions in splenectomized mice. We, therefore, analysed the histological features of the liver tissue from different groups and correlated the histopathological reactions with persistence of parasitemia. Examination of liver tissues demonstrated a good correlation between the levels and persistence of recrudescent parasitemia and two pathologic changes, namely, leucocytic infiltrates surrounding portal veins and ischemic necrosis of hepatic parenchyma. In mice, which have cleared the infection (that is, splenectomized mice given undepleted or T cell depleted transfers), the liver histology was normal except for residual pigment deposition in Kuppfer cells and occasional lymphoid aggregates in hepatic sinuses (Figure. 3C). In contrast, splenectomized mice which had not received any cell transfer had massive leucocytic infiltrates and ischemic necrosis (Figure. 3A). The midzonal necrosis of the parenchymal tissue most likely

resulted from anoxia and perfusion deficits accompanying the severe episodes of anemia. Interestingly, livers of B cell depleted transfer recipients, which experienced low grade persistent parasitemia exhibited a similar degree of leucocytic infiltrates in the portal veins but showed no evidence of necrotic lesions (Figure. 3B.). These results indicate that low levels of parasitemia are sufficient to induce the leucocytic aggregates. The cellular infiltrates were composed predominantly of plasmacytes and macrophages, and occasionally granulocytes and small lymphocytes.

DISCUSSION

We have examined the requirement for an intact spleen in the induction and expression of the immune response to non-lethal *Plasmodium chabaudi* AS in C57BL/6 mice. Our results clearly demonstrate that an intact spleen is required for the development of an effective immune response to this parasite. However, the immune cells do not require an intact splenic tissue for their effector function to be expressed. Earlier studies by Weidanz and colleagues [12] showed that splenectomized mice given dispersed normal spleen cells did resolve rescrudescent *P. chabaudi adami* parasitemia. However, as we have shown in the present study, transfer of dispersed immune spleen cells allowed splenectomized recipients to clear their infection similar to spleen intact mice. This indicates that immune cells (present in immune spleens but absent in normal spleens) capable of suppressing recrudescent parasitemia fail to develop in the absence of an intact spleen. The present results are reminiscent of earlier studies done by Playfair and colleagues [19] on the protective immunity against lethal *P. yoelii* induced by vaccination. In this study, mice vaccinated with fixed parasitized red cells and *Bordetella pertussis* were protected from an otherwise lethal infection. Splenectomy

prior to but not after the vaccination protocol abolished the protective effect. An intact spleen is, therefore, required for the induction but not the effector phase of protective immunity to intraerythrocytic malarial parasites.

Transfer of protective immunity to splenectomized recipients is abrogated by the depletion of B cells but not T cells in the immune cells transferred. Only those splenectomized mice which received immune B cells were capable of suppressing and clearing parasitemia. Our findings are consistent with the previous observations that anti-IgM treated mice, although capable of suppressing patent parasitemia, fail to completely resolve *P. chabaudi adami* infections [6]. More recently, it has been shown that both T cells and B cells must be transferred into *scid* mice in order for them to control and clear *P. chabaudi* AS infections [8]. Since in these studies a clear presence or absence of parasite reactive IgG corresponded with the ability or inability to resolve infections, it was concluded that anti-*P. chabaudi* IgG mediated the resolution. The inability of splenectomized mice to clear parasitemia cannot be explained by the mere absence of parasite reactive IgG. Splenectomized mice without transfer or with B cell depleted transfer eventually develop high titers of IgG but fail to resolve parasitemia. This would imply that subtle qualitative differences may exist between the antibody repertoire that develops in the presence or the absence of the spleen. By Western blot analysis, no obvious difference in the Ag recognition patterns of sera from different groups of mice was detected. This is consistent with findings of a recent study which investigated the difference between protective and non-protective antibodies to *P. yoelii* [20]. It was found that when hyperimmune sera were fractionated according to isotypes, the protective fraction resided in

the IgG2a fraction, which is the major complement fixing and cytophilic IgG subclass in mice. However, analysis of the antigen recognition patterns of protective and non-protective isotypes by immunoprecipitation revealed an identical pattern. A recent study in human populations also indicates that clinical immunity correlated with the isotype distribution rather than the antigen recognition patterns [21]. Cytophilic IgG1 and IgG3 were associated with clinical immunity. Non-protected subjects have either low levels of all antibody isotypes or a disproportionate representation of non-cytophilic IgG2 and IgM. Furthermore, IgG from non-protected individuals blocked the efficacy of IgG from protected individuals in an in vitro antibody-dependent cellular inhibition assay.

