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OF PLASMODIUM FALCIPARUM

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NATURALLY ACQUIRED IMMUNITY TO MALARIA MEASURED IN VITRO AGAINST THE ERYTHROCYTIC CYCLE OF PLASMODIUM FALCIPARUM

by

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ABSTRACT

NATURALLY ACQUIRED IMMUNITY TO MALARIA MEASURED IN VITRO AGAINST THE ERYTHROCYTIC CYCLE OF PLASMODIUM FALCIPARUM

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Naturally acquired immunity to malaria in the human host is well documented but not well understood. Experimental evidence suggests that both humoral and cell-mediated immune responses may be essential. The in vitro cultivation of Plasmodium falciparum provides a means of analyzing the antimalarial activities and mechanisms found in immune sera. We have studied antimalarial activity of immune sera against P. falciparum in vitro and have measured two distinct inhibitory activities. One activity, is an antibody-mediated mechanism, merozoite invasion inhibition (MII), and a second is a non-antibody serum component that inhibits intraerythrocytic development of the parasite and yields crisis-forms (CFF) and which may be a secretory component of cellular immunity.

There has been considerable variation in the reports of these antimalarial activities and genetic differences have been incriminated. We have examined the sera from two epidemiologically distinct populations in Sudan, Africa, a

semi-immune (Sugar Factory, SF) and immune (Sundus Village,SV) population. Significant differences in the mean values of nearly all in vitro parameters measured were found. The SF semi-immune population could be characterized by a predominant CFF antimalarial immunity and the SV immune population could be characterized by a predominant MII antimalarial immunity. In addition, infected SF semi-immune individuals showed an increase in MII and CFF activity suggesting a specific immune response. In contrast, infected SV immune individuals demonstrated no such immune stimulation to infection.

Passive protection provided to the newborn is also an important facet of this immunity. I have examined paired maternal and cord sera from Damazin, Sudan, hyperendemic for falciparum malaria and have found maternal sera to contain both high MII and CFF activity. In paired cord sera there was also high MII, but virtually no CFF activity.

Our results suggest that in vitro assays can measure quantitatively and qualitatively components of naturally acquired immunity to malaria. MII and CFF may be significant components of this immunity and the relative expression of each may be epidemiologically and immunologically dependent. In addition, MII appears to be a major component of passive protection to the newborn, whereas CFF does not appear to cross the placental barrier to participate in this protection.

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Introduction

Immunity to malaria, which develops among most individuals living in malarious regions, is well documented. 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 is inherently limited in specificity and sensitivity, especially under field conditions, but has been the only available indicator 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 extremely complicated due to the nature of investigations involving

human subjects, inaccessibility to modern laboratory facilities, and the consequent inability to 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 review is to explane some aspects of acquired immunity to malaria in individuals indigenous to malarious regions.

Natural, or innate immunity among those indigenous to 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 regions is characterized by both age and exposure dependence. It is these dependencies to which immunological parameters need to be compared, since they are the principal measurements 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* which 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 techniques.

Innate resistance

Introduction. Under the selective pressure of malarial disease, especially that caused by faciparum 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 nonlethal. Heterozygous carriers of the sickle cell gene are less susceptible to Plasmodium falciparum infection than are 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. (59) and were finally demonstrated in vitro by Friedman et al. (37,38) and Pasvol et al. (85). 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 and thus conferring a certain innate resistance in sickle cell carriers (59). 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 deficiency. The presence of glucose-6-phosphate dehydrogenase deficiency in erythrocytes is a genetic trait found with relatively high frequency 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 (57,60). The mechanism of protection against malaria in the heterozygote is not well understood. According to Luzzato et al. (61), parasites which invade erythrocytes deficient in the enzyme 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 (81,84).

Duffy blood group. In addition to the genetic red cell abnormalities described which interfere with parasite development, a red cell surface component associated with the Duffy blood group antigen apparently determines erythrocytic susceptibility to P. vivax invasion. This was first observed by Boyd and coworkers (5) 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 P. vivax infections (77). 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 (76).

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 (31)

observed that native children suffered more acutely from malaria as measured by fevers than did native adults. Splenomegally rates among children were also observed to be much higher than rates among adults (31,50). Further observations on indigenous populations found an age dependent expression of this immunity, that is, if children survived they appeared to develop a tolerance to infection despite detectable parasitemia (50). Spleen rates and rates of infection were also found to decrease with age (17,19,62,91) but a predictive pattern of acquired immunity was not observed due to differences in geographic location, parasite species and endemicity (19).

Since these early observations, investigators have worked under relatively consistent conditions by taking advantage of the stable malaria, predominantly P. falciparum, in many regions of Africa. Chloroquine was also 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 led to the identification of a predictive pattern of acquired immunity.

Clinical-epidemiological observations. McGregor (66), consolidated the research done in the past with that of his

own experience in the Gambia, and proposed that acquired immunity could be divided into successive stages, depending upon age and exposure. Since this work, many investigators have confirmed and expanded upon 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 (75). The placenta sequesters a tremendous quantity of parasites (11,39). However, in holoendemic areas, transplacental transmission of infection is rare, but becomes more frequent in areas of lower malaria endemicity (29), suggesting an immune response.

The second stage of immunity is considered to be the passive transfer of protection from the mother to the 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 other age groups of children. Furthermore, when infections do occur, parasitemias are low and symptoms mild (3,11,46,66,72). The nature of such protection is not known, but the period of protection roughly correlates with the duration of maternal antibodies in the infant's serum (15,68). However, the presence of fetal hemoglobin, which is inhibitory to parasite development, is also considered to play a protective role (84).

As the passive immunity declines, susceptibilty increases signalling the third stage of immunity.

Beginning at approximately six 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 (3,11,23,69).

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 (3,46,66,69,73). Finally, a stable immunity is reached sometime between 15 to 30 years (3,19,66,69,73). 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 principal means of elucidating the immune mechanism(s) operating against the disease.

Premunition immunity. The increasing protection seen 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 limitations of diagnosis, adults may carry subclinical or chronic infections which go undetected. Consequently, the degree of immunity is difficult to ascertain. Sergent et al. (92) 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 immuntiy 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 resurgence of disease when control measures were terminated, malaria returned but at a lower incidence than before control measures even though infected mosquito inoculation rates returned to precontrol values. Based on this criterion, the duration of acquired immunity decreases steadily but has been shown to prevail in the absence of infection possibly for up to 11 years (28,34). However, as it is difficult to determine the degree of protection in a population, much less an individual, the duration of protection using this criterion is not easily assessed. More predictable observations have been made from immune individuals who have left malarious regions and were radically cured of the disease and freed 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 (63). Because so little data are available on humans, it is not known whether premunition immunity is the result of maintenance of 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 respnses 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 Sotiriades (94) 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 reductions in both parasitemia and fever. In later experiments, Lorando and Sotiriades (58) 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. (20) 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 hours and some, but not all, recovered without the aid of chemotherapy. Following a similar procedure in Nigeria, Edozian and coworkers (36) 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 (70,113) but serum IgG from Malaysia failed to protect West Africans (24). Human immune IgG was found to partially protect Aotus monkeys from acute falciparum infections (33). From these studies it is apparent that IgG can have significant influence on the course of infection. Since parasitemias decrease, the IgG appears to be affecting the erythrocytic cycle (20,24). 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 be expressing a 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 the 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 immunoglbulin 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 (69). Among adults, it was found that chemoprophylaxis reduced the elevated IgG concentration (41). Cohen and McGregor (21) found that among African adults in malarious areas IgG synthesisand catabolism were nearly seven times those of European controls. This hypergammaglobulinemia was found in all age groups with detectable parasitemias, and IgG concentrations increased directly with spleen size through 15 years of age (69,73). However, only during the first two years were IgM concentrations higher among parasitized children (73). No differences were observed between parasitized and nonparasitized adults (64,73). No consistent relationships could be demonstrated for IgA, IgD or IgE concentrations among any age group (67).

