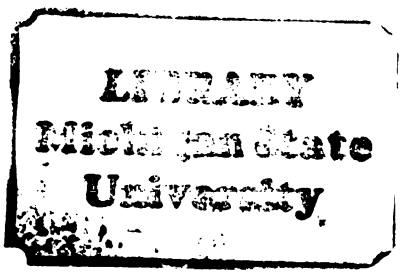


This is to certify that the

thesis entitled

LONGITUDINAL STUDIES ON THE IMMUNE RESPONSE TO PLASMODIUM FALCIPARUM IN SUDAN MEASURED IN VITRO

presented by

John Alan Vande Waa

has been accepted towards fulfillment
of the requirements for

M.S. degree in Microbiology

James B Jensen
Major professor

Date May 17, 1984

LONGITUDINAL STUDIES ON THE IMMUNE RESPONSE TO *PLASMODIUM FALCIPARUM* IN SUDAN MEASURED *IN VITRO*

By

John A. Vande Waa

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1984

ABSTRACT

LONGITUDINAL STUDIES ON THE IMMUNE RESPONSE TO *PLASMODIUM FALCIPARUM* IN SUDAN MEASURED *IN VITRO*

By

John A. Vande Waa

Inhibition of *Plasmodium falciparum in vitro* by human immune serum provides needed information in understanding antimalarial immune mechanisms. Longitudinal, dry season to wet season, changes in antimalarial activities were studied in sera isolated from 62 individuals living in an area of hyperendemic but unstable malaria. Highly synchronous cultures of *P. falciparum* were used to distinguish and quantitate two antimalarial activities, merozoite invasion inhibition and intraerythrocytic parasite retardation. In 54% of the individuals, intraerythrocytic parasite retardation activity increased significantly, nearly 3-fold, in wet season sera as compared to dry season sera. Merozoite invasion inhibition activity was moderate and did not change seasonally. Merozoite invasion inhibition was, however, correlated to parasite-specific IgG titers, and total serum IgG concentration. These results confirm earlier studies which demonstrate two antimalarial activities in Sudanese sera and provides evidence that intraerythrocytic parasite retardation activity plays a role in antimalarial immunity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

The Immune Response to *Plasmodium falciparum*

Introduction

Immunity to malaria, which develops among most individuals living in malarious regions, is a well documented phenomenon. In areas of high or consistent malaria endemicity, where there is little, if any, implementation of control measures, such immunity provides considerable protection from serious malarial disease. Although well documented, the majority of evidence which supports the occurrence and efficacy of this immunity, has been obtained from clinical and epidemiological observations. The use of clinical parameters as indicators of immunity are inherently limited in specificity and sensitivity, especially under field conditions, but have been the only available indicators of protection. Characterization of the immune mechanisms responsible for the observed protection has been attempted using a variety of techniques and immunological markers. Interpretation of experimental findings has been severely complicated due to the nature of investigations involving human subjects, inaccessibility to modern laboratory facilities, and the consequent inability to adequately correlate *in vivo* and *in vitro* observations. Although the mechanisms of protective immunity are not well understood, a great deal is known about the immunological components and parameters associated with extensive exposure to malaria infections. It is the aim

of this paper to investigate some aspects of acquired immunity to malaria in individuals indigenous to malarious regions.

Natural, or innate immunity, among indigenous of the tropics, although not actively acquired, does exist due to selective pressure exerted by the disease. It is important to emphasize these factors since they require consideration when interpreting epidemiological and immunological results. Immune protection acquired in malarious areas is characterized by both age and exposure dependence. It is these dependencies to which immunological parameters need to be compared, since they are a principle measurement of protection. Parasite-specific and non-specific serum components have been extensively examined and correlated with acquired immunity. More recently, parasite-specific inhibition by immune serum has been analyzed *in vitro*, has provided much needed information about mechanisms and target antigens. Lastly, cell-mediated immune responses are reviewed in regard to their correlation with acquired immunity in light of the recent advances in *in vitro* culture.

Innate resistance

Introduction. Under the selective pressure of malarial disease, especially falciparum malaria, some individuals from the tropics have developed a natural resistance to severe infection. This natural, or innate, immunity generally interferes physiologically with parasite development, or host-cell invasion. As obligate intracellular parasites, *Plasmodium spp.* are dependent upon erythrocytes for continuous asexual reproduction. Accordingly, three abnormal blood diseases have evolved in man under selective pressure from malaria that, while pathogenic to man are even more so to the parasite. These blood diseases are sickle cell

anemia, the thalassemias and glucose-6-phosphate dehydrogenase deficiency.

Sickle cell anemia. The most well-known of these conditions is sickle cell anemia. Sickle cell hemoglobin (Hb) results from the presence of the sickle cell gene S and may be expressed as HbSS (homozygous), which is lethal, or HbAS (heterozygous), which is non-lethal. Heterozygous carriers of the sickle gene are less susceptible to Plasmodium falciparum than individuals without this gene. The correlation between HbS and resistance to malaria was first suggested by Allison (1). Mechanisms through which HbS confers resistance were studied by Luzzato et al. (45) and finally demonstrated in vitro by Friedman et al. (30,31) and Pasvol et al. (68). Since sickling occurs more readily in low oxygen concentrations and when the red cell cytosol becomes acidified, and since the presence of the parasite induces both of these conditions, erythrocytes containing HbS undergo sickling when parasitized killing the parasite, thus conferring a certain innate resistance to sickle-cell carriers (45). Thus, the selective pressure by the parasite on the heterozygote has maintained this gene in the population despite the lethality of homozygosity.

Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase deficiency in erythrocytes is a genetic trait found with relatively high frequencies in malarious populations. Allison (2) suggested that the Gd^- gene was selected through the generations by infections of P. falciparum. The most protected by this deficiency are females heterozygous for the gene, Gd^+/Gd^-, whereas males are either Gd^+ or Gd^- since the gene is sex-linked (43,46). The mechanism of protection against malaria in the heterozygote is not understood. According to Luzzato et al. (47), parasites which invade deficient erythrocytes in vitro are

developmentally inhibited and only a fraction are able to complete the asexual cycle.

Thalassemias. Similarly, thalassemia syndromes also have inhibitory effects on the intracellular growth of the parasite, presumably due to the retention of fetal hemoglobin or reduced iron, two conditions that do not favor parasite development (67,65).

Duffy blood group. In addition to the genetic red cell abnormalities described which interfere with parasite development, a red cell surface component associated with Duffy blood group antigen, apparently determines erythrocytic susceptibility to P. vivax invasion. This was first observed by Boyd et al. (4) who noted that American blacks were often refractory to P. vivax infections. Further studies demonstrated that Duffy positive individuals were susceptible whereas Duffy-negative individuals were resistant to vivax infections (62). It is not clear how Duffy blood group antigens interact with the parasite; in vitro, merozoites can attach to Duffy-negative cells but cannot subsequently complete the invasion process (61).

Such natural resistance to malaria infections needs to be differentiated from acquired immunity during investigations of immune mechanisms. Despite the potential interference that innate factors could cause in the interpretation of immune responses, these factors are seldom considered.

Acquired immunity

Introduction. Some of the earliest reports of an acquired immunity to malaria were made from malarious regions among indigenous populations. Daniels (24) observed that native children suffered more acutely from malaria, as measured by fevers, than did native adults. Spleen rates

among children were also observed to be much higher than rates among adults (24,38). Further observations on indigenous populations found an age dependent expression of this immunity, where if the children survived they appeared to produce a tolerance to infection despite detectable parasitemia (38). Spleen rates and rates of infection were also found to decrease with age (71,12,48,14) but a predictive pattern of acquired immunity was not observed due to differences in geographic location, parasite species and endemicity (14).

Since these early observations, investigators have worked under relatively consistent conditions by taking advantage of the stable malaria, predominately P. falciparum, in many regions of Africa. Chloroquine also was available, as were effective insecticides, which provided a certain amount of control over exposure and, consequently, unexposed groups from one village could be compared to villages under high rates of exposure. These relatively controlled conditions plus more consistent diagnosis, clinical description and quantitation of parasitemia provided consistency among investigators which lead to the identification of a predictive pattern of acquired immunity.