The significance of the differences in kinetics and distribution of IgG isotypes that we observed among different groups of mice remains conjectural. In all likelihood, the reasons for the inability of splenectomized mice and splenectomized mice with B cell depleted transfers to control and resolve infections are different. In splenectomized mice, there is a generalized delay in the IgG response and protective IgG2a is not well represented. This could be due to lack of a splenic microenvironment for the appropriate cognate cellular interactions to occur. Given that the IgG response to *P. chabaudi* is absolutely T cell dependent [8,10], a paucity of competent T helper cells may explain the low levels of antibodies formed. It is also possible that the splenic microenvironment provides a more conducive milieu for the activation and maturation of parasite-reactive B cells. Similar to B cell sufficient transfer recipients, splenectomized mice which received B cell depleted transfers develop high levels of IgG and are able to prevent the parasitemia from becoming patent. However, they are unable to clear the parasitemia.

Interestingly, there is a higher representation of IgG2a in the B cell sufficient compared to B cell deficient transfer recipients. Since activated macrophages preferentially upregulate Fc receptors for IgG2a [22], it could be argued that the IgG2a is protecting the mice by opsonizing parasites, while the non-protective isotypes are blocking the efficacy of IgG2a in the B cell deficient transfers. However, it is equally probable that these patterns may have evolved as a result of the divergence in the levels of parasitemia. Further studies using in vivo depletion of specific isotypes and functional characterization of these isotypes would be required to resolve this question.

The differences in isotype profiles between protective and non-protective antibody responses probably reflects T/accessory cell cytokine regulation of Ig isotype switching and selection in the B cell compartment [23]. It is known that different cytokines may skew IgG isotype responses. For instance, IFN-γ is known to induce IgG2a while IL-4 induces IgG1 and IgE and TGF-β induces IgG2b. Thus, the persistence of IgG2a and IgG2b in protected mice probably reflects the presence of IFN-γ and TGF-β, respectively. Conversely, the decrease in IgG1 may indicate that IL-4 production does not persist. The remarkable increase in IgG2b in B cell deficient transfers may indicate overproduction of TGF-β. The cytokine profile of the T cell response could have been altered because of the redistribution of immune responses to other lymphoid organs. It is known that different lymphoid organs show characteristic bias in the Th1/Th2 profiles [24]. The influence of accessory cell heterogeneity in different lymphoid organs on the isotype profile of nascent and established antibody responses needs to be investigated further [25, 26].

In the absence of the spleen, certain arms of the immune response

continue to be effectively expressed. The primary and subsequent peaks of parasitemia were rapidly, albeit temporarily, decreased in splenectomized mice. These rapid decreases in parasitemia are thought to be induced by T cell-dependent macrophage activation. It has been suggested that T cells of the Th1 type secrete IFN-γ and other macrophage activating lymphokines which induce macrophages into a parasiticidal state. Consistent with this notion is the observation that these rapid decreases in recrudescent patent parasitemia are not observed in T cell deficient mice infected with *P. chabaudi*. [8, 10, 15]. It would appear that this putative effector mechanism remains essentially intact in the absence of the spleen. Also, in the splenectomized mice which had been given B cell sufficient transfers, the antibody response effectively resolved the infection. It has been suggested previously that antibodies against *Plasmodium* act by opsonization of parasitized red cells and free merozoites, rather than by simple neutralization [9, 21]. The spleen is considered to be a major site for immune phagocytosis and parasite killing. It would appear from our studies that other reticuloendothelial organs such as the liver effectively subsume the phagocytic and parasiticidal functions of the spleen. All this being said, there are functional deficits in splenectomized mice transferred immune cells which could be attributed to the absence of the splenic tissue microenvironment. For instance, spleen-intact mice transferred immune spleen cells experience low level parasitemia, whereas similarly transferred splenectomized mice suffer from higher levels of primary parasitemia. Conceivably, the transferred cells home and differentiate more efficiently in the splenic tissue. Furthermore, rechallenge of splenectomized mice transferred immune cells results in parasitemia, which is not observed in spleen intact mice (our unpublished data).