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 (71,106). 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 (35). 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 with spleen size and, although uncommon among African adults, splenomegaly was also correlated to I.F.A. titers here (40). Little is known about IgM I.F.A. titers among malarious populations but, much like IgM serum concentrations in primary infections, IgM titers are the first to appear and gradually diminish while IgG titers rise (26). Sustained IgM I.F.A. titers were found to be a distinctive feature of tropical splenomegaly syndrome (30). 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 (28). Other methods for measuring parasite-specific antibody titers, such as passive agglutination methods using parasite antigens coated on red blood cells, can be sensitive but have not been useful for epidemiological work (109). Some of the more recent enzyme-linked immunosorbent assays (ELISA) have been used successfully and have given results similar to those seen with I.F.A. titers (107). However, with low antibody titers this method does not appear to be as sensitive as I.F.A. (108). 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 (108).

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 of these studies were those of McGregor and Wilson (74). 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 increased until approximately 10 years of age when they became stable (74). 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 (28). 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 (114,115). The importance of this circulating antigen to the maintenance of infection has yet to be confirmed. Schmidt-Ullrich et al. (89) demonstrated antibodies in sera from immune Gambians which react with infected red cell glycoproteins. The ring-infected erythrocyte surface antigen (RESA), MW

155,000 (SDS-PAGE), has also been identified (86). Titers of antibodies to RESA in sera from endemic areas has been shown to be correlated strongly with the inhibition of merozoite invasion by P. falciparum in vitro (86,110,111). However, further studies by other investigators found higher variability and consequent poor predictability of RESA titers for in vitro inhibition (27,25). Several investigators have demonstrated antibodies from individuals from endemic areas to a group of merozoite antigens called pecursors to major merozoite surface antigens (PMMSA), which have SDS-PAGE identification of 185 - 195 kD, (47,48,45). The role of these antibodies which react to the antigens described above in aquired immunity remain to determined .

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 (78). 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 activity have been utilized.

Phillips et al. (87) 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 (116). These early studies were conducted before continuous *in vitro* cultures of *P. falciparum* were achieved, and thus, the degree of serologically induced inhibition could not be assessed reliably. The development of successful culture techniques for continuous *in vitro* propagation of *P. falciparum* developed by Trager and Jensen (98), has provided a much needed tool for the analysis of antiparasitic mechanisms found in human 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 (42,88). Brown and coworkers (7) 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 (8). 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 (9).

The mechanisms through which serum or IgG is acting on the parasite appeared to be via inhibition of merozoite invasion (43,105,110,111,86,10,90). Strain specificity of serum antibodies from immune individuals is well documented and necessitates the use of falciparum strains isolated from the areas of study or strains closely related geographically to the areas of study (105,9,49). In addition to the variations due to strain specificity, some of the observed variations between IgG, *in vitro* inhibition and immunoprecipitation may also be the result of methodology, but this remains to be determined.

Jensen *et al.* (51) 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 and analyzed similarly showed only inhibition of merozoite invasion, with no intracellular growth inhibition activity (53). While further characterization of these two antiparasitic activities are needed, the intracellular growth inhibition found in the serum does not appear to be antibody and appears to be associated with clinical immunity (52). These authors have suggested that the nonantibody, antiparasitic factor associated with intraerythrocytic parasite inhibition may be a product of cell-mediated immunity (52).

Since the studies of Jensen et al. (51), many investigators have reported CFF or CFF-like in vitro inhibition of P. falciparum but there has been considerable variation in the quantitative and qualitative measurements of these activities. This activity has been reported by Butcher et al. (12) and Tharavani et al. (97) in acute falciparum infections from Papua New Guinea (PNG) and Thailand, respectively, and also by Nkuo and Deas (80) in infected individuals in Cameroon. However, further studies by Butcher et al. (13), did not identify any nondialysable CFF-type activity from crossectional studies in Papua New Guinea, but did find CFF activity which was dialyzable. Marsh et al. (65) failed to demonstrate any CFF-type inhibition in sera from the Gambia, in an area with high malaria transmission without chemotherapy or vector control. It has been speculated that genetic, immunological and epidemiological variables may be responsible for this variability (53,102,103).

Cell-mediated immune response. The study of cell-mediated immune responses in man have been severely limited by 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 for a general reduction in peripheral T-cells (117,44,96). Changes in B-cells, null cells and K-cells have been noted, but such changes vary

unpredictably (44,112,117). In studies from Nigeria, Ojo-amaise et al. (82) found a positive correlation between parasitemia and both gamma-interferon and natural killer cell activity in malarious children. Peripheral blood lymphocytes from individuals living in endemic areas proliferate in vitro when exposed to P. falciparum antigens, and the responding cells were T cells (55,118,14). The T cells from clinically immune individuals give a much higher, longer lasting and consistent antigen induced proliferative response than those from individuals with limited malaria exposure or those who are acutely ill, which respond poorly (4,99). It has been proposed that immune individuals may be less susceptible to the suppressive effect of falciparum antigen preparations (101).

The activation of T cells which is found in immune individuals may result in B cell proliferation and consequent antibody production, cytokine secretion, and may also activate macrophages. Interferon gamma is considered a good indicator of cell mediated immunity and is produced in great amounts from T cells in vitro from clinically immune individuals (100), although it has no direct effect upon falciparum cultures (54). Interferon gamma has been shown to activate monocyte derived macrophages which can induce crisis forms when cocultured with P. falciparum in vitro (83), and oxygen-independent mechanisms were indicated consistent with secretion of parasite inhibitory cytokines. These results suggesting cytokine participation

in parasite inhibition may be similar to those seen with the crisis form factor found in malarious sera (51), however, further characterization of these serum factors and inhibitory activities is needed.

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 may represent an antibody-dependent cellular cytotoxicity response to malaria (6,44). Polymorphonuclear leukocytes (neutrophils) and monocytes from nonimmunes were found to ingest infected red cells in culture in the presence of IgG from individuals living in endemic areas (16). Similar opsonization activity was found by Khusmith and Druilhe (56) using nonimmune monocytes and immune IgG, but here, merozoites and not infected cells were ingested. The many studies of antiparasitic activities associated with specific antibodies combined with 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 remain at present obscure.

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CHAPTER ONE

EPIDEMIOLOGICAL AND IMMUNOLOGICAL VARIABLES AFFECTING BOTH QUANTITATIVE AND QUALITATIVE INHIBITION OF *PLASMODIUM FALCIPARUM IN VITRO*.

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ABSTRACT

We have examined the serum of from two distinct populations in Sudan, Africa for antimalarial activity measured *in vitro*, against the erythrocytic cycle. One population was primarily semi-immune from sugar cane fields (SF) in unstable hyperendemic areas near Sennar and the second population was from Sundus Village (SV) near the Ethiopian border, an area holoendemic for malaria. Significant differences in mean values of nearly all parameters measured were observed. Semi-immune sera (SF) contained low, (mean:12.7%) antibody-dependent merozoite invasion inhibition (MII), low IgG IFA titers but high (mean:43.6%) antibody-independent intraerythrocytic inhibition known as crisis form factor (CFF). In contrast, immune sera from SV contained low CFF activity (mean:24.8%) but high (mean:45.6%) MII and IgG IFA titers. The MII and CFF activity, IgG IFA and IgG RESA titers were significantly increased in SF patients who were infected with *Plasmodium falciparum* at the time of sampling compared to those who were not. However, no significant differences were observed with sera from SV patients. Our results suggest that inhibition of the erythrocytic cycle in vitro may characterize qualitatively and quantitatively a facet of naturally acquired immunity to falciparum malaria, and these measurements are thus affected by epidemiological and immunological variables.