Clinical-epidemiological observations. McGregor (51), consolidating the research done in the past with that of his own experience in the Gambia, proposed that acquired immunity could be divided into successive stages, depending upon age and exposure. Since this work, many investigators have confirmed and expanded McGregor's observations. The first stage of immunity is the protection from malarial infections which the mother presumably provides for the developing fetus. The mechanisms through which the mother confers this protection are not known but the placenta is thought to be a major factor (60). The placenta sequesters a tremendous quantity of parasites (9,32). However, in holoendemic areas

transplacental transmission of infection is rare, but becomes more frequent in areas of lower malaria endemicity 922) which suggests an immune response. The second stage of immunity is considered to be the passive transfer of protection from mother to newborn. The evidence for this protection is derived from the many observations that infants, within the first 2-3 months, suffer malaria much less frequently than do the other age groups of children, and when infections do occur parasitemias are low and symptoms mild (3,9,51,57,37). The nature of such protection is not known, but the period of the protection roughly correlates to the duration of maternal antibodies in the infant's serum (53,10). However, the presence of fetal hemoglobin, inhibitory to parasite development, is also considered to play a protective role (67). As the passive immunity declines, susceptibility increases, signaling the 3rd stage of immunity. Beginning at approximately 6 months and lasting through 2 years of age children suffer through the most dangerous period of exposure to malaria infection. Frequency of infection progressively increases, parasitemias rapidly rise and the infections become increasingly virulent, peaking at 2 years. Anemia, fever and splenomegaly, all severe and often fatal, are characteristic symptoms of malaria during this period (3,9,54,18). Following the period of greatest morbidity and mortality, by 3 years of age, children begin to demonstrate some degree of resistance. This stage of immunity is characterized by a tolerance to infection, with a reduction in clinical symptoms despite marked parasitemias. Beginning at 3 years, children usually have the frequency of infection and high parasitemias characteristic of 2 year olds, but anemia, fever and mortality are significantly reduced. The protection gradually increases with age and in addition to the maintenance of tolerance, there is a steady decrease in parasitemias, frequency of

infection and splenomegaly through ages 5-10 (3,54,51,58,37). Finally, a stable immunity is reached sometime between 15 to 30 years 3,14,54,51,58). By this time, infections when they do occur are usually mild, with low parasitemias, are often subclinical, and self-limiting. Investigations of the parameters of this stable immunity in adults have become the principle means of elucidating the immune mechanism(s) operating against the disease.

Premunition immunity. The increasing protection with age and exposure suggests a slowly acquired immunity. When a stable immunity is reached in adults the protection is not absolute. Adults infrequently suffer from malaria although the infections are usually mild. Because of the limitation in diagnosis, adults may carry subclinical, or chronic infections, which go undetected. Consequently, the degree of immunity is difficult to ascertain. Sergent et al. (72) were the first to propose that maintenance of immunity to Sporozoa was an immunity to superinfection, which he termed "premunition" immunity. Epidemiological studies on the duration of this immunity have provided evidence to support this suggestion. In areas where malaria incidence had been dramatically reduced or virtually eliminated by chemoprophylaxis and insecticides, followed by a resurgence of disease when control were measures were terminated, malaria returns but at a lower incidence than before control even though infected mosquito inoculation rates return to precontrol values. Based on this criteria, the duration of acquired immunity decreases steadily but has been shown to prevail in the absence of infection possibly for up to 11 years (21,27). However, as it is difficult to determine the degree of production in a population, much less an individual, the duration of protection using these criteria is not easily assessed. More predictable observations have been made from

immune individuals who have left malarious regions, were radically cured of the disease, and were free of possible reinfection. After only six months abroad, when such individuals return to malarious regions they again become susceptible to malaria infections, which may be severe (49). Because so little data is available on humans, it is not known whether premunition immunity is the result of maintenance of a subclinical infection or repeated exposure and self-cure. Due to the limitations in determining subclinical infections, sterilizing immunity in humans has yet to be determined.

Characterization of the immune response

Introduction. The immune mechanisms responsible for protection in humans are not well understood, due, in part, to difficulties in determining and defining protection, and then differentiating the protective from nonprotective immune responses. The determination of protection ultimately requires verification *in vivo* by controlled parasite challenge. Obviously, work of this nature cannot be accomplished routinely, and consequently, protection can only be defined by clinical and epidemiological correlations. In general, the assumption is made by most investigators that every adult from a malarious region is immune. Adults who have recently experienced a malaria episode are often considered to be hyperimmune. Immune responses of these "protected" individuals were first studied by passive transfer experiments, but most work has been focused on correlating immunological and serological parameters to clinical and epidemiological observations. The emphasis of most of this work has been on humoral immune responses. Evidence that cell-mediated immune responses also participate has not been conclusive due to the difficulty of conducting appropriate studies

using infected humans. Recently, *in vitro* inhibition techniques have provided a great deal of information concerning antiparasite immune responses, both humoral and cell-mediated.

Passive transfer. The first passive transfer experiments were those of Sotiriadès (74), who injected blood from adults who had recently recovered from a malaria episode into an individual with an acute infection. One to two days later the recipient showed signs of recovery, demonstrating reduction in both parasitemia and fever. In later experiments, Lorando and Sotiriadès (44) controlled for possible cross-infectivity from the blood by adding quinine to the sample prior to injection. Although the quinine concentration received by the injection was not therapeutic, the immune response may have been altered. To investigate which serum component(s) were responsible for passive protection, Cohen *et al.* (15) obtained pooled serum from adult Gambians, purified the IgG fraction, and gave several injections of this IgG to children with acute falciparum infections. Parasitemias were reduced significantly, usually within 48 hrs, and some, but not all, recovered without the aid of chemotherapy. Following a similar procedure in Nigeria, Edozian *et al.* (29) isolated the IgG fraction from umbilical cord blood and adult serum which separately could reduce parasitemia and symptoms in children with acute infections. Using IgG from adults, cross-protection was observed from East to West Africa (55,82) but serum IgG from Malaysia failed to protect West Africans (19). Human immune IgG was found to partially protect *Aotus* monkeys from acute falciparum infections (26). From these studies it is apparent that IgG can have significant influence upon the course of infection. Since parasitemias decrease the IgG appears to be affecting the erythrocytic cycle (15,19). Further characterization of the immune mechanisms could not be done using

these methods. It was also difficult to assess the degree of protection provided by the IgG since the recipients were at an age where they could already express a certain degree of tolerance. Thus, the partial protection observed was probably the result of both the donated IgG and the children's own immune response.

Serology. The evidence that serum, as IgG, could provide partial protection by passive transfer led to many investigations involving analysis of serum components found among individuals exposed to malaria. Several serological studies have measured both non-parasite-specific immune components, such as total serum immunoglobulin concentrates, as well as parasite-specific immune components, including immunofluorescence titers and immunoprecipitation to parasite antigens.

Non-specific response. The non-specific serological studies have demonstrated that total immunoglobulin concentrations among malarious populations are significantly elevated. Children who have been exposed to malaria have greater concentrations of IgG than children under chemoprophylaxis (54). Among adults it was found that chemoprophylaxis reduced the elevated IgG concentration (34). Cohen and McGregor (16) found that among African adults in malarious areas IgG synthesis and catabolism were nearly seven times those of European controls. This hypergammaglobulinemia was found in all age groups with detectable parasitemias and Ig concentrations increased directly with spleen size through 15 years of age (54,58). However, only during the first two years were IgM concentrations higher among parasitized children (58). No differences were observed between parasitized and nonparasitized adults (50,58). No consistent relationships could be demonstrated for IgA, IgD or IgE concentrations among any age group (52).

Parasite-specific response. Parasite-specific serological studies have shown greater correlations with acquired immunity among populations. Parasite-specific immunoglobulins could be detected by immunofluorescence in the cord blood and serum of the neonate and were determined to be of maternal origin (77,56). From 2 months to about 1 year, immunofluorescent antibody titers (I.F.A.) decreased, but after this period titers progressively increased until they stabilized among the 20-30 year olds (28). These changes in I.F.A. titers correlated quite closely with the clinical-epidemiological observations of the development of acquired immunity. In young children the I.F.A. titers correlated well to spleen size and, although uncommon among African adults, splenomegaly was also correlated to I.F.A. titers (33). Little is known about IgM I.F.A. titers among malarious populations but much like IgM serum concentrations and primary infections, IgM titers are the first to appear and gradually diminish while IgG titers rise (20). Sustained IgM I.F.A. titers were found to be a distinctive feature of tropical splenomegaly syndrome (23). Although I.F.A. titers are relatively stable in adults, over several years of malaria control measures Ig titers in all age groups significantly decreased (21). Other methods for measuring parasite-specific antibody titers, such as the passive agglutination methods using parasite antigens coated on red cells, can be sensitive but have not been useful for epidemiological work (80). Some of the more recent enzyme linked immunosorbent assays (ELISA) have been used successfully and have given similar results to I.F.A. titers (78). However, with low antibody titers this method does not appear to be as sensitive as I.F.A. (79). Despite the association between I.F.A. titers and acquired immunity, the relationship is far from absolute and does not

predict the degree of protective immunity of an individual, but simply represents the degree of exposure received by a population (79).

Immunoprecipitation. Antigens used for I.F.A. determinations are usually infected red cells and as such have limited specificity with regard to protective antigens. A more specific analysis of the antibody response in acquired immunity is achieved by immunoprecipitation of plasmodial antigens. The first such studies were those of McGregor and Wilson (59). Using gel diffusion techniques they demonstrated that, much like I.F.A. titers, the number of precipitated antigens increased with age. When parasite fractions were used they found a heat labile fraction that responded more specifically and was correlated with the epidemiology of acquired immunity. Both the number of individuals with precipitating antibodies and the number of heat labile antigens precipitating increased with age. These antibodies were present in the sera of newborns, their titers decreased during the first year and then increased until approximately 10 years of age when they became stable (59). It was also found that both the number of individuals with precipitating antibodies and the number of precipitated antigens decreased after malaria control measures were introduced (21). A heat stable parasite fraction could be found circulating in the serum of infected individuals which demonstrated a considerable degree of serological diversity, and has recently been used for serotyping Plasmodium isolates (84,83). The importance of this circulating antigen to the maintenance of protection has yet to be confirmed.