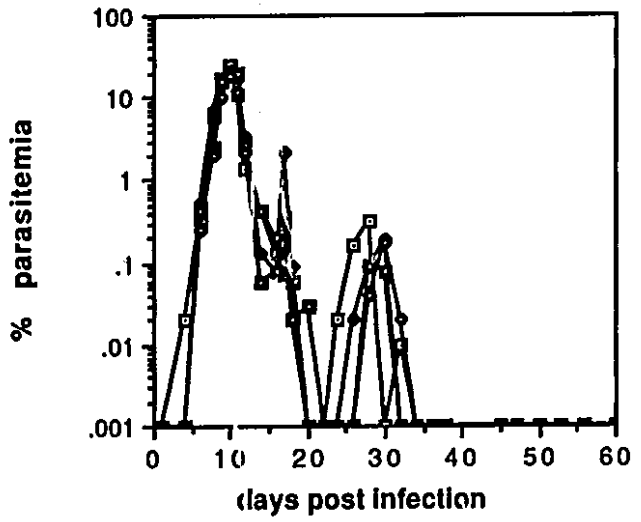
The liver has been shown to be a site of immune activation during malaria, especially in splenectomized mice [19]. Previously, Sayles and Wassom [27] observed mononuclear cell infiltrates in liver tissue of splenectomized mice infected with *P. yoelii*. These infiltrates were observed in both mouse strains which survive and die. Our findings that these aggregates were chronically present only in livers of unprotected mice would indicate that they are induced by the persistence of the parasites, regardless of the level of parasitemia. It is possible that these periportal infiltrates are formed secondary to endothelial damage due to circulating immune complexes. Plasma cells were massively prominent in these infiltrates. It would be interesting to investigate whether they secrete antibodies against parasite or host determinants. Preliminary phenotypic characterization of hepatic $CD4^+$ T cells from splenectomized mice show evidence of recent activation, that is, 60-90 per cent of $CD4^+$ cells were CD 45 RB^{lo} (our unpublished data). We speculate that these T cells may behave like Th2 type cells, secreting B cell growth and differentiation factors which induce plasma cell hyperplasia [28]. Furthermore, the chronic inflammatory and regenerative processes in the liver may induce hepatocytes and other cell types to secrete TGF-β, thereby favoring the increase in IgG2b.

Finally, we would like to point out that a seemingly parallel situation may exist in humans with respect to the role of the spleen in malaria. In a case report study of non-immune and clinically immune, splenectomized, Thai patients infected with *P. falciparum* and *P. vivax*, the clearance of parasitemia was delayed only in the nonimmune patient. Therefore,the processes leading to parasite clearance in partially immune, splenectomized patients did not require the presence of an intact spleen [29].

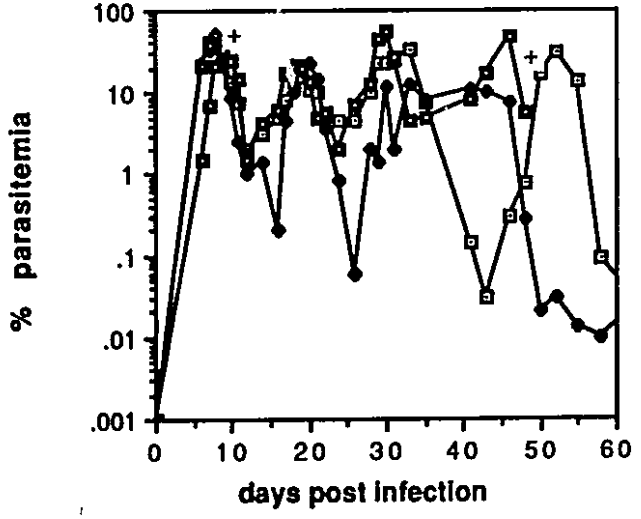
Figure 1.

Course of blood stage infection of *Plasmodium chabaudi* AS in control, spleen intact mice (A), splenectomized mice, SPLX (B), splenectomized mice transferred with immune spleen cells, SPLX + T-B (+) (C), and splenectomized mice transferred with immune spleen cells depleted of T cells (SPLX + T(-), closed symbols) or depleted of B cells (SPLX + B(-), open symbols) (D). Each symbol represents an individual mouse. Four mice were included in each group. All mice (C57BL/6 strain) were infected by intraperitoneal injection with 10^6 parasitized red cells.