INTRODUCTION

The mechanisms responsible for acquired immunity to malaria are not well understood. The *in vitro* assays remain one of means of studying antimalarial immune mechanisms, notwithstanding considerabe variation in both qualitative and quantitative measurements of these mechanisms. Previously, we have studied antimalarial activity of immune sera against the erythrocytic stages and cycle of *Plasmodium falciparum in vitro* and have determined two distinct inhibitory activities (1,2,). One, an antibody mediated mechanism, inhibits the successful invasion of the host red blood cell by merozoites (merozoite invasion inhibition, MII), and the second, a non-antibody, nondialyzable serum component that inhibits intraerythrocytic growth and development of the parasite ultimately yielding crisis-formsknown as crisis form factor (CFF). It has been speculated that this second type of inhibition, CFF, may be a secretory component of cellular immunity. Variation has been reported by several laboratories in the measurement of CFF and similar *in vitro* activity in serum from individuals exposed to or infected with malaria (2,3,4,5,6,7). There have also been several studies which have failed to demonstrate any detectable amounts of CFF-like activity in serum samples from endemic areas (8,9).

The aim of this study was to compare sera collected from epidemiologically distinct populations to determine and describe some variables which may affect the acquired immune response as measured against the erythrocytic cycle of the parasite *in vitro*. Within our logistical confines, in the Sudan, we were able to study and compare two relatively distinct populations: a semi-immune population, transiently exposed to considerable *P. falciparum* challenge and an immune population previously endemic for malaria without known malaria control intervention. From these populations we determined serum IgG and IgM indirect fluorescent antibody (IFA), IgG and IgM ring-infected erythrocyte surface antigen (RESA), merozoite invasion inhibition and CFF activity. We have found significant differences in serum antibodies, antimalarial activities and antimalarial immune profiles between these two epidemiologically distinct populations.

MATERIALS AND METHODS

SERA Serum samples, obtained from peripheral blood were prepared as previously described (2,3).

Sugar Factory (SF) sera were obtained in an area adjecent to sugarcane fields and processing plant, near Sennar, Blue Nile Province. This population lived in an area having extensive irrigation where strict malaria control with chemotherapy and domicile spraying with insecticides had been maintained for several years prior to

our study. However, due to the use of outdated insecticides and unprepared clinical support, at the time the samples were collected, September 1984, the SF area was undergoing a serious falciparum epidemic. Samples were obtained crossectionally from the population which included patients seeking treatment at the local clinic. Of the samples included in this study 35% were infected with P. falciparum at the time of sampling as determinded by Geimsa stained thick and thin films. The principal complaint in nearly all patients was fevers and generalized weakness. There were no other predominant parasitic infections or other malarial species present during this period of sampling as detected by examination of blood, urine and feces.

Sundus Village (S.V.) sera were obtained from individuals living in the north eastern part of the Blue Nile province, several hundred kilometers southeast of Gedaref. This remote area adjacent to the Atbara river near the Ethiopian border is usually holoendemic for malaria and has no control measures and limited availability of antimalarial drugs. Transmission at the time of sampling, October 1984, was unusually low for this area as a result of the extensive drought but prevalence remained elevated due in part to the lack of treatment. In this cross-section, 30% of the individuals were thick-film positive during the sampling, 66% of these cases were P. falciparum and 33% were P. malariae. Interestingly, none of the individuals from this population complained of

fever, malaise nor any other constitutional symptoms typical of malaria. However, nearly all sampled had evidence of infection with Onchocerca volvulus (skin snip and slit-lamp confirmed).

Parasite cultures. Cultures of P. falciparum were maintained in vitro using the candle jar method of Jensen and Trager (10). The P. falciparum strain tested against all of the sera was the FCR-MSU1 previously isolated from the Blue Nile province of Sudan (3). This parasite strain, cultured in O+ red blood cells was used for all assays. Parasite cultures synchronized for all assays by using a combination of sequential 5% sorbitol (11) and 0.5% gelatin floatation methods (12).

Assays for malarial parasite antibody. Antibodies specific for falciparum parasites were determined from each sera for specificity against schizont and RESA antigens using the FCR-MSU1 isolate. Both IgG and IgM serum titers were measured using an appropriate IgG and IgM specific conjugate (Cappel, Organn Teknika Co. and Vector Laboratories). Schizont IFA titers were determined by the method of Hall et al (13), in synchronized parasites and O+ RBCs, as described previously (2,3). Ring-infected erythrocyte surface antigen (RESA) IFA titers were determined by the method of Perlmann et al.(14). IgM RESA IFA titers were the only unique modification to these

established methods and were measured using anti-IgM conjugate.

Merozoite invasion inhibition. Inhibition of merozoite invasion by individual sera was measured as previously described (3). In addition to direct measurement of parasitemia by Giemsa-stained thin films as described, quantitation of parasitemia was also measured by the incorporation of[3H]hypoxanthine. Briefly, synchronized segmenting shizonts are added to uninfected O+ rbcs to a 10% parasitemia, and dispenced into 96-well microtiter plates (Linbro). Each well contained 1.5 ul of cells, 100 ul of RPMI-1640 containing 5% (v/v) pooled nonimmune sera (RP-5) and 25% (v/v) dialysed immune test serum (25-IS). The plates were incubated in a candle jar at 37'C for 4 h, allowing for release of merozoites and subsequent invasion into rbcs, in the presence of test sera. After 4 h, thin films were made from one set of triplicate wells for visual parasitemia determination and a second set of triplicate wells are processed for isotope incorporation. Incorporation of label was accomplished in a stepwise manner. First, 75 ul of the incubation media is removed, without removal of cells, replaced with 5% aqueous sorbitol, and incubated at 37 C for 15 min to lyse remaining, mature parasites. After 15 min, the cells were settled by gentle centrifugation of the microculture plates, and the sorbitol removed by repeated washing with 200 ul/well RP-5 and centrifugation. Finally, the newly

invaded parasites were cultured in RP-5 contaning 2 uCi/well of [3H]hypoxanthine (10-25 Ci/mmole;New England Nuclear) and incubated and 37 C for an additional 30 h; harvested onto glass fiber filters with Bellco Microharvester, and parasitemia determined by liquid scintillation spectrometry. The amount of merozoite invasion inhibition was assessed by comparing the number of newly invaded rbc (thin films) or [3H]hypoxanthine incorporation in nonimmune control sera versus test sera. Inhibition was normalized and expressed as percentage of nonimmune control sera.

Intraerythrocytic inhibition. The inhibition of intraerythrocytic growth and development of the parasite, CFF activity, was measured as described (3). Using cultures synchronized at the young ring stage, the incorporation of [3H]hypoxanthine into parasite nucleic acids reflects parasite development and when morphologically confirmed by thin films can reliably assess intraerythrocytic growth and development. Inhibition of intraerythrocytic growth by the test sera was determined bycomparing the incorporation of labeled nucleic acids by parasites cultured in nonimmune control sera versus sampled test sera. Inhibition of parasite development was confirmed morphologically by equivalent Giemsa-stained thin films. Inhibition was normalized and expressed as percentage of nonimmmune control sera.

RESULTS

A comparison of Sugar Factory and Sundus Village sera for malaria specific antibodies and antimalarial activities *in vitro* is presented in **Table 1.** The number of samples, mean and range are given for each parameter measured and mean values were statistically different for all parameters between the two populations except age ($P<0.05$. Student's t test). Sera collected from residents living near the Sugar Factory were characterized by low IgG and IgM IFA and RESA endpoint dilutions, poor inhibition of merozoite invasion but high inhibition of intraerythrocytic growth (CFF activity). In contrast, sera from Sundus Village were characterized by relatively high IgG and IgM IFA and RESA endpoint dilutions, high inhibition of merozoite invasion but low inhibition of intraerythrocytic growth.

In a further attempt to characterize and compare the antimalarial immune profiles between these two populations, correlation coefficients between the antimalarial parameters measured were determined. The correlation coefficients for the SF sera samples were determined and summarized in **Table 2.** Significant correlations existed between IgG and IgM IFA ($P<0.001$), IgG and MII ($P<0.001$), IgM and MII ($P<0.05$), IgG RESA and MII ($P<0.05$) and between MII and CFF ($P<0.001$). In comparison, **Table 3** shows the same correlations made between the antimalarial parameters from the SV sera samples. In SV sera, significant

Table 1. Comparison of Sugar Factory and Sundus Village sera, malaria specific antibodies and antimalarial activities *in vitro*.