In vitro inhibition. The serological information, most notably I.F.A. titers and immunoprecipitation, are reasonably associated with clinical-epidemiological observations of acquired immunity. However, these associations are derived from population studies, and when applied

to individuals such associations and their predictive values for protection become diminished (63). In order to analyze the immune response of an individual it is obvious that a great deal of clinical information concerning malaria must be obtained. Even with extensive clinical information there is still uncertainty about how protected the individual may be. Since protection for each individual cannot be realistically determined by challenge *in vivo*, *in vitro* assays for antiparasitic activities have been utilized.

Phillips *et al.* (69) first used human immune serum from adult Gambians on cultures of *P. falciparum*. Of the 15 sera tested, only two significantly inhibited parasite multiplication. Similar results were later reported by Wilson and Phillips (85). These early studies were conducted before continuous *in vitro* cultures of *P. falciparum* were achieved, and thus, the degree of serological induced inhibition could not be reliably assessed. The development of successful culture techniques for continuous *in vitro* propagation of *P. falciparum*, developed by Trager and Jensen (76), has provided a much needed tool for analyzing antiparasitic mechanism found in human immune serum. Since then many investigators have used *in vitro* inhibition of *P. falciparum* to study immune mechanisms and have attempted to associate this inhibition with acquired immunity. Inhibition of parasite multiplication has been observed by many investigators using adult immune serum obtained from endemic areas. However, not all sera from endemic areas are inhibitory to cultured parasites, and since many studies of immune mechanisms have been conducted using pooled serum samples, the results may be of questionable value. For example, a positive correlation between IgG I.F.A. titers and *in vitro* parasite inhibition has been reported by some,

but not all investigators (70,35). Brown et al. (6) found inhibition of cultures by IgG which also precipitated schizont antigens. Attempts to determine the protective antigens and the parasite strain specificity of those antigens by the inhibitory IgG have not been conclusive (7). Some sera which had no antiparasitic activity in vitro immunoprecipitated many of the same, and in some cases, exactly the same antigens as the inhibitory IgG (8). The mechanism through which serum, or IgG, is acting on the parasite appeared to be inhibition of merozoite invasion, but this was not conclusive. Much of the observed variations between IgG, in vitro inhibition and immunoprecipitation may be the result of methodology, but this remains to be determined. Jensen et al. (39) used highly synchronous cultures of P. falciparum and demonstrated two different antiparasitic activities in most adult immune serum from Sudan--antimerozoite and intracellular growth inhibition. Serum obtained from Indonesia analyzed similarly demonstrated only inhibition of merozoite invasion, but these sera had no intracellular growth inhibition activity (41). Further characterization of these two antiparasitic activities are needed, but the intracellular growth inhibition found in the serum dose not appear to be antibody, yet in the Sudanese sera appears to be associated with clinical immunity (40). These authors have suggested that the nonantibody, antiparasitic factor associated with intraerythrocytic parasite inhibition may be a product of cell-mediated immunity (40).

Cell-mediated immune response. The studies of cell-mediated immune responses in man have been severely limited because of the inability to control and manipulate cellular factors in vivo in man. Consequently, most work in this area has not been conclusive when applied to acquired immunity in malaria. There are measurable changes in lymphocytes during

malarial infections, but no consistent patterns have been recognized except a general reduction in peripheral T-cells. Changes in B-cells, null cells and K-cells have been noted, but such changes vary unpredictably (86,36,81). Ojo-amaise et al. (66) found a positive correlation between parasitemia and both gamma-interferon and natural killer cell activity in malarious children. In vitro inhibition of P. falciparum has also been used to study peripheral effector cells. Peripheral lymphocytes from immune individuals significantly inhibited the multiplication of P. falciparum but only in the presence of malaria antibody, which represents an antibody-dependent cellular cytotoxicity response to malaria (36,5). Polymorphonuclear leukocytes (neutrophils) and monocytes from nonimmunes were found to ingest infected red cells in culture in the present of IgG from individuals living in endemic areas (11). Similar opsonization activity was found by Khusmith and Druilhe (42) using nonimmune monocytes and immune IgG upon the ingestion of merozoites, but not infected cells. The many studies of antiparasitic activities associated with specific antibodies and those using various cellular components of the immune system suggest that acquired immunity to malaria is complex, involving both humoral and cellular immune mechanisms. However, the interactions of these various components and their relationship to clinical immunity is still obscure.

LONGITUDINAL STUDIES ON THE IMMUNE RESPONSE TO *PLASMODIUM FALCIPARUM* IN SUDAN MEASURED *IN VITRO*

John A. Vande Waa and James B. Jensen

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824-1101

ABSTRACT

Inhibition of Plasmodium falciparum in vitro by human immune serum provides needed information in understanding antimalarial immune mechanisms. Longitudinal, dry season to wet season, changes in antimalarial activities were studied in sera isolated from 62 individuals living in an area of hyperendemic but unstable malaria. Highly synchronous cultures of P. falciparum were used to distinguish and quantitate two antimalarial activities, merozoite invasion inhibition and intraerythrocytic parasite retardation. In 54% of the individuals, intraerythrocytic parasite retardation activity increased significantly, nearly 3-fold, in wet season sera as compared to dry season sera. Merozoite invasion inhibition activity was moderate and did not change seasonally. Merozoite invasion inhibition was, however, correlated to parasite-specific IgG titers, and total serum IgG concentration. These results confirm earlier studies which demonstrate two antimalarial activities in Sudanese sera and provides evidence that intraerythrocytic parasite retardation activity plays a role in antimalarial immunity.

INTRODUCTION

Acquired immunity to malaria in the human host is well documented but not well understood (20,22). Evidence is available suggesting that both humoral (4,6) and cell-mediated (1,8) immune responses are essential components of this immunity. The in vitro cultivation of Plasmodium falciparum, as described by Trager & Jensen (23) provides a means of analyzing serum antimalarial components and mechanisms of this immunity. Data from in vitro inhibition assays substantially improves our understanding of malaria immunology, heretofore based on clinical observations (18).

It was previously shown that both purified IgG and a non-immunoglobulin serum component, found in persons living in malarious endemic areas of Sudan, inhibited P. falciparum in vitro (13). The latter component has been termed crisis-form factor (C.F.F.), since it retards intraerythrocytic parasite development generating typical crisis-forms in vitro (11). Thus, sera from malarious regions in the Sudan demonstrate two distinct antimalarial activities, merozoite invasion inhibition, which has been demonstrated to be antibody dependent (5), and intraerythrocytic parasite retardation, an activity suspected to be mediated through non-antibody mechanisms or components (13). In the present experiments using highly synchronous cultures of P. falciparum, we have been able to distinguish and specifically quantitate these two antimalarial activities in individual sera. We have determined the longitudinal changes in these two activities in paired sera obtained during the low transmission dry season and malarious wet season, respectively, from subjects living in an area of unstable hyperendemic

falciparum malaria. We have found profound seasonal changes in crisis-form activity but could not demonstrate any changes in merozoite invasion inhibition.

MATERIALS AND METHODS

Subjects

All 62 individuals studied were adult male volunteers, ages 20-66 years old, residents of the Blue Nile Province, Sudan. In this area, malaria is hyperendemic but unstable. Malaria incidence during the dry season, particularly in early June, is almost undetectable except in young children where the incidence is approximately 1-5%, as determined by thick films stained with Giemsa. However, the incidence of malaria for the entire population profoundly increases after the rains begin in June and July, reaching a peak during October and November of over 50% (unpublished observations). The population in this area is exposed primarily to P. falciparum, with rare infections of P. vivax and P. malariae. Samples were collected from Sennar army camp and the local villages of Sheihk Talha, Um Shoka, Wad Hashim and Ismail, all within 80 kilometers of Sennar and situated along the Blue Nile River.

Serum

Paired serum samples were obtained from each subject, the first sample during the end of the dry season, June, 1982, and the second sample following the peak of transmission in october or November, 1982. A third sample was obtained from nineteen subjects during the following dry season, June, 1983. Blood samples were taken by venipuncture in siliconized vacutainers (Becton-Dickenson), allowed to clot, refrigerated overnight at $4^{o}C$ and serum separated from formed elements by

centrifugation. Once separated, the sera were immediately frozen and kept at -20°C until transported to our laboratory at Michigan State University, where they arrived still frozen as described previously (12), and stored at -20°C until analyzed. All paired serum samples were dialyzed concurrently to remove any chloroquine or other drugs and to equilibrate them with the parasite culture medium. Dialysis was conducted at 4°C in 10,000-12,000 MW cutoff membranes (Spectropor), first in 0.015 M phosphate-buffered saline, pH 7.2, for two 1:100 dilutions and finally 1:100 in RPMIs 1640 supplemented with 25 mM HEPES and 0.21% sodium bicarbonate, dialysate was changed at 24 h intervals. After dialysis, the paired sera were filter sterilized through 0.45 m pore membranes (Schleicher & Schull) and heat inactivated at 56°C for 30 min before testing for antiparasitic activity.