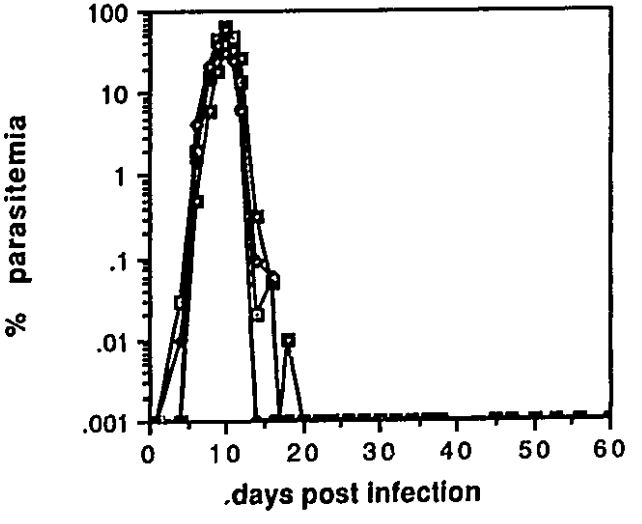
A. spleen intact



B. SPLX



C. SPLX + T-B(+)



D. SPLX + T(-): SPLX + B(-)

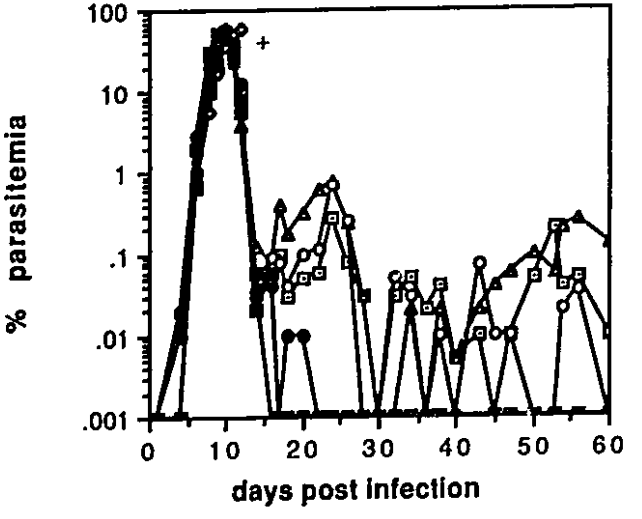


Figure 2.

Anti-*Plasmodium chabaudi* AS IgG responses in intact and splenectomized mice with or without various cell transfers. Optical density values are shown for serum dilution of 1: 100 on day 17 (black), day 43 (striped) and day 56 (stippled). OD values for normal mouse serum (NMS) and immune mouse serum (IMS) are also shown for reference.

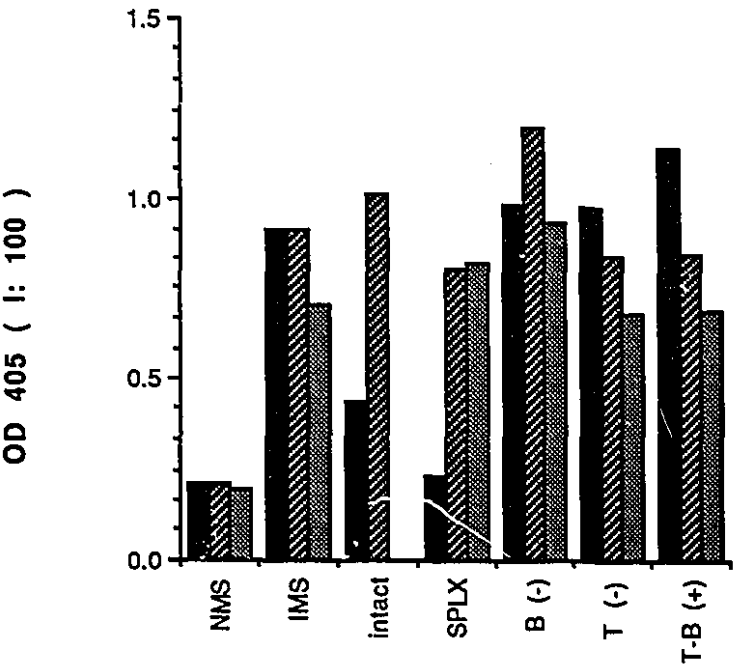

OD 405 (I: 100)
1.5
1.0
0.5
0.0
NMS
IMS
intact
SPLX
B (-)
T (-)
T-B (+)

Figure 3.