	Sugar Factory			Sundus Village			
	n	mean	range	n	mean	range	significance[e]
IgG IFA[a]	112	1.80	0-8	44	5.70	1-9	P<0.001
IgM IFA[a]	112	0.70	0-6	44	2.00	0-6	P<0.001
IgG RESA[b]	108	1.79	0-11	43	4.14	0-9	P<0.001
IgM RESA[b]	108	0.55	0-7	43	1.86	0-7	P<0.01
MII (%)[c]	112	12.71	-30.3-74.9	44	45.60	21.1-79.4	P<0.001
CFF (%)[d]	112	43.60	-17.1-95.7	44	24.80	-6.1-78.4	P<0.001
Age (years)	110	20.75	6-70	34	28.50	11-76	NS

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
[e]Student's t-test used to determine levels of significance between means.
NS = not significant, P>0.05.

Table 2. Correlation coefficients between parasite specific antibodies and antimalarial activities in vitro.

Sugar Factory Sera

	IgG[a] IFA	IgM[a] IFA	IgG[b] RESA	IgM[b] RESA	MII[c]	CFF[d]
IgG IFA	-	0.704*	0.622*	-0.003	0.458*	0.090
IgM IFA	-	-	0.015	0.198	0.246*	-0.020
IgG RESA	-	-	-	-0.016	0.279*	-0.013
IgM RESA	-	-	-	-	-0.012	-0.152
MII	-	-	-	-	-	0.382*

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
*P<0.05 (Student's t-test).

Table 3. Correlation coefficients between parasite specific antibodies and antimalarial activities in vitro.

Sundus Village

	IgG[a] IFA	IgM[a] IFA	IgG[b] RESA	IgM[b] RESA	MII[c]	CFF[d]
IgG IFA	-	0.680*	0.527*	0.317*	-0.176	-0.540*
IgM IFA	-	-	0.442*	0.060	-0.288	-0.241
IgG RESA	-	-	-	-0.214	-0.046	-0.353*
IgM RESA	-	-	-	-	-0.211	-0.035
MII	-	-	-	-	-	0.079

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[b]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
*P<0.05 (Student's t-test).

correlations were determined between IgG and IgM IFA ($P<0.001$), IgG IFA and IgG RESA ($P<0.001$), IgG IFA and IgM RESA ($P<0.05$), IgG RESA and IgM IFA ($P<0.01$), a negative correlation between IgG IFA and CFF ($P<0.001$), negative between IgG RESA and CFF ($P<0.05$).

Samples from each population were subdivided into patients with known active malarial infections and those who were not thick film positive at the time of sampling. In **Table 4**, the comparisons between malaria-infected versus uninfected individuals from the two populations for IgG and IgM IFA and RESA antibodies, merozoite invasion inhibition and CFF activity. Significant differences in mean values between noninfected and infected were found for IgG IFA ($P<0.05$), IgG RESA ($P<0.01$), MII ($P<0.001$) and CFF ($P<0.05$) from the Sugar Factory samples. In contrast, the equivalent samples from Sundus Village showed no significant differences between the noninfected and infected with the exception of IgM RESA ($P<0.05$). **Figure 1** and **Figure 2** graphically demonstrate the distribution of MII and CFF, respectively, in nonnfected and infected from Sugar Factory sera.

Table 4. Comparison between malaria infected and noninfected serum antibodies and antimalarial activities from Sugar Factory and Sundus Village sera.

	Sugar Factory			Sundus Village		
	mean noninfected	mean infected	signif.[e]	mean noninfected	mean infected	signif.[e]
IgG IFA[a]	1.51	2.20	P<0.05	6.47	6.00	N.S.
IgM IFA[a]	0.68	0.81	N.S.	2.16	1.77	N.S.
IgG RESA[b]	1.54	2.39	P<0.01	4.74	3.11	N.S.
IgM RESA[b]	0.65	1.23	N.S.	1.89	0.22	P<0.05
MII (%)[c]	5.71	27.40	P<0.001	46.62	48.60	N.S.
CFF (%)[d]	40.28	52.21	P<0.05	24.22	15.11	N.S.

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
[e]Student's t-test, N.S. = not significant P>0.05.

Figure 1. The comparison between malaria infected and noninfected individuals from the Sugar Factory area for serum merozoite invasion inhibition *in vitro*. Means for the two groups represented by dashed line (------). Inhibition of merozoite invasion determined by the reduction of ring stages from synchronized segmenting schizonts incubated in 25% immune as compared to 25% nonimmune control sera. All data points from each serum sample represent mean inhibition values from 3 experiments (S.D. ±10% inhibition).