Parasite strain

The paired sera were all tested for antiparasitic activity against the FCMSU-1/Sudan strain, isolated in the area of study and transported to Michigan State University (Divo, Vande Waa & Jensen, manuscript in preparation). Stock cultures were maintained in O+ erythrocytes using the candle jar method (9).

Merozoite reinvasion inhibition

Parasite cultures, grown to 10-15% parasitemia, were synchronized to 6-8 h age differential using a combination of the sorbitol lysis (15) and gelatin flotation methods (10) as previously described (12). At the time of segmentation and merozoite release, the gelatin-concentrated schizont-infected cells were diluted to 25% parasitemia using freshly washed O+ erythrocytes and immediately dispensed into 96-well microtiter plates (Linbro). Each well contained 1.5 µl cells and 100 µl of RPMI 1640

containing 10% v/v pooled nonimmune human serum (RP-10) and 25% v/v dialysed test serum. The plates were incubated in a candle jar at 37°C for 4 h to allow merozoites to invade the fresh cells. After 4 h, thin films from each well were made and stained with Giemsa to determine the degree of merozoite invasion inhibition by comparing the number of newly invaded ring-stage parasites in the test serum to the number observed in identical cultures exposed to an equal concentration of dialyzed nonimmune serum.

Intraerythrocytic retardation

Parasites were presynchronized as described above. Merozoite invasion was allowed to occur for 4 h, and was terminated by lysing all remaining schizonts with 5% aqueous sorbitol, producing a culture of young ring-stage parasites with a 4 h age differential. These ring-stage parasites were diluted to 2-3% parasitemia with fresh O+ erythrocytes and dispensed into 96-well microtiter plates. Each well contained 2 µl cells, 200 µl 25% immune sera in RP-10 and 2 µCi of [^{3}H]hypoxanthine (10 Ci/mmole, New England Nuclear). These plates were incubated in a candle jar at 37°C for 40 h, and then harvested onto glass-fiber filters using a Bellco Microharvester. The incorporation of [^{3}H]hypoxanthine into parasite nucleic acids was measured by liquid scintillation spectrometry as described (12). Thin films were also made from wells in which [^{3}H]hypoxanthine was omitted and stained with Giemsa. Parasite retardation was morphologically determined by comparing the extent of parasite development in test serum to development in dialyzed nonimmune serum (11).

Serum immunoglobulins

Indirect fluorescent antibody (I.F.A.) titers were determined using trophozoite and schizont stage parasites of the FCMSU-1/Sudan strain according to the methods of Hall et al. (7). Class-specific, fluorescein conjugated anti-human antibodies (Cappel laboratories) were used to determine specifically the IgG, IgM and IgA titers for each serum.

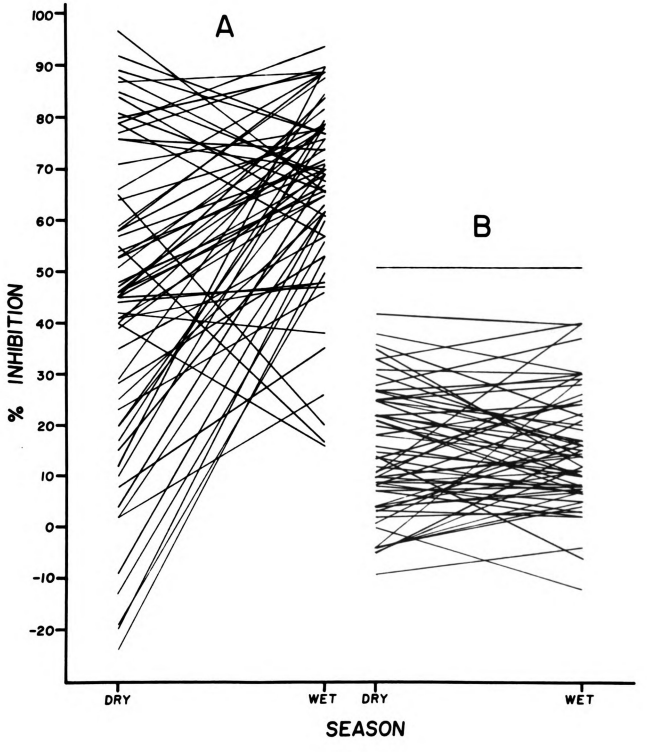
Total serum concentrations of IgG, IgM and IgA were determined by single radial immunodiffusion (R.I.D.) using Endoplate Immunoglobulin Test Kits (Kallestad Labs). Ring diameters were measured using the endpoint method described by Mancini, Carbonara & Heremans (1965).

RESULTS

Intracellular parasite retardation, merozoite invasion inhibition, and parasite-specific and non-specific antibody responses were compared for paired dry season (DS and wet season (WS) serum samples from 62 individuals.

Data describing the degree of intracellular parasite retardation and merozoite invasion inhibition in individual immune sera are presented in Figure 1. The mean difference in parasite developmental retardation from dry to wet season, 39% in DS vs 66.5% WS, was highly significant ($P < 0.001$). Intracellular retardation profoundly increased from the DS to the WS in nearly 55% of the subjects. In this high responding group, the mean intracellular parasite retardation activity increased nearly 3-fold, from 24.5% to 69.0% inhibition. In 35% of the subjects, intracellular parasite retardation activity did not significantly change (two standard deviations above or below the mean, S.D. $\pm$ 10% inhibition); most of these had elevated inhibitory serum at both sample times. In less than 10% of

Fig. 1. Antimalarial activities of paired serum samples collected in dry and wet seasons on Plasmodium falciparum in vitro from 62 individuals. (A) Inhibition of intraerythrocytic parasite development (crisis-form activity) as determined by the reduction of [^{3}H]hypoxanthine incorporation into synchronized parasites grown in the presence of 25% immune serum as compared to parasites grown in 25% nonimmune sera. (B) Inhibition of merozoite invasion as determined by the reduction of newly formed ring stages from segmenting schizont culture incubated in 25% nonimmune serum. All data points from each serum sample represent mean inhibition values from 3 experiments (S.D. $\pm$ 10% inhibition).


A
B
100
90
80
70
60
50
40
30
20
10
0
-10
-20
% INHIBITION
DRY
WET
DRY
WET
SEASON

the subjects parasite development retardation activity decreased from DS to WS. The data shown were obtained in experiments using [^{3}H]hypoxanthine incorporation as a measure of parasite maturation, the retardation of growth by was confirmed morphologically in Giemsa-stained thin films.

In contrast to the significant increase in intracellular retardation activity seen in the WS sera, the degree of merozoite invasion inhibition did not change from DS (15.7%) to WS (15.4%). Most sera collected had low levels of merozite invasion inhibition. A third serum sample was obtained from 19 of the same individuals during the following DS, June, 1983. Antiparasite activities of these DW-WS-DS samples were tested together in the same microtiter plate to minimize inter-experiment variability. These data, shown in Figure 2, demonstrated again a highly significant increase in parasite retardation activity that returned to approximately the same degree of inhibition measured in the sera from the previous dry season (21.4% to 66.5% to 23.8%, DS-WS-DS, respectively).

A comparison of total mean DS and WS parasite inhibition and antibody concentrations measured in these sera is shown in Table 1. Only intracellular parasite retardation changed significantly from the DS to WS ($P < 0.001$).

To investigate the relationships between merozoite invasion inhibition, intracellular parasite retardation and humoral immune factors, correlation coefficients were determined comparing each parameter among individuals. These data are summarized in Table 2. Intracellular parasite retardation was not significantly correlated with merozoite invasion inhibition or humoral immune factors, with the exception of a weak correlation between intracellular parasite retardation and I.F.A. titers. As expected, there was a significant ($P <$

Fig. 2. Inhibition of intraerythrocytic parasite development of serum samples longitudinally collected from 19 individuals during the dry season, 1982, wet season, 1982, and dry season, 1983. All data points from each serum represent mean inhibition values from 3 experiments (S.D. $\pm$ 10% inhibition).

Table 1. Immunoglobulin concentrations and antimalarial activities in serum samples collected from the same individuals during dry and wet seasons, 1982.

	DRY SEASON			WET SEASON			
	n	mean	range	n	mean	range	significance*
IgG I.F.A.[a]	62	688	20 - 2560	62	1152	20 - 5120	NS
IgM I.F.A.[a]	17	56	0 - 320	17	72	0 - 320	NS
IgA I.F.A.[a]	17	7	0 - 80	17	15	0 - 80	NS
IgG (mg/dl)[b]	62	1360	941 - 2275	62	1411	988 - 2357	NS
IgM (mg/dl)[b]	62	124	34 - 362	62	129	34 - 314	NS
IgA (mg/dl)[b]	62	181	69 - 318	62	183	83 - 363	NS
Merozoite invasion inhibition (%)	62	15.7	-9.1 - 51.0	62	15.4	-12.5 - 51.1	NS
Intraerythrocytic parasite development inhibition (%)	62	39.0	-24.1 - 97.0	62	66.5	16.7 - 93.6	(P < 0.001)

[a]Reciprocal endpoint titers
[b]Serum immunoglobulin concentrations as determined by R.I.D.
*Student's t-test used to determine levels of significance
N.S. = Not significant, P > 0.05

Table 2. Correlation coefficients between parasite-specific immunoglobulin classes, total serum immunoglobulin concentrations by classes, merozoite invasion inhibition, and inhibition of intraerythrocytic parasite development (crisis-form activity).