Hepatocyte necrosis (left and right side, see triangles) and leucocytic infiltrate surrounding vessel (middle) in liver of splenectomized mouse not given immune cells (A); Liver tissue of splenectomized mouse given B cell depleted immune cells, showing massive leucocytic infiltration in portal vein without hepatocyte necrosis (B); Liver tissue of splenectomized mouse given whole immune cells, showing normal parenchyma with pigment deposition in Kuppfer cells and occasional leucocytes (C). Magnification: 120 X.

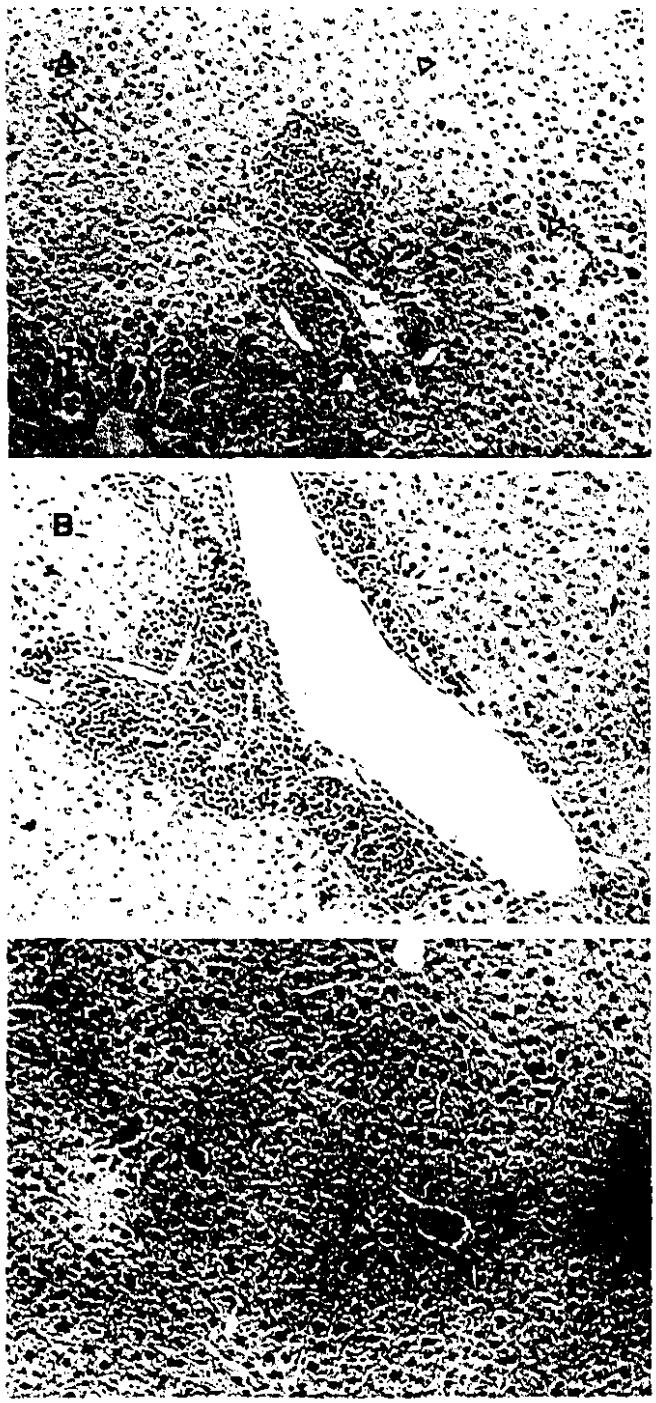

A
B

Table 1. Anti-*Plasmodium chabaudi* AS Isotypic Responses in Intact and Splenectomized Mice with or without Immune Spleen Cell Transfers

	OD Units[a]			
	IgG1	**IgG2a**	**IgG2b**	**IgG3**
day 17				
intact	0.04	0.15	0.07	0.11
SPLX	0	0	0	0
SPLX + B(-)	0.22	0.33	0.39	0.42
SPLX + T(-)	0.17	0.48	0.42	0.38
SPLX +T-B(+)	0.17	0.52	0.43	0.39
day 43				
intact	0.31	0.39	0.73	0.60
SPLX	0.26	0.22	0.24	0.21
SPLX + B(-)	0.42	0.43	1.77	0.52
SPLX + T(-)	0.09	0.57	0.77	0.18
SPLX +T-B(+)	0.15	0.38	0.52	0.19
day 56				
intact	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
SPLX	0.29	0.26	0.45	0.83
SPLX + B(-)	0.12	0.44	0.90	0.72
SPLX + T(-)	0.03	0.40	0.45	0.07
SPLX +T-B(+)	0	0.30	0.42	0.04