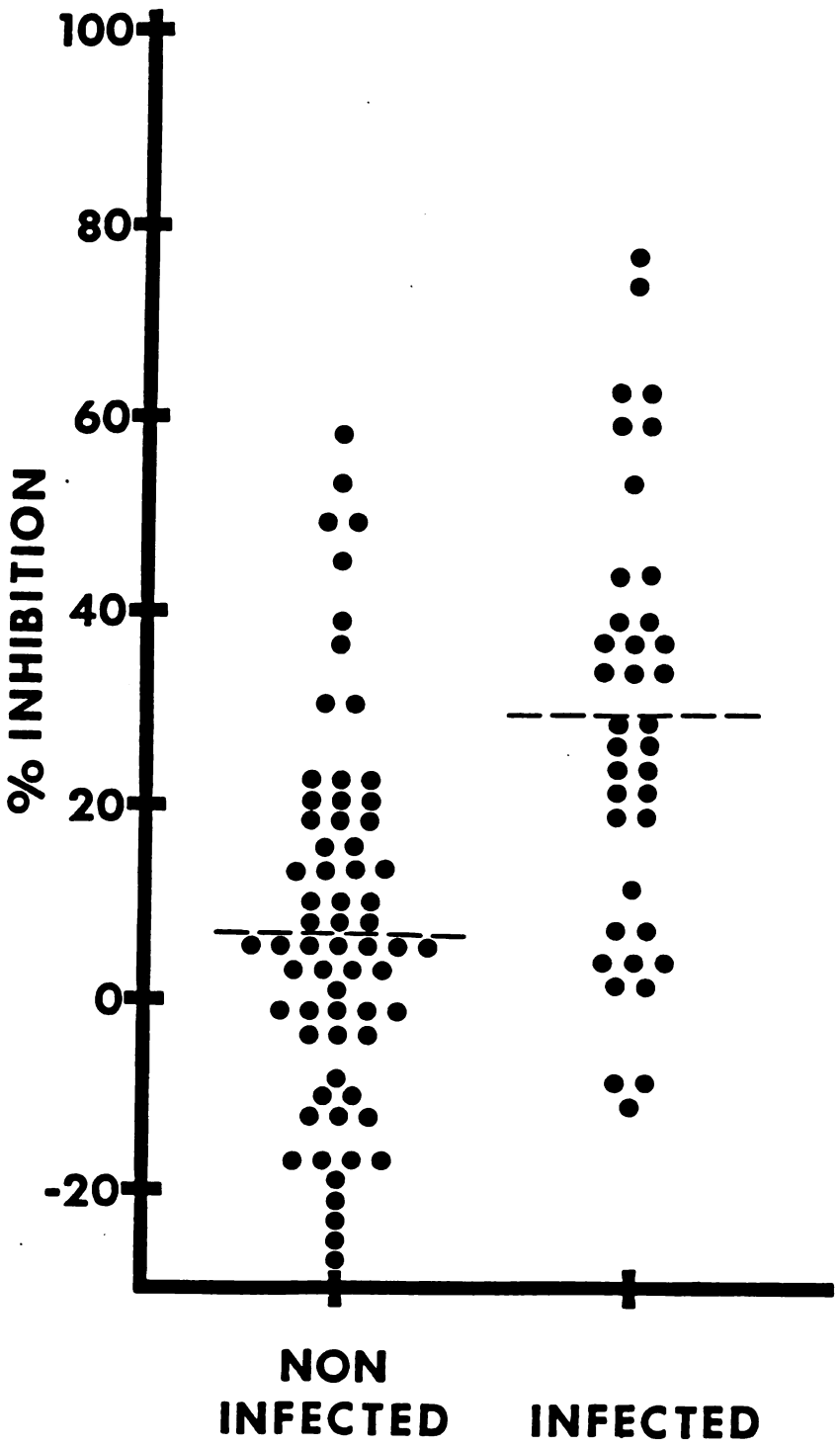
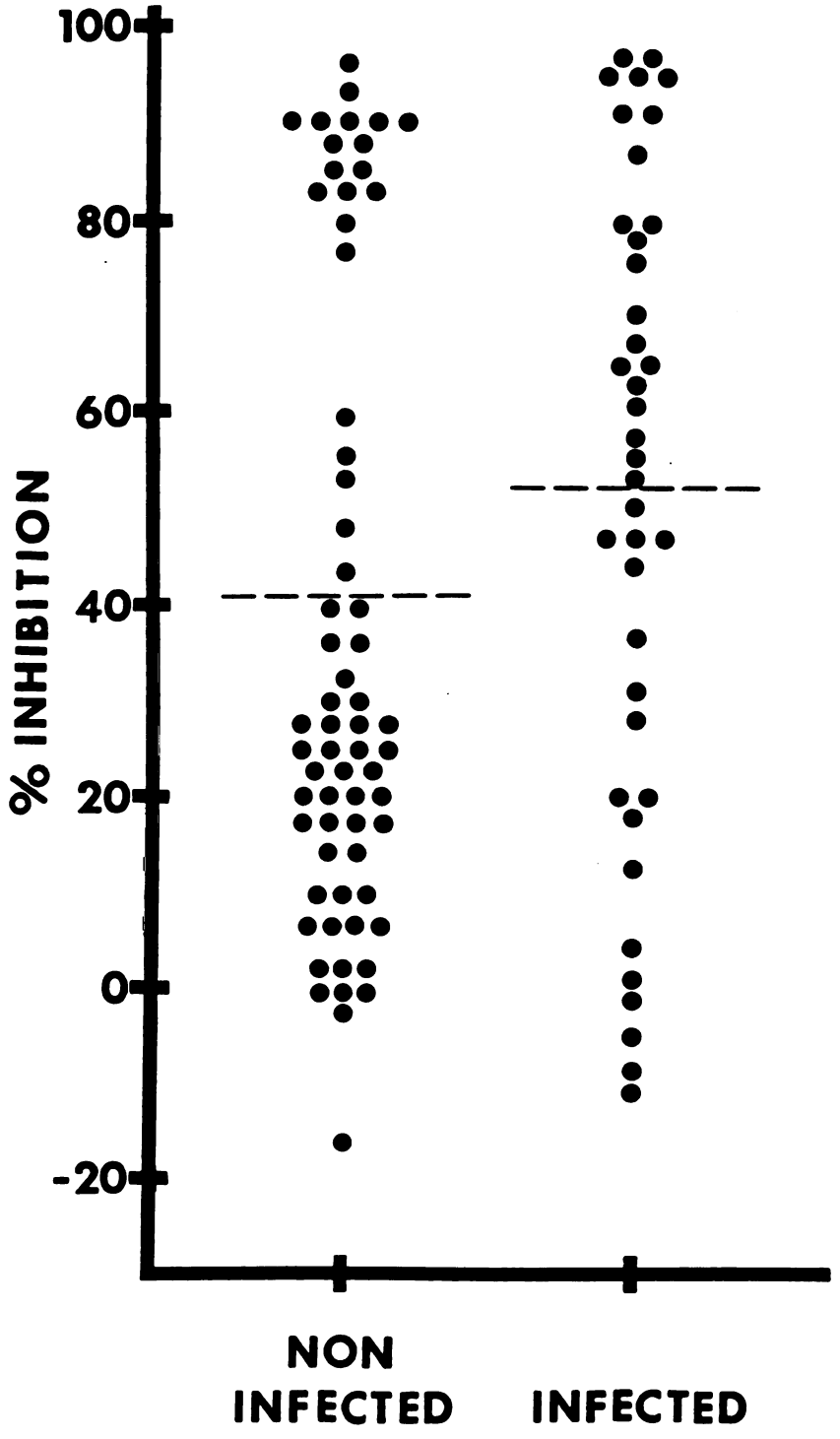

100
80
60
40
20
0
-20
% INHIBITION
NON INFECTED
INFECTED

Figure 2. The comparison between malaria infected and noninfected individuals from the Sugar Factory area for serum CFF activity *in vitro*. Means for the two groups represented by dashed line (------). Inhibition of intraerythrocytic development, CFF, determined by the reduction of [3H]hypoxanthine incorporation into synchronized parasites cultured in 25% immune as compared to 25% nonimmune sera. All data points from each serum sample represent mean inhibition values from 3 experiments (S.D. ±10% inhibition).


100
80
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NON INFECTED
INFECTED

DISCUSSION

We have compared and contrasted the naturally acquired immunity to malaria, as measured *in vitro*, between two epidemiologically different populations from Sudan. We have found significant differences in malaria specific antibodies and antimalarial activities between these two populations.

The two populations studied differed greatly with respect to falciparum transmission, endemicity, malaria control efforts and immune status. Although the area of Sennar surrounding the Sugar Factory is usually hyperendemic for malaria, the SF populations immune status may be considered poorer than one would expect for this endemicity due to the influence and of previously well maintained malaria control measures and chemotherapy. Due to an unfortunate combination of circumstances, this poorly immune population was experienceing a severe malaria epidemic at the time of our study. Consequently, the SF samples may represent an incomplete or immature immunity that was boosted by abrupt *P. falciparum* transmission and immune challenge. In contrast, the SV samples may represent complete or mature acquired immunity to falciparum malaria that is usually under persistent malaria challenge, however, at the time of our study there was little transmission and the infections we observed most likely were long standing. Unlike the SF samples, the

acquired immunity of the SV samples had not been attenuated by control or chemotherapy. The Sundus area was also endemic for P. malariae and O. volvulus, two potentially important factors to consider, however, the effect of these additional infections upon the acquired immunity is unknown. In addition, there were some genetic differences between the two populations, which cannot be ruled out as a contribution to the observed differences, although these populations were not as genetically diverse as those compared from Sudan and Indonesia as described by Jensen et al (8).

In addition to the epidemiological characteristics, our speculation that the SF samples may represent an incomplete immunity is further supported by the antimalarial immune profile, where IFA, RESA and antimerozoite activity of the sera is remarkably low, consistant with low levels of parasite exposure. In contrast, the acquired immunity of the SV samples was reflected by relatively high antimalarial antibody as determined by IFA and RESA and merozoite invasion inhibition activity, consistant with persistant parasite endemicity. An equally important and differentiating component of the antimalarial immune profile was the CFF activity, a retardation of intraerythrocytic development. The SF samples, with their apparently incomplete immunity, demonstrated, as an acutely challenged population, high CFF activity, consistant with previous studies with similar epidemiology (1,2,3). Interestingly, the S.V. samples with their more mature

acquired immunity had as a population little intraerythrocytic inhibition activity. We hypothesize that these distinct antimalarial immune profiles may represent different stages in the process of acquiring immunity to malaria. The incomplete immunity of the primarily adult population of SF may be similar to the preadolescent immunity in endemic areas such as SV. Our study included few children and we could not demonstrate any age-dependent differences in the immune profiles from these populations. Preliminary evidence from other studies have demonstrated such an age-dependent relationship between antibody, CFF and acquired malaria immunity (manuscript in preperation).