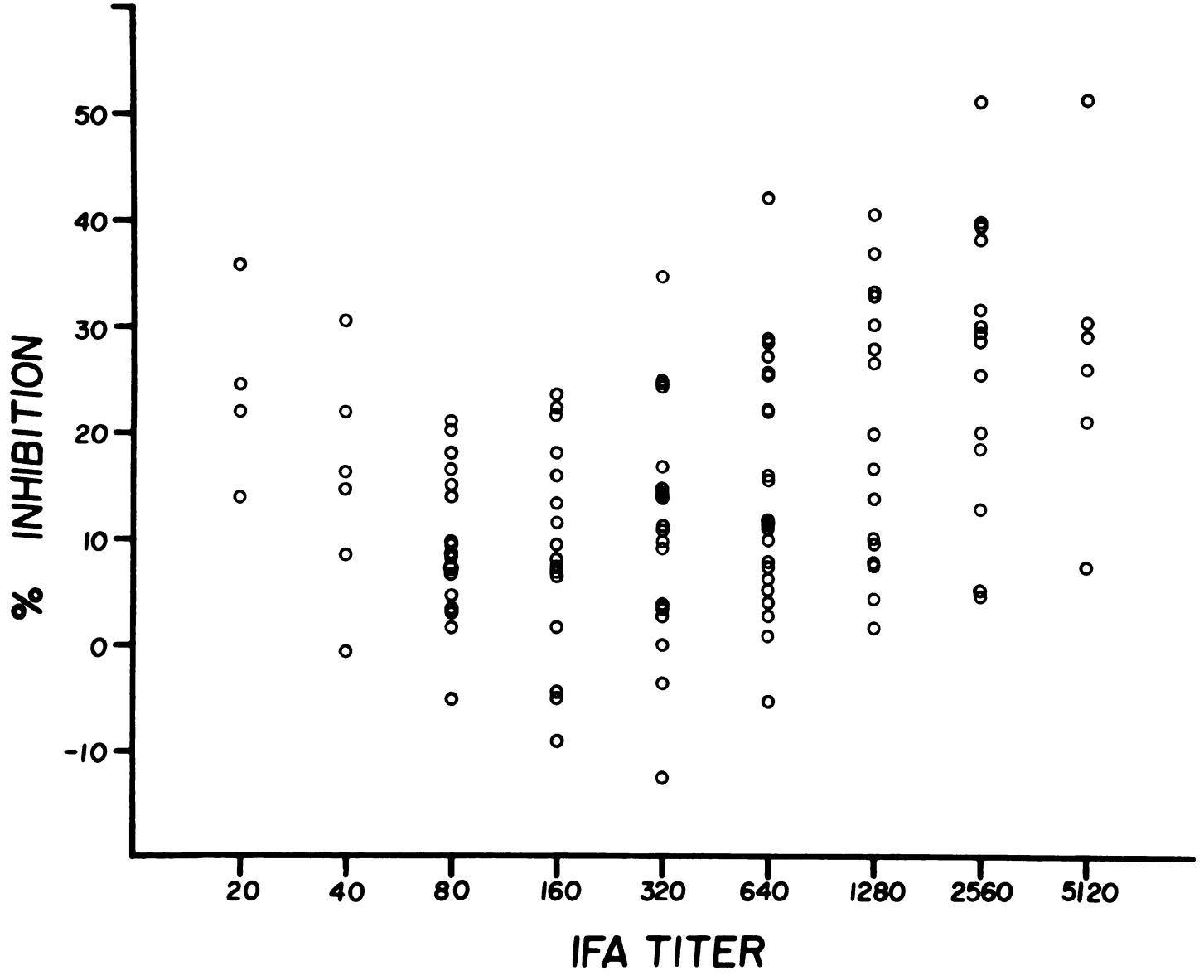
	IgG I.F.A.	IgM I.F.A.	IgA I.F.A.	IgG level	IgM level	IgA level	Merozoite[a] inhibition	C.F.F.[b] activity
IgG I.F.A.	-	0.43*	0.03	0.31*	0.20	0.07	0.45*	0.23*
IgM I.F.A.	-	-	0.02	0.62*	0.49*	0.15	0.25	-0.10
IgA I.F.A.	-	-	-	0.04	-0.10	-0.09	0.01	0.03
IgG (mg/dl)	-	-	-	-	-0.04	0.35*	0.21@	0.03
IgM (mg/dl)	-	-	-	-	-	-0.06	0.09	0.03
IgA (mg/cl)	-	-	-	-	-	-	0.14	0.07
Merozoite[a] inhibition	-	-	-	-	-	-	-	0.06

[a]Merozoite invasion inhibition activity
[b]Intraerythrocytic parasite development inhibition (crisis form factor activity)
*P < 0.001 (student's t-test)
@P < 0.05

0.001) correlation between serum IgG I.F.A. titers and merozoite invasion inhibition. However, when these data are presented graphically (Figure 3), it is clear that the large individual variation prevents the use of IgG I.F.A. titers against falciparum schizonts as a reliable index of merozoite inhibition. Merozoite invasion inhibition was also correlated with total serum IgG levels, though not as strongly. Statistically significant relationships also existed between IgG I.F.A. titers and IgM I.F.A. titers (0.43), IgG I.F.A. titers and total serum IgG concentration (0.31), and between total serum IgG concentration and total serum IgA concentration (0.35). In addition, significant correlations existed between IgM I.F.A. titers and total serum IgG concentration (0.62), and between IgM I.F.A. titers and total serum IgM concentration (0.40).

To assess our populations exposure to other parasitoses we surveyed the villages for urinary and intestinal protozoa and helminths. The actual incidence of these parasitoses were not determined at the time of our survey, however, the relative frequencies of each parasite could be determined, primarily among the children. The comparative frequencies of the intestinal protozoa were Entamoeba coli > Giardia lamblia > Entamoeba histolytica > Chilomastix mesnili > Endolimax nana. The comparative frequencies of urinary or intestinal helminths found were Schistosoma haematobium > Enterobius vermicularis > Hymenolepis nana > Taenia saginata > S. mansoni(rare). The frequencies of all these parasites varied extensively from village to village, especially S. haematobium where in one village over 85% of the children were passing S. haematobium eggs while in another only 5% of 200 children were infected. No association could be made between these parasitoses and the seasonal antimalarial immune responses of the individuals in our study. Furthermore the soldiers who contributed more than 25% of the paired

Fig. 3. Distribution pattern of *in vitro* merozoite invasion inhibition as related to falciparum parasite-specific IgG I.F.A. titer. Although correlation coefficient (Table 2; $P < 0.001$) between IgG I.F.A. titers and antimerozoite activity was positive, the distribution demonstrated that one could not accurately predict the degree of merozoite invasion inhibition as a function of IgG I.F.A. titer.


% INHIBITION
50
40
30
20
10
0
-10
20
40
80
160
320
640
1280
2560
5120
IFA TITER

serum samples examined were essentially free of parasites, except for occasional G. lamblia. Nonetheless, these individuals had as much malaria as did the villagers and their sera varied in antiplasmodium activity from DS to WS to the same extent as sera from villagers with significant prevalent parasitoses.

DISCUSSION

We have found that sera from Sudanese adults contained both merozoite invasion inhibition and intracellular parasite retardation activities. From dry season to wet season, intracellular parasite retardation activity increased significantly ($P < 0.001$), whereas merozoite invasion inhibition did not change. During the following dry season, 1983, intracellular parasite retardation activity, hereafter called crisis-form activity, returned to levels corresponding to the previous dry season, 1982. This seasonal rise and fall in serum concentration of crisis-form factor was apparently due to exposure to falciparum malaria, because in our parasitological surveys of this population, we did not find any other parasite or infectious agent the transmission of which fluctuated with changes in rainfall. Although there were differences in the relative frequencies of other parasite infections from village to village, there were no differences in antimalarial activity between villages. Furthermore, preceding the rainy season, 1983, the study area was extensively sprayed to control mosquito populations and the crisis-form activity in sera collected during October and November 1983, remained uniformly low (unpublished observations).

Some of the sera did not change seasonally, but maintained reasonably high concentrations of crisis-form factor in dry and wet

seasons. We do not yet know the actual kinetics or serum half-life of this activity, so we can only speculate as to how long C.F.F. remains active in the serum. These individuals could possibly have been exposed to malaria during the dry season or alternatively could harbor subclinical malaria infections, but the latter possibility is less likely in this population since splenomegaly is rare and IgG I.F.A. titers uniformly low (14). Sera from a few individuals decreased in crisis-form activity during the wet season, which could be explained by a malaria infection that occurred during the dry season but not in the wet season.

In this population crisis-form activity was correlated with IgG I.F.A. titers. However, since schizont-specific I.F.A. titers are more of an indicator of the degree of exposure, or recent exposure to falciparum infections (17) rather than a reliable index of protection (13), we postulate that the correlation between IgG I.F.A. titers and crisis-form activity results from parasite exposure which induced the production of crisis-form factor.