[a] The values represent net readings determined by ELISA. Wells of a 96-well assay plate were coated with *P. chabaudi* AS antigen, and 1:10 dilutions of serum samples were incubated in coated, washed wells. Peroxidase anti-isotype reagents were added to wells before development with ABTS substrate. Each value represent the mean of 2 to 4 samples of serum from different groups of mice. The OD value for preimmune mouse serum has been subtracted from each isotype determination.

[b] Not Done.

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CHAPTER 6
GENERAL DISCUSSION
AND
CLAIMS TO ORIGINALITY

Plasmodium chabaudi AS infections in inbred mouse strains follow a lethal and non-lethal course depending upon the strain of the mouse infected (Stevenson, 1989). The phenotype of resistance or susceptibility has been defined mainly by the criterion of mortality. Hence, upon infection with 10^6 PRBC, 100% of animals of resistant mouse strains survive while greater than 75% of mice of susceptible strains die within 14 days. Genetic analysis has indicated that a major, dominant, autosomal locus or closely linked loci control resistance to this parasite. Understanding exactly how this gene or set of genes exerts its protective effect awaits, among other things, the physical localization and determination of the sequence of the gene and the identity of the encoded product. In the absence of this genetic information, I have attempted to understand the phenotype of resistance and susceptibility by studying the evolution of the infection and the associated pathophysiological changes in resistant C57BL/6 and susceptible A/J mice. Because the nature of this type of analysis is largely correlative, its main drawback is the difficulty, if not impossibility, of establishing causation. It is difficult to ascertain how far removed the observed phenotype is from the gene. Nonetheless, I believe that studies on the evolution of the infection and pathophysiology, such as those embodied in this thesis, provide a framework for thinking about how the resistance gene may operate. The study of the phenotype *per se* is warranted. After all, genotype provides only a blueprint, but cannot predict the sequence of events and interactions which occur within the organism and with the environment leading to the generation of phenotype. Phenotype, by definition, is epigenetic.

In this last section of the thesis, I would like to briefly address several issues which need further clarification or reiteration.

The Cause of Death in Susceptible Mice

As death defines the phenotype of susceptibility, it is important to determine the cause of death. Death in susceptible mice is associated with a high level of peak parasitemia and is preceded by severe anemia, weight loss, hypothermia and systemic shock. The terminal stage of severe malaria in A/J mice undoubtedly involves multiorgan dysfunction and systemic shock. The ability of blood transfusion to prevent mortality emphasizes the importance of severe anemia in precipitating other pathological changes which lead to death. Although the exact course leading to death is uncertain, severe anemia could easily lead to hypoxic and ischemic /reperfusion injury in the liver and kidney (Kremsner et al., 1992). An acute respiratory distress syndrome (ARDS)-like pathology with pleural leucocyte accumulation and oedema has been documented in terminal malaria in the mouse (Clark et al., 1987). Acute respiratory and cardiac failure secondary to hypoxia, metabolic acidosis, fluid and electrolyte imbalance are probable immediate causes of death (Holloway et al., 1991; White and Ho, 1992).

The Cause of Anemia

The etiology of anemia in *P. chabaudi* AS infected mice is largely hemolytic in nature and parasite-induced. Calculation of daily hematocrits, considering the geometric increases in the percentage of parasitemia and the daily rupture of red cells during the process of merozoite release, could easily account for the severe anemia with hematocrits of less than 20% developing in 4-5 days after patency. It is, therefore, unnecessary to invoke an autoimmune component to anemia, at least in the acute phase.