The parasitological and immunological specificity of the antimalarial immune profiles and the differences between these two populations are further reflected in the comparisons between malaria-infected and noninfected groups within each population. The SF samples showed significantly elevated antiparasite activity, both merozoite inhibition and intraerythrocytic inhibition, in the infected group. It may be that the incomplete immunity, as indicated by the low parasite exposure and low antibody response, when challenged responds with a CFF-type activity because the antibody mediated antimalarial activity may not be sufficient to control the pathogenic process. As a consequence, CFF-type activity may be elicited as an acute inflamatory protective response in the host. This antimalarial response to infection may result from specific immune challenge or be a component of a

nonspecific acute phase type reaction, the relative importance of either response remains unknown at present.

In contrast, no significant differences in antiparasite activity between infected versus noninfected groups were measured from the SV samples. All parameters of immunity except intraerythrocytic inhibition were elevated, independent of infection with malaria. This lack of measurable response to infection in the SV population may reflect the relative maturity of their acquired immunity. It may be that the lack of intraerythrocytic inhibition in this immune population may be a consequence of their degree of immunity sufficient to prevent malaria symptoms, despite infection, in contrast to the symptomatic infections seen at the Sugar Factory. These observations suggest that CFF may not be a component of the acquired immune response but a product of an inflamatory mechanism triggered by infection in the non- or semi-immune individuals. Additional studies will be required to resolve the inductive mechanisms, their relative importance and other contributing factors, such as concomitant O. volvulus and P. malariae.

Lastly,the data from this study clearly demonstrate the different presentations of immunologic responses to malaria between these two populations, as measured in vitro. The quantitative and qualitative differences in antimalarial activity found in the sample sera between these populations is easily appreciated. Of particular interest is the difference in relative amounts of intraerythrocytic

inhibition activity as discussed above. The epidemiological variables presented most likely account for the contrasting immune reponses and similar differences in this intraerythrocytic activity reported by various authors in sera from immune populations which differ epidemiologically as well as geographically. There have been several authors who have measured the acquired immune response to P. falciparum in vitro and have measured quantitatively CFF or CFF-like intraerythrocytic inhibition. This activity was found by Butcher et al(4) and Tharavani et al(6) in acute falciparum from Papua New Guinea (PNG) and Thailand respectively, Nkuo and Deas (7), in Cameroon also from infected individuals. However, Butcher et al (5) failed to identify any nondialysabe CFF-type activity from crossectional studies in PNG which is similar to the findings of Jensen et al (8) in Indonesia. Marsh et al (9), could not demonstrate any CFF-like inhibition in sera from the Gambia, in an area with high malaria transmission without chemotherapy or vector control, consistant with our findings from SV.

The differences in demonstrating CFF-type inhibitory activity is apparent from these studies. Such differences between studies may be due to the differences in epidemiology, immunology, parasitology and possibly genetic differences. Unfortunately, the nature of these types of studies and logistical confines limiting controls greatly reduces the ability to make direct comparisons between studies. The role of the CFF-type inhibition in acquired

immunity remains uncertain. Further studies aimed at controlling for the multiple variables described would aid considerably in our understanding of this inhibitory activity. In addition to further epidemiological and serological studies, the identification of the CFF-like component(s) and development of more sensitive assays would greatly improve our ability elucidate further the variables contributing to the development of naturally acquired immunity to malaria.

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CHAPTER TWO

A COMPARISON OF PLASMODIUM FALCIPARUM IN VITRO MEROZOITE INVASION INHIBITION AND CRISIS-FORM ACTIVITY IN MATERNAL VERSUS PAIRED UMBILICAL CORD SERA FROM SUDAN.

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ABSTRACT

The components of passive protection from malaria conveyed by mother to newborn are not completely understood. Antibody mediated mechanisms, such as merozoite invasion inhibition, and cell-mediated mechanisms such as crisis-form activity, have been demonstrated. This study compares the relative contribution of each of these mechanisms in maternal and umbilical cord sera. Sera were obtained from individuals living near Damazin, Sudan, an area with unstable, hyperendemic Plasmodium falciparum malaria. Serum samples were examined for anti-schizont IFA, RESA, in vitro merozoite invasion inhibition and crisis-form activity measured against FC-MSU1, a strain isolated from the same province. The results of this study showed a significant correlation between maternal and paired umbilical cord sera for IgG IFA (r=0.960), IgG RESA (r=0.810), and merozoite invasion inhibition (r=0.715). Mean crisis-form activity in maternal sera was 60% (±19 %) inhibition as compared to 10% (±18 %) inhibition for umbilical sera. No significant correlation between maternal and paired umbilical cord sera was found for crisis-form activity (r=-0.149). These results confirm previous reports of the importance of merozoite invasion inhibition in passive protection and suggest that crisis-form activity may not cross the placental barrier and does

not contribute to the passive protection in this population.

INTRODUCTION

The passive transfer of protection to malaria in the newborn has been clinically and epidemiologically well documented and the period of protection roughly correlates to the duration of maternal antibodies (1,2,3,4). However, the antimalarial mechanisms through which the mother confers her naturally acquired immunity to her newborn is not completely understood. Studies on the antimalarial activity of paired sera are limited (5,). In past investigations, we have studied the antimalarial activity of immune sera from adults against P. falciparum in vitro and have measured two distinct inhibitory activities (8,9). One, an antibody mediated mechanism, merozoite invasion inhibition (MII); the second is a non-antibody, nondialyzable serum component that inhibits intrerythrocytic growth and development of the parasite called crisis-form factor (CFF). We have also demonstrated this activity in several cord sera taken from semi-immune indivdiuals, living in unstable, hyperendemic malarious areas (9). It has been speculated that this nonantibody inhibition may result from a secretory component of cellular immunity and might also contribute to the protection of the newborn (9). No other studies to date have examined the antimalarial activities of paired maternal and cord sera against P. falciparum in vitro, although several studies have demonstrated nearly

equivalent levels of antimalarial antibodies in maternal and paired cord sera (5,6,7).

The aim of this study was to examine some of the elements of passive transfer of maternal immunity to the newborn by determining the antimalarial activities in *in vitro* inhibition assays found in paired maternal and umbilical cord sera at parturition. We have studied a population living in an unstable hyperendemic area for *P. falciparum* in Damazin, Blue Nile Province, Sudan. Our findings demonstrate a strong correlation between maternal and cord antimalarial antibodies, indirect fluorescent antibody titers (IFA) and ring-infected erythrocyte surface antigen (RESA), and inhibition of merozoite invasion *in vitro*. However, there was little evidence to support an apparent transfer of CFF-type activity from mother to newborn in this population.

MATERIALS AND METHODS

Sera. Serum samples were obtained from individuals living in areas in or near Damazin, located in the southern part of the Blue Nile province in Sudan. This is an area hyperendemic and unstable for falciparum malaria which usually increases in transmission following the raining season in July through October. However, at the time of sampling, November and December, there had been no seasonal rains due to the drought and as a consequence malaria transmission was greatly reduced.

Cord samples were collected at the time of parturition and the matching maternal sample collected within 24 hrs post-delivery by venopuncture. The geometric mean age of this maternal population was 25.91 with a range of 17 - 42 years. The geometric mean number of prior pregnancies in this population was 4.58 with a range of primagravida to 13 previous pregnancies. All serum samples were identically prepared as in previous studies (9,10).

Parasite cultures. Cultures of P. falciparum were maintained in vitro using the candle jar method of Jensen and Trager (11). The FCR-MSU1 strain of P. falciparum, previously isolated from the Blue Nile province of Sudan, was used for all assays (10) and cultured in O+ red blood cells from the same nonimmune donor. Cultures were synchronized using a combination of sequential 5% sorbitol (12) and 0.5% gelatin floatation methods (13).

Schizont and RESA IFA. Serum IFA titers were determined from each serum sample for antimalarial specificity to schizont and RESA antigens. Both IgG and IgM serum titers were determined using anti-IgG and anti-IgM specific conjugates (Cappel,Organin Teknika Co. and Vector Laboratories). Schizont IFA titers were determined by the method of Hall et al (14), in synchronized parasites as described previously (10). Ring-infected erythrocyte surface antigen (RESA) titers were determined by the method of Perlmann et al (15).