The seasonal, and apparently parasite-specific, induction of crisis-form activity indicates that C.F.F. may be an integral part of the immune response to malaria in the individuals studied. The ability to produce or maintain high concentrations of C.F.F. and the resultant suppression of malaria infections requires further characterization. However, Jensen et al. 913) have recently demonstrated an association between serum crisis-form activity and clinical immunity. Although our data suggest a parasite-specific dependence of the production of C.F.F., the triggering or induction mechanisms are not known. Nonetheless, the degree of malaria exposure that these people experience, primarily during the wet season, is sufficient to induce highly inhibitory concentration of C.F.F. when necessary. Since crisis-form activity was correlated to IgG I.F.A.

titers, which in turn reflects the degree of exposure to malaria infections, crisis-form activity would predictably be greater in holoendemic areas of Africa.

The source of crisis-form factor is not know. However, some investigators working with animal models have suggested that crisis-forms result from the action of mononuclear cell secretions, i.e. monokines, lymphokines, lymphotoxins or tumor necrosis factor (2,3,21,24), and that these factors may be T-cell regulated (1,8).

In this population most of the sera only moderately inhibited merozoite invasion, with few individuals inhibiting invasion 40% or more. These results are similar to those reported previously where only 2 of 12 sera collected from holoendemic southern Sudan had appreciable antimerozoite activity (13). Furthermore, they support the observations of Philips et al. (19) who found only 2 of 15 Gambian sera inhibitory to short-term cultures of P. falciparum. However, it was the impression of these researchers that the inhibition may have been directed against the late developmental stages of the parasite. They reported that in the presence of the inhibitory sera, the segmenters appeared abnormal, some were lysed, and there was a measurable reduction in the incorporation of radiolabeled precursors into parasite macromolecules. Although these observations were made before the development of techniques for continuous cultivation of P. falciparum, they parallel our own experience with sera collected in different regions of Sudan.

In contrast to the situation in Sudan, many sera obtained from an Indonesian population inhibited merozoite invasion over 80%, but these sera had 8 to 15 times more parasite-specific antibody and no C.F.F. activity (14). Our assay for antimerozoite activity is based on assessing new ring formation and does not determine if merozoites allowed

to invade in the presence of antibody are subsequently damaged. If damaged, the inhibition of the parasite by this mechanism would obviously be enhanced. Furthermore, this population generally experiences P. falciparum infections only during the wet season and the degree of such exposure is reflected in the relatively low IgG I.F.A. titers and, subsequently, low merozoite invasion inhibition activity. Presumably, greater antigenic stimulation in areas of higher malaria incidence would produce higher parasite specific IgG I.F.A. titers and merozoite invasion inhibition.

Our finding that merozoite invasion inhibition did not change with seasonal parasite exposure was somewhat unexpected, especially since it was measured against a parasite strain isolated from one of the villages included in our study. As a primary antiparasitic defense, antimerozoite antibody titers would be expected to rise anamnestically to the exposure from an infection during the wet season, but such was not observed. The triggering of antibody production by antigenic stimulation, in this case antibodies responsible for merozoite invasion inhibition, is well understood. However, there are many factors which can suppress an antibody response during a malaria infection (22). The rapid rise in crisis-form activity may be a key factor in suppressing the increase of antimerozoite antibody production by rapidly clearing the infection and reducing antigenic stimulation.

Merozoite invasion inhibition was correlated with IgG I.F.A. titers, as would be expected from an antibody-mediated activity. However, the use of whole trophozoites and schizonts as antigens in the I.F.A. assay limits the specificity of this test which does not distinguish inhibitory from noninhibitory antibodies. Such lack of specificity was demonstrated by several sera with high IgG I.F.A. titers that did not

appreciably inhibit merozoite invasion. Clearly, a purer antigen preparation would be required to improve the predictive value of IgG I.F.A. titers for merozoite invasion inhibition. It is also important to note that there was no correlation between merozoite inhibition and C.F.F. activity. This lack of correlation supports the validity of our assay system using whole sera, in that a 4 h incubation period apparently is sufficient to allow for reinvasion inhibition without interference with intraerythrocytic parasite development.

There was a significant correlation between merozoite invasion inhibition and total serum IgG concentration. Hypergammaglobulinemia, to varying degrees, is a common characteristic of people living in malarious regions (18) and the population examined in this study was no exception. These moderately elevated serum IgG concentrations were also correlated with parasite-specific IgG I.F.A. titers and this in turn was probably responsible for the merozoite invasion inhibition observed.

No correlation was found between parasite-specific IgM and IgA I.F.A. titers and merozoite invasion inhibition. The reliability of this data is, however, limited since there were so few individuals who demonstrated IgM or IgA I.F.A. titers, all of which were comparatively low. A larger, or younger, population with a greater range of IgM or IgA I.F.A. titers may have provided more definitive results. Interestingly, the one individual who had the highest antimerizoite activity not only had a high IgG I.F.A. titer, as expected, but also had a uniquely high IgM I.F.A. titer. Whether this was due to both immunoglobulin classes remains to be determined. Other statistically significant correlations existed between the class-specific I.F.A. titers and total serum immunoglobulin concentrations, but their relationship, if any, to parasite inhibitory activities are unclear.

In summary, the combined action of two antimalarial activities, merozoite invasion inhibition and crisis-form activity, could provide a substantial inhibitory effect on parasite growth and multiplication. Our data strongly support the hypothesis that production of crisis-form factor is positively correlated with exposure to falciparum malaria and thus, may play a significant role in the acquired immunity to malaria in Sudan.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Alan A. Divo for his assistance in the field and the staff of The Malaria Training Center for their assistance and logistical support. This research was sponsored by NIH, grant no. AI-16312, under the auspices of Michigan State University - Ministry of Health "Collaborative Research on Parasitic Diseases in Sudan" project and contract AID/DSPE-C-0067 of the U.S. Agency for International Development. This paper is listed as article #11165 of the Mighican Agricultural Experiment Station.

REFERENCES

1. Allison A.C. & Eugui E.M. 1983. The role of cell-mediated immune responses in resistance to malaria, with special reference to oxidant stress. Ann. Rev. of Immunol. 1:361-392.
2. Clark I.A., Allison A.C. & Cox F.E. 1976. Protection of mice against Babesia and Plasmodium with B.C.G. Nature 259:309-311.
3. Clark I.A., Virelizier J.L., Carswell E.A. & Wood P.R. 1981. Possible importance of macrophage-derived mediators in acute malaria. Infec. Immun. 32:1056-1066.
4. Cohen S., McGregor I.A. & Carrington S. 1961. Gamma globulin and acquired immunity to human malaria. Nature 192-733-737.
5. Cohen S. & Butcher G.A. 1970. Properties of protective malarial antibody. Immunology 19:369-383.
6. Deans J.A. & Cohen S. 1983. Immunology of malaria. Ann. Rev. Microbiol. 37:25-49.
7. Hall C.L., Haynes J.D., Chulay J.D. & Diggs C.L. 1978. Cultured Plasmodium falciparum used as antigen in a malaria indirect antibody test. Am. J. Trop. Med. Hyg. 17:849-851.
8. Jayawardena A.N. 1981. In Parasitic Disease, Vol. 1. The Immunology, p. 89-91, Marcel Dekker Inc, New York.
9. Jensen J.B. & Trager, W. 1977. Plasmodium falciparum in culture: use of outdated erythrocytes and description of the candle jar method. J. Parasitol. 63:883-886.
10. Jensen J.B. 1978. Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of Plasmodium falciparum. Am. J. Trop. Med. Hyg. 17;1274-1276.

11. Jensen J.B., Boland M.T. & Akood M. 1982a. Induction of crisis forms in cultured Plasmodium falciparum with human immune serum from Sudan. Science 216:1230-1233.

12. Jensen J.B., Boland, M.T., Hayes J. & Akood M. 1982b. Plasmodium falciparum: rapid assay for in vitro inhibition due to human serum from residents of malarious areas. Exp. parasitol. 54:416-424.

13. Jensen J.B., Boland M.T., Allan J.S., Carlin J.M., Vande Waa J.A., Divo A.A., & Akood M. 1983. Association between human serum-induced crisis forms in cultured Plasmodium falciparum and clinical immunity to malaria in Sudan. Infec. Immun. 41:1302-1311.

14. Jensen J.B., Hoffman, S.L., Boland M.T., Akood M., Laughlin L.W., Kurniawan L. & Marwoto H.A. 1984. Comparison of immunity to malaria in Sudan and Indonesia: crisis form versus merozoite-invasion inhibition. Proc. Nat. Acad. Sci. 81:-.

15. Lambros C. & Vanderberg J.P. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65:418-420.

16. Mancini G., Carbonara A.O. & Heremans J.F. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235-254.

17. McGregor I.A., Williams K., Voller A. & Billewicz W.Z. 1965. Immunofluorescence and the measurement of immune response to hyperendemic malaria. Trans. R. Soc. Trop. Med. Hyg. 59:395-414.

18. McGregor I.A., Rowe D.S., Wilson M.E. & Billewicz W.Z. 1970. Plasma immunoglobulin concentrations in an African (Gambian) community in relation to season, malaria and other infections and pregnancy. Clin. Exp. Immunol. 7:51-74.