Kinetics and Regulation of Erythropoiesis in Murine Malaria

Numerous studies have analyzed the erythropoietic response of different inbred mouse strains to different rodent malarial parasites. Taken together, several general conclusions can be made regarding the nature of the erythropoietic response to malaria in mice. First, the erythropoietic response is shifted from the bone marrow to the spleen (Maggio-Price et al., 1985; Silverman et al., 1987). This occurs by a process of increased recruitment and migration of BFU-E and other myeloid progenitors from the marrow to the spleen (Weiss et al., 1989; Yap and Stevenson, 1992). The shift to splenic erythropoiesis is distinctly more pronounced in non-lethal vs. lethal, and in resistant vs. susceptible mice (Nawa et al., 1992; Villeval et al.,1990; Yap and Stevenson, 1992). Second, an adequate erythropoietin response occurs in lethal malaria or in susceptible mice (Yap and Stevenson, 1992). Third, a non-erythropoietin-dependent increase in splenic CFU-E and BFU-E has been observed in reticulocytophilic but not in normocytophilic malarial parasites (Weiss et al., 1989). Of special interest is a report on the ability of H-2 D molecules purified from *P. yoelii* 17X PRBC to induce reticulocytosis and monocytosis (Mogil et al., 1988). Fourth, an adequate reticulocytic response is not observed until the parasite population has been controlled. The detection of erythroid-specific inhibitory mediators released by marrow and splenic cell populations may represent an underlying mechanism (Miller et al., 1989; Yap and Stevenson, 1994).

The Relationship between Impaired Splenic Erythropoiesis and Susceptibility to Malaria

Is the impaired erythropoietic response in the spleen observed in A/J mice the cause or the consequence of susceptibility to *Plasmodium chabaudi* AS? I am presently equivocal but inclined to elect the consequence option for the following reasons: First, death precedes any appreciable release of and increase in reticulocytes in the periphery. Therefore, death preempts any possible contribution of differences in splenic erythropoietic amplification to survival and death. Second, splenectomy together with immune cell reconstitution allows resistant C57BL/6 mice to clear and survive the infection. Therefore, splenic erythroid amplification is in itself not essential for red cell recovery and the survival of resistant mice. It is unclear at present, whether marrow erythropoiesis compensates entirely or whether ectopic hepatic erythropoiesis is reactivated. Thirdly, the enhanced ability of a particular mouse strain to respond erythropoietically is conditionally expressed depending upon the virulence of the parasite (Nawa, 1992). Fourth, consideration must be given for the influence of the general health of the host on its capacity to respond erythropoietically. Dietary restriction and stress-related endocrine changes could blunt the erythropoietic response (Allipi et al., 1979).

On the Nature of Crisis

Current thinking on the mechanisms of parasitologic crisis invokes the cooperation of T cells and macrophages in the spleen for the induction of crisis forms (Melancon-Kaplan and Weidanz, 1989). The requirement for T cells and an intact spleen is inconsistent with the occurrence of crisis in T cell deficient and in splenectomized mice. A host of non-antigen specific factors has been shown to induce crisis forms in vitro and in vivo (Jensen,

1989). Lipid peroxides, cytokines, and crisis form factor have been shown to inhibit parasite multiplication and induce crisis forms in vitro. However, the relationship between these factors and crisis as it occurs in vivo is unclear. I have argued in chapter 4 that anemia and the systemic shock which follows is required to precipitate or maintain crisis. It is envisioned that during the anemic phase, mediators are released systemically which retard parasite maturation. This would have entailed the demonstration of such an inhibitory activity towards homologous or heterologous parasites in sera of anemic, infected mice but not in sera of transfused, infected mice. Attempts were made towards this end, but were thwarted by my inability to maintain *P. chabaudi* AS in vitro. Fortunately, similar experiments have been performed by Butcher and Clark (1990) and their results are particularly cogent. They detected an activity in sera of mice infected with *P. chabaudi* capable of inhibiting *P. falciparum* in vitro. Inhibitory activity was detectable only in mice which were ill and had at least 50 percent parasitemia. This is consistent with the notion that a host of non-specific mechanisms, such as soluble mediators and fever, control parasitemia or effect crisis. As overproduction or dysregulation of these mechanisms is likely to be deleterious to the host as it is inimical to the parasite, tight controls are necessary either in the levels of mediators or in the development of protective mechanisms (such as, antioxidants) in host tissues. The idea of non-specific immunity effecting crisis is, however, difficult to reconcile with the apparent species and even strain-specificity of the mechanisms that effect crisis as reported by Jarra and Brown (1989).