Merozoite invasion inhibition. Inhibition of merozoite invasion by prepared sera was measured according to the methods previously described (10). In addition to direct measurement of parasitemia by Giemsa stained thin films as described, quantitation of parasitemia was measured by incorporation of [3H]-hypoxanthine according to the method of Vande Waa et al (17). Briefly, merozoite invasion inhibition was determined by allowing synchronized schizonts to release merozoites in the presence of 25% (v/v), followed by the removal of remaining schizonts and determining the amount of successful invasion by comparing [3H]-hypoxanthine incorporation and confirmed by thin films, in nonimmune control sera versus immune sera. Inhibition was normalized and expressed as percentage of control sera values from three experiments.

Intraerythrocytic inhibition. The inhibition of intraerythrocytic growth and development of the parasite, CFF activity, was determined as described (10). Using synchronized cultures, the incorporation of [3H]-hypoxanthine into parasite nucleic acids correlates with parasite development, and can be used as an index of inhibition of intraerythrocytic growth in the presence of immune serum samples. Thus, CFF activity was determined by comparing the incorporation of [3H]-hypoxanthine into nucleic acids by parasites cultured in nonimmune control sera versus imune sera and confirmed by equivalent Giemsa

stained thin films. Inhibition was normalized and expressed as percentage of nonimmune control sera.

RESULTS

A summary of the measurable antimalarial activity from the serum samples is presented in **Table 1**, where quantitation of malaria specific antibodies and antimalarial activities *in vitro* from maternal and umbilical cord sera are compared. There was no significant difference between mean maternal and cord serum values of IgG IFA and IgG RESA antimalarial antibodies or between merozoite invasion inhibition activity ($P<0.05$, Student's t-test). Both IgM IFA and IgM RESA antibodies to the FCR-MSU1 falciparum strain were demonstrated from the maternal serum in this population sampled. However, no antimalarial IgM antibodies were found in the cord sera samples. Inhibition of intraerythrocytic growth, CFF activity, was demonstrated in both maternal and cord sera in this population and the mean value for this activity was significantly greater in the maternal sera ($P<0.001$).

In order to further characterize the antimalarial profile of the maternal sera, correlation coefficients between the antimalarial parameters measured were determined. In **Table 2** the correlation coefficients between parasite specific antibodies and antimalarial actvities from maternal sera are summarized. Significant correlations were demonstrated between all of the IFA and

Table 1. Comparison between maternal and cord serum malaria specific antibodies and *in vitro* antimalarial activities.

	Maternal sera			Cord sera			
	n	mean	range	n	mean	range	significance[e]
IgG IFA[a]	39	3.18	1-8	39	2.82	1-6	N.S.
IgM IFA[a]	39	1.26	0-5	39	0.00	0-0	-
IgG RESA[b]	38	2.24	0-11	38	1.34	0-9	N.S.
IgM RESA[b]	38	0.97	0-6	38	0.00	0-0	-
MII (%)[c]	39	43.01	13.00-71.70	39	38.72	1.00-74.40	N.S.
CFF (%)[d]	39	59.03	20.40-89.01	39	10.56	-18.00-66.72	P<0.001
Age (years)	22	25.91	17-42				

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
[e]Students t-test used to determine levels of significance between means.
NS = not significant, P>0.05.

Table 2. Correlation coefficients between parasite specific antibodies and antimalarial activities *in vitro*.

Maternal sera

	IgG[a] IFA	IgM[a] IFA	IgG[b] RESA	IgM[b] RESA	MII[c]	CFF[d]
IgG IFA	-	0.793*	0.469*	0.439*	0.268	-0.349*
IgM IFA	-	-	0.564*	0.778*	0.179	-0.299
IgG RESA	-	-	-	0.604*	-0.012	-0.222
IgM RESA	-	-	-	-	0.168	-0.329*
MII	-	-	-	-	-	0.251

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
*$P<0.05$ (Student's t-test).

RESA antibodies measured ($P<0.05$). No statistically significant correlation could be found between the malaria specific antibodies and merozoite invasion inhibition. In comparison, there was a significant negative correlation between IgG IFA and CFF activity and also between IgM RESA and CFF activity ($P<0.05$).

To further characterize the antimalarial profile of the cord sera, the correlation coefficients between parasite specific antibodies and antimalarial activities are presented in **Table 3.** Significant correlations were demonstrated between IgG IFA and IgG RESA, however there was also significance between IgG IFA and merozoite invasion inhibition ($P<0.05$), not found in the maternal sera.

Table 4 shows the correlation coefficients between maternal and paired umbilical cord sera for parasite specific antibodies and antimalarial activities. Consistent with a transplacental transfer of immunoglobulin, there was nearly a one to one relationship between maternal and cord serum IgG IFA, IgG RESA and merozoite invasion inhibition activity ($P<0.001$).

Figure 1 shows graphically the distribution and relationship between maternal and paired cord sera merozoite invasion inhibition. There was no detectable transfer of IgM IFA or IgM RESA in any of the cord sera samples. There was no significant correlation between maternal and cord CFF activity, ($P>0.05$), r -0.149). For

Table 3. Correlation coefficients between parasite specific antibodies and antimalarial activities in vitro.

Cord Sera

	IgG[a] IFA	IgM[a] IFA	IgG[b] RESA	IgM[b] RESA	MII[c]	CFF[d]
IgG IFA	-	0	0.462*	0	0.495*	0.023
IgM IFA	-	-	0	0	0	0
IgG RESA	-	-	-	0	0.143	0.184
IgM RESA	-	-	-	-	0	0
MII	-	-	-	-	-	-0.204

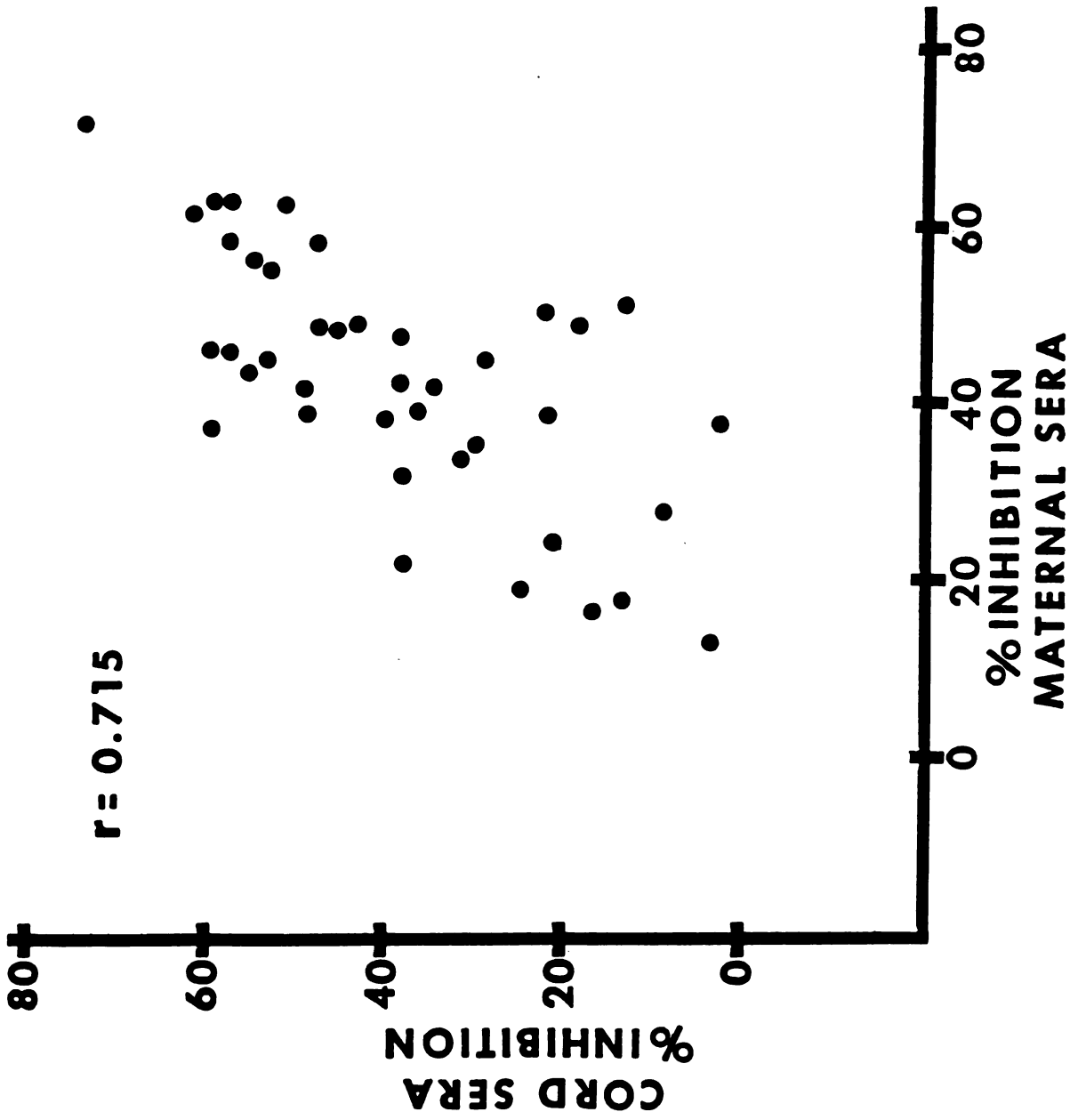
[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
*P<0.05 (Student's t-test).