19. Phillips. R.S., Trigg P.I., Scott-Finigan T.J. & Bartholomew R.K. 1971. Culture of Plasmodium falciparum in vitro: a subculture

technique used for demonstrating antiplasmodial activity in serum from some Gambians, resident in an endemic malarious area. Parasitology 65:525-535.

20. Playfair J.H.L. 1982. Immunity to malaria. Br. Med. Bull. 38:153-159.

21. Taverne J., Dockrell H.M. & Playfair J.H. 1981. Endotoxin induced serum factor kills malarial parasites in vitro. Infect. Immun. 33:83-89.

22. Taylor D.W. & Siddiqui W.A. 1982. Recent advances in malarial immunity. Ann. Rev. Med. 33:69-96.

23. Trager W. & Jensen J.B. 1976. Human malaria parasites in continuous culture. Science 193:673-675.

24. Wozencraft A.O., Dockrell H.M., Taverne J., Targett G.A.T. & Playfair J.H.L. 1984. Killing of human malaria parasites by macrophage secretory products. Infect. Immun. 43:664-669.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Allison, A.C. (1954). Protection afforded by sickle-cell trait against subtertian malaria infection. Br. Med. J. 1:290.

2. Allison, A.C. (1960). Glucose-6-phosphate dehydrogenase deficiency in red blood cells of East Africans. Nature 186:531.

3. Barber, M.A., Olinger, M.T. and Putnam, P. (1931). Studies on malaria in Southern Nigeria. Ann. Trop. Med. 25:461.

4. Boyd, M.F., Stratman-Thomas, W.K. (1933). Studies on benign tertian malaria IV. On the refractoriness of negroes to inoculation with Plasmodium vivax. Am. J. Trop. Med. Hyg. 18:485.

5 Brown, J. and Smalley, M.E. (1980). Specific antibody-dependent cellular cytotoxicity in human malaria. Clin. Exp. Immunol. 41:423.

6. Brown, G.V., Anders, R.F., Stace, J.D. and Mitchell, G.F. (1981). Immunoprecipitation of biosynthetically-labeled proteins from different Papua New Guinea Plasmodium falciparum isolates by sera from individuals in the endemic area. Parasite Immunol. 3:283.

7. Grown, G.V., Anders, R.F., Mitchell, G.F. and Heywood, P.F. (1982). Target antigens of purified human immunoglobulins which inhibit growth of Plasmodium falciparum in vitro. Nature 297:591.

8. Brown, G.V., Anders, R.F., and Knowles, G. (1983). Differential effect of immunoglobulin on the in vitro growth of several isolates of Plasmodium falciparum. Infec. Immun. 39:1228.

9. Bruce-Chwatt, L.J. (1952). Malaria in African infants and children in Southern Nigerian. Ann. Trop. Med. Parisitol. 46:173.

10. Campbell, C.C., Martinez, J.M., Collins, W.E. (1980). Seroepidemiological studies of malaria in pregnant women and newborns from coastal El Salvador. Am. J. Prop. Med. Hyg. 29:151.

11. Celada, A., Cruchand, A. and Pevrin, L.H. (1983). Assessment of immune phagocytosis of Plasmodium falciparum infected red blood cells by human monocytes and polymophonuclear leukocytes. A method for visualizing infected red blood cells ingested by phagocytes. J. Immunol. Meth. 63:263.

12. Christophers, S.R. (1924). The mechanism of immunity against malaria in communities living under hyperendemic conditions. Indian J. M. Res. 12:273.

13. Christophers, S.R. (1949). Endemic and epidemic prevalence. In Malariology, ed. M.F. Boyd, pp. 298-721, Saunders, Philadelphia, Pennsylvania.

14. Clark, H.C. (1944). The age level for the peak of acquired immunity to malaria as reflected by labor forces. Am. J. Trop. Med. Hyg. 24:159.

15. Cohen, S., McGregor, I.A. and Carrington, S.P. (1961). Gamma-globulin and acquired immunity to human malaria. Nature (London) 192:733.

16. Cohen, S. and McGregor, I.A. (1963). Gamma-globuin and acquired immunity to malaria. In "Immunity to Protozoa", eds. Garnham, Pierce & Roitts, pp. 123, Blackwell, Oxford.

17. Cohen, S., Butcher, G.A. and Crandall, R.B. (1969). Action of malarial antibody in vitro. Nature 223:368.

18. Cohen, S., and Butcher, G.A. (1971). Serum antibody in acquired malarial immunity. Trans. Roy. Soc. Trop. Med. Hyg. 65:125.

19. Cohen, S. and Butcher, G.A. (1972). The immunologic response to Plasmodium. Am. J. Trop. Med. Hyg. 21:713.

20. Collins, W.E., Contacos, P.G., Skinner, J.C., Harrison, A.J. and Gell, L.S. (1971). Patterns of antibody and serum proteins in experimentally induced human malaria. Trans. Roy. Soc. Trop. Med. Hyg. 65:43.

21. Cornille Brogger, R., Mathews, H.M., Storey, J., Ashkar, T.S., Brogger, B. and Molineaux, L. (1978). Changing patterns in the humoral immune response to malaria before, during and after the application of control measures: a longitudinal study in the West African savanna. Bull. W.H.O. 56:579.

22. Covell, G. (1950). Congenital malaria. Top. Dis. Bull. 47:1147.

23. Crane, G.G., Gardner, A., Hudson, P., and Voller, A. (1977). Malaria antibodies in tropical splenomegaly syndrome in Papua New Guinea. Trans. Roy. Soc. Trop. Med. Hyg. 71:308.

24. Daniels, C.W. (1901). Notes on blackwater fever in British Central Africa. Rep. Mal. Com. Roy. Soc., fifth series, 44.

25. Davidson, G. (1955). Further studies of the basic factors concerned in the transmission of malaria. Trans. Roy. Soc. Trop. Med. Hyg. 49;339.

26. Diggs, C.L., Wellde, B.T., Anderson, J.S., Weber, R.M. and Rodriguez, E. (1972). The protective effect of African immunoglobulin G in Aotus Trivigatus infected with Asian Plasmodium falciparum. Proc.. Helmith. Soc. Wash. 39 (special issue):449.

27. Draper, C.C., Lelijveld, J.L.M., Matola, Y.G. and White, G.B. (1972). Malaria in the Pare area of Tanzania IV. Malaria in the human population 11 years after the suspension of residual insecticide spraying, with special reference to the serological findings. Trans. Roy. Soc. Trop. Med. Hyg. 66:905.

28. Draper, C.C. Voller, A. and Carpenter, R.G. (1972). The epidemiological interpretation of serologic data in Malaria. Am. J. Trop. Med. Hyg. 21:696.

29. Edozien, J.C., Gilles, H.M. and Udeozo, I.O.K. (1962). Adult and cord-blood gamma globulin and immunity to malaria in Nigerians. Lancet 2:951.

30. Friedman, M.J. (1978). Erythrocytic mechanism of sickle-cell resistance to malaria. Proc.. Natl. Acad. Sci. 75:1994.

31. Friedman, M.J. (1979). Oxidant damage mediates variant red cell resistance to malaria. Nature 280:245.

32. Garnham, P.C.C. (1938). The placenta in malaria with special reference to reticulo-endothelial immunity. Trans. Roy. Soc. Trop. med. Hyg. 32:13.

33. Gebbie, D.A.M., Hamilton, D.J.S., Hutt, M.S.R., Marsdeu, P.D., Voller, A. and Wilks, N.E. (1964). Malaria antibodies in idiopathic splenomegaly in Uganda. Lancet 2:392.

34. Gilles, H.M., McGregor, I.A. (1961). Studies on the significance of high serum gamma-globulin concentrations in Gambian Africans. III. Gamma-globulin concentrations of Gambian women protected from malaria for two years. Ann. Trop. Med. Parasitol. 55:463.

35. Golenser, J., Miller, J., Avraham, H., and Spira, D.T. (1983). The inhibitory effect of human immune sera upon the in vitro development of Plasmodium falciparum. Trop. Geogr. Med. 35:15.

36. Greenwood, B.M., Oduloju, A.J. and Stratton, D. (1977). Lymphocyte changes in acute malaria. Trans. Roy. Soc. Trop. Med. Hyg. 71:408.

37. Hendrickse, R.G., Hasan, A.H., Olumide, L.O. and Akinkunmi, A. (1971). Malaria in early childhood. Ann. Trop. Med. Parisitol. 65:1.

38. James, S.P. (1920). Malaria at home and abroad. London, John Bale, Sons and Danielson, Ltd.

39. Jensen, J.B., Boland, M.T., and Akood, M. (1982). Induction of crisis forms in cultured P. falciparum with human immune serum from Sudan. Science 216:1230.

40. Jensen, J.B., Boland, M.T., Allan, J.S., Carlin, J.M., Vande Waa, J.A., Divo, A.A., and Akood, M. (1983). Association between human serum-induced crisis forms in cultured Plasmodium falciparum and clinical immunity to malaria in Sudan. Infec. Immun. 41:1302.