Role of the Spleen in the Development of Immunity to Malaria

The results presented in chapter 5 highlight the unique role of the spleen as a site of activation, differentiation and interaction of immune cells

(van den Eertwegh et al., 1992). Deficits in both T cell and B cell immunity can be deduced from the data presented in chapter 5. T cell help for antibody synthesis was clearly lacking and was complemented by the B cell-depleted (T cell-enriched) transfer. Endogenous B cells cooperated with the transferred T cells to mount an antibody response, which controlled but did not clear the parasitemia. Immune splenic B cells (depleted of immune T cells) transferred into splenectomized mice allowed for the control and clearance of parasitemia. The nature of the qualitative difference in the antibodies present in spleen-intact and splenectomized mice has not been fully elucidated, but may involve differences in isotype distribution. In the absence of an intact spleen, several parasitologic crises, occuring with remarkable periodicity, were observed during the chronic patent parasitemias observed in splenectomized mice. Clearly, the host response to malaria is layered, consisting of antigen specific and non-specific components, the development and expression of which show differential requirements for an intact spleen.

Possible Sites of Action of the Resistance Gene

The survival or death of an individual parasitized animal is determined by a fine balance between host and parasite factors. Parasite factors, such as growth rate, cellular tropism, cytoadherence, the capability to induce harmful and protective host immune responses and host factors, such as innate resistance and acquired immune responses, nutritional and endocrine status interact, in a complex manner, to determine the tempo and peak levels of parasitemia, the type of pathology, the success of the immune response in controlling and sterilizing the invading parasite population and ultimately, host survival.

In the murine malarial infection studied in this thesis, rapid parasite multiplication during patency leads to a critical level of parasite load and concomitant decrease in red cell mass. During crisis, the parasite population and the red cell mass decreases precipitously and the host either survives or dies. The level of peak parasitemia and the subsequent severity of anemia appear to be the main determinants of host survival. Thus, parasitemias greater than 50% or hematocrits of less than 15% almost invariably lead to death. Thus, the innate resistance gene could act by controlling the rate of parasite multiplication or the peak levels of parasitemia. The resistance gene, in this case, could be expressed at the level of an effector cell (e.g., the macrophage) or a regulatory cell (e.g., the T helper cell). Previous observations have, however, indicated that resistant mice survive the infection even when higher inocula were used and higher levels of parasitemia were attained (Stevenson et al., 1982). It could be envisaged that the resistance gene might act by protecting host tissues and organs from injury and failure during the period of crisis. Understanding the mechanism by which the resistance gene to *Plasmodium chabaudi* AS exerts its protective effect awaits the molecular cloning of this gene.

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CLAIMS TO ORIGINALITY

Chapter 2 demonstrates a deficiency in splenic erythropoiesis in susceptible A/J mice during *Plasmodium chabaudi* AS infection.

(1) During *P. chabaudi* AS infection, the shunting of erythropoiesis from the bone marrow to the spleen occurs to an earlier and greater extent in resistant C57BL/6 mice than in susceptible A/J mice.
(2) Erythropoietin production is not deficient in susceptible A/J mice.
(3) The mobilization of committed erythroid progenitors (BFU-E) is not defective in susceptible A/J mice.

Chapter 3 demonstrates the production by bone marrow and spleen cells of a soluble activity which inhibits erythropoietin-induced proliferation of late erythroid progenitors during the course of *P. chabaudi* AS infection.

(1) Inhibitory activity increases with the rise in parasitemia, but is host cell mediated.
(2) Inhibitory activity is produced by bone marrow and spleen cells in both susceptible and resistant mice
(3) Inhibition is specific for the late stage of the erythroid lineage
(4) Inhibition is not caused by IL-1, TNF or IFN-γ.

Chapter 4 demonstrates the importance of anemia and associated pathophysiological alterations in the mortality of susceptible A/J mice and in the induction and/or maintenance of parasitologic crisis in both strains of mice..

(1) Blood transfusion rescues susceptible A/J mice but retards the resolution of patent parasitemia in both resistant and susceptible mice.
(2) Parasitologic crisis in *P. chabaudi* AS-infected mice is not associated with "opening" of splenic barrier cells as previously reported in *P. berghei* infected rats..
(3) The incidence of gametocytes is increased by reticulocyte transfusion of infected mice.

Chapter 5 demonstrates the requirement for an intact spleen for the induction but not for the effector function of acquired immunity to *P. chabaudi* AS.

(1) Parasitologic crises, but not resolution and clearance of parasitemia, occur in the absence of an intact spleen.
(2) Immune B cells, but not T cells, are required for the transfer of the capability to clear infection in splenectomized mice.
(3) The anti-*Plasmodium* IgG response is impaired in splenectomized mice.