41. Jensen, J.B., Hoffman, S.L., Boland, M.T., Akood, M., Laughlin, L.W., Kurniawin, L., and Marwoto, H.A. (1984). Comparison of immunity to malaria in Sudan and Indonesia: crisis forms versus merozoite invasion inhibition. Proc.. Nat. Acad. Sci. 81:922.

42. Khusmith, S. and Druilhe, P. (1983). Antibody-dependent ingestion of P. falciparum merozoites by human blood monocytes. parasite Immunol. 5:357.

43. Livingstone, F.B. (1971). Malaria and human polymorphisms. Ann. REv. Genet. 5:33.

44. Lorando, N. and Sotiriades, D. (1936). Immunity in malaria: Therapeutic results obtained from subcutaneous injection of immunized blood. J. Trop. Med. Hyg. 39:197.

45. Luzzato, L., Nwachuku-Jarrett, E.S., and Reddy, S. (1970). Increased sickling of parasitized erythrocytes as mechanism of resistance against malaria in the sickle-cell trait. Lancet 1:319.

46. Luzzato, L. and Testa, U. (1978). Human erythrocyte glucose-6-phosphate dehydrogenase: structure and function in normal and mutant subjects. Curr. Top. Hematol. 1:1.

47. Luzzato, L., Sodeinde, O., and Martin, G. (1983). Genetic variation in the host and adaptive phenomena in P. falciparum infection, p. 159-173, in Malaria and the Red Cell. Pitman, London (Ciba Foundation Symposium 94).

48. Macdonald, G. (1931). The mechanism of infection with malaria in children living under endemic and hyperendemic conditions. Indian J.M. Research 18:1347.

49. Macgraith, B.G. (1974). In Medicine in the tropics pp. 27-73, A.W. Woodruff, ed. Churchill Livingstone, Edinburgh.

50. McFarlane, H. and Voller, A. (1966). Studies on immunoglobulins of Nigerians II. Immunoglobulins and malarial infections in Nigerians. J. Trop. Med. Hyg. 69:104.

51. McGregor, I.A. (1960). Demographic effects of malaria with special reference to the stable malaria of Africa. West Afr. Med. J. 9:260.

52. McGregor, I.A. (1971). Immunity to plasmodial infections; consideration of factors relevant in man. Inter. Rev. Trop. Med. 4:1.

53. McGregor, I.A. (1972). Immunology of malarial infection and its possible consequences. Brit. Med. Bull. 28:22.

54. McGregor, I.A., Gilles, H.M., Walters, J.H., Davies, A.H., and Pearson, F.A.P. (1956). Effects of heavy and repeated malarial infections on Gambian infants and children: effects of erythrocytic parasitization. Brit. Med. J. 2:686.

55. McGregor, I.A., Carrington, S.P., and Cohen, S. (1963). Treatment of EAst African P. falciparum malaria with West AFrican human gamma globulin. Trans. Roy. Soc. Trop. Med. Hyg. 57:170.

56. McGregor, I.A., Williams, K., Voller, A., and Billewicz, W.Z. (1965). Immunofluorescence and the measurement of immune response to hyperendemic malaria. Trans. Roy. Soc. Trop. Med. Hyg. 59:395.

57. McGregor, I.A. Williams K. Billewicz, W.Z., and Thomson, A.M. (1966). Haemoglobin concentration and anemia in young West AFrican (Gambian) children. Trans. Roy. Soc. Trop. Med. Hyg. 60:650.

58. McGregor, I.A., Rowe, D.S., Wilson, M.E., and Billewicz, W.Z. (1970). Plasma immunoglobulin concentrations in an African (Gambian) community in relation to season, malaria and other infections, and pregnancy. Clin. Exp. Immunol. 7:51.

59. McGregor, I.A. and Wilson, R.J.M. (1971). Precipitating antibodies and immunoglobulins in Plasmodium falciparum infections in the Gambia, West Africa. Trans. Roy. Soc. Trop. Med. Hyg. 65:136.

60. McGregor, I.A., Wilson, M.E., and Billewicz, W.Z. (1983). Malaria infection of the placenta in the Gambia, WEst Africa; its incidence and relationship to stillbirth, birth weight, and placental weight. Trans. Roy. Soc. Trop. Med. Hyg. 77:232.

61. Miller, L.H., Mason, S.J., Dvorak, J.A., McGinniss, M.H., and Rothman, J.K. (1975). Erythrocyte receptors for Plasmodium knowlesi malaria: Duffy blood group determinants. Science 189:561.

62. Miller, L.H., Mason, S.J., Clyde, D.F., and McGinniss, M.H. (1976). The resistance factor to Plasmodium vivax in blacks. The Duffy blood group genotype, FyFy. N. Engl. J. Med. 295:302.

63. Mitchell, G.F., Anders, R.F., Brown, G.V., Handman, E., Roberts-Thomson, I.C., Chapman, C.B., Forsyth, K.P., Kahl, L.P., and Cruise, K.M. (1982). Analysis of infection characteristics and antiparasitic immune responses in resistant compared with susceptible hosts. Immun. Rev. 61:137.

64. Nandin, E.H., Nussenzweig, R.S., McGregor, I.A. and Brylan, Hill. (1979). Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. Science 206:597.

65. Nurse, G.T. (1979). Iron, the thalassemias and malaria. Lancet 2:938.

66. Ojo-Amaize, E.A., Salimoner, L.S., Williams, A.I.O., Akinwolere, O.A.O., Shabo, R., Alm, G.V. and Wigzell, H. (1981). Positive correlation between degree of parasitemia, interferon titers, and Natural Killer cell activity in Plasmodium falciparum-infected children. J. Imunol. 127:2296.

67. Pasvol, G., Weatherall, D.J., Wilson, R.J.M., Smith, D.H. and Gilles, H.M. (1976). Fetal haemoglobin and malaria. Lancet 1:1269.

68. Pasvol, G., Weatherall, D.J., Wilson, R.J.M. (1978). Cellular mechanism for the protective effect of haemoglobin S against Plasmodium falciparum malaria. nature 274:701.

69. Phillips, R.S., Trigg, P.I., Scott-Finnigan, T.J. and Bartholomew, R.K. (1972). Culture of Plasmodium falciparum in vitro: a subculture technique used for demonstrating antiplasmodial activity in serum from some Gambians resident in an endemic malarious area. Parasitology 65:525.

70. Reese, R.I., Motyl, H.R. and Hofer-Warbiner, R. (1981). Reaction of immune sera with components of the human malaria parasite Plasmodium falciparum. Am. J. Trop. Med. Hyg. 30:1168.

71. Schuffner, W.A.P. (1919). Twee onderwerpen uit de malaria epidemiologie. Meded. Burgerlijk. Geneesk. Dienst. Nederl.-Indie 9:1.

72. Sergent, Ed., Donatien, A.L., Parrot, F.C., Plantureux, E., and Rougeleief, H. (1924). Etudes experimentales sur les piroplasmoses bovines d'Algeriae.

73. Shortt, H.E. and Garnham, P.C.C. (1948). The pre-erythrocytic development of Plasmodium cynomolgi and Plasmodium vivax. Trans. Roy. Soc. Trop. Med. Hyg. 41:785.

74. Sotiriadès, D. (1917). Essais de sero-therapie dans la malaria. Grèce Méd. 19:27.

75. Sotiriadès, D. (1936). Passive immunity in experimental and natural malaria. J. Trop. Med. 39:257.

76. Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science 193:673.

77. Voller, A. and Bray, R.S. (1962). Fluorescent antibody staining as a measure of malaria antibody. Proc. Soc. Exp. Biol. Med. 110:907.

78. Voller, A., Bartlett, A., and Bidwell, D.E. (1976). Enzyme immunoassays for parasitic diseases. Trans. Roy. Soc. Trop. Med. Hyg. 70:96.

79. Voller, A., Cornille-Brogger, R., Story, J., and Molineaux, L. (1980). A longitudinal study of Plasmodium falciparum malaria in the West African savanna using the ELISA technique. Bull. W.H.O. 58:529.

80. Voller, A. and Draper, C.C. (1982). Immunodiagnosis and seroepidemiology of malaria. Brit. Med. Bull. 38:173.

81. Wells, R.A., Pavanand, K., Zolyomi, S., Permpanich, B., and McDermott, R.P. (1979). Loss of circulating T lymphocytes with normal levels of B and "null" lymphocytes in Thai adults with malaria. Clin. Exp. Immunol. 35:202.

82. Wilson, R.J.M. (1970). Antigens and antibodies associated with Plasmodium falciparum infections in WEst AFrica. Trans. Roy. Soc. Trop. Med. Hyg. 64:547.

83. Wilson, R.J.M. (1980). Serotyping Plasmodium falciparum malaria with S-antigens. Nature 284:451.

84. Wilson, R.J.M., McGregor, I.A., Hall, R., Williams, K., and Bartholomew, R. (1969). Antigens associated with Plasmodium falciparum infections in man. Lancet ii:201.

85. Wilson, R.J.M. and PHillips, R.S. (1976). Method to test inhibitory antibodies in human sera to wild populations of Plasmodium falciparum. Nature 263:132.

86. Wyler, D.J. (1976). Peripheral lymphocyte subpopulations in human falciparum malaria. Clin. Exp. Immunol. 23:471.