Table 4. Correlation coefficients between maternal and cord sera parasite specific antibodies and antimalarial activities.

Maternal	Cord	r	Significance*
IgG IFA[a]	IgG IFA	0.960	P<0.001
IgM IFA[a]	IgM IFA	0	
IgG RESA[b]	IgM RESA	0.810	P<0.001
IgM RESA[b]	IgM RESA	0	
MII[c]	MII	0.715	P<0.001
CFF[d]	CFF	-0.149	P>0.05

[a]Endpoint dilutions, negative = <1, 1 = 1:20 dilution.
[b]Endpoint dilutions, negative = <1, 1 = 1:5 dilution.
[c]Merozoite invasion inhibition, % inhibition of nonimmune sera.
[d]CFF activity, % inhibition of intraerythrocytic growth of nonimmune sera.
*Student's t-test.
r = correlation coefficient.

Figure 1. Relationship between paired maternal and cord sera for merozoite invasion inhibition (MII).


r= 0.715
80
60
40
20
0
CORD SERA
%INHIBITION
0
20
40
60
80
%INHIBITION
MATERNAL SERA

comparison, Figure 2 shows the distribution and lack of any relationship between maternal and cord CFF activity.

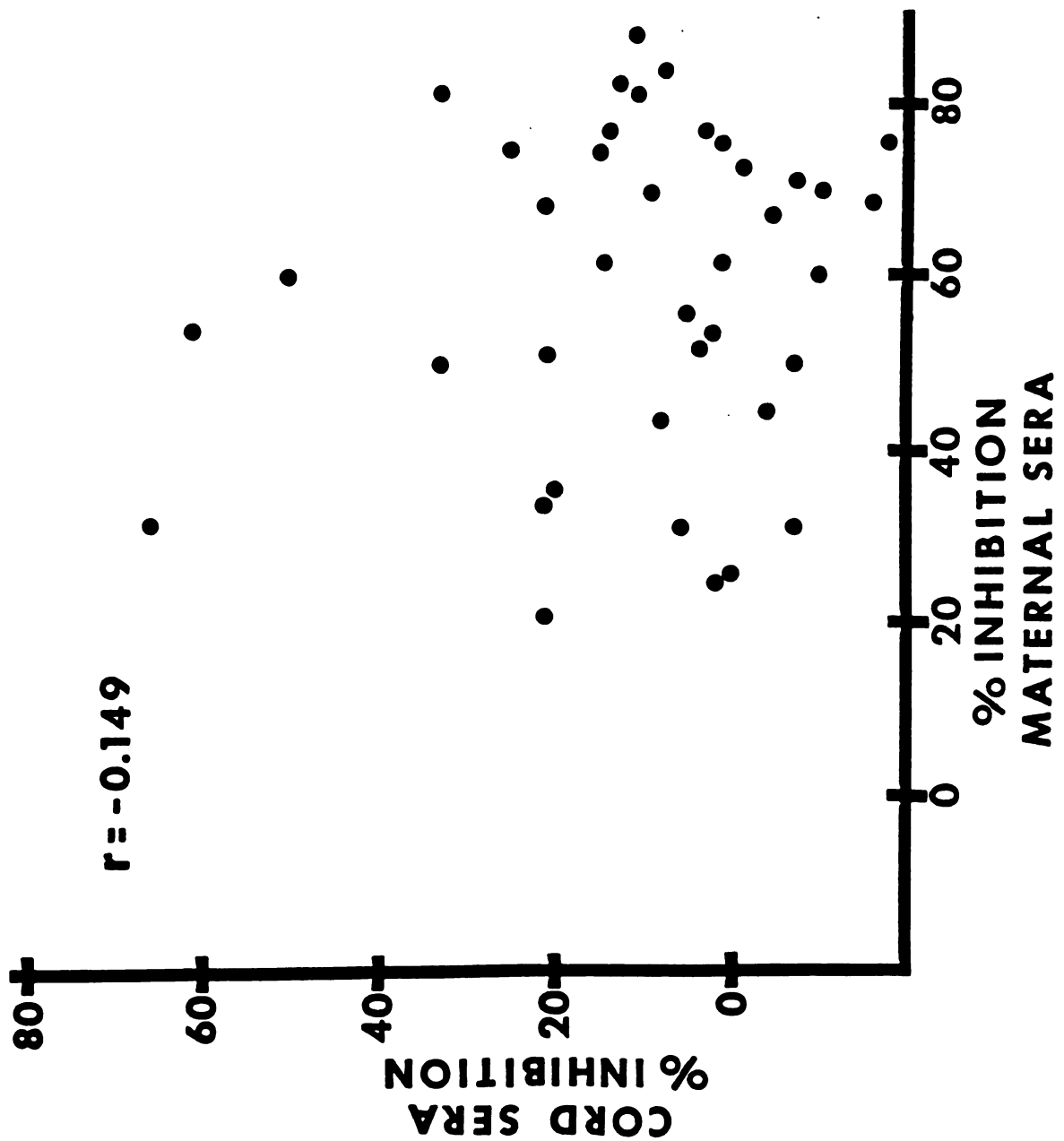
DISCUSSION

We have examined antimalarial antibodies and antiparasitic activity *in vitro* in maternal and paired umbilical cord sera from individuals living in an area of hyperendemic, unstable falciparum malaria. Our data demonstrate both strong similarities and distinct differences in the antimalarial immunity profile between maternal and paired umbilical cord sera which may indicate some components of passive protection transmitted to the newborn.

The maternal serum was characterized by moderate titers of IgG and IgM antibodies to falciparum parasites as determined by IFA and RESA assays. These sera also had moderate to high merozoite invasion inhibition (MII). These indices are somewhat higher but consistent with our previous findings in adults from other hyperendemic, unstable malarious regions in Sudan (9,10,17).

In addition to the substantial MII activity there was also high CFF activity in these sera, a result which differs from our previous findings (9,10,17). However, the relationship between specific antibodies and antimalarial activities for the maternal sera was similar to previous findings (9,10). There was no significant correlation between antibodies and merozoite invasion inhibition,

Figure 2. Relationship between paired maternal and cord sera for inhibition of intraerythrocytic development (CFF activity).


r = -0.149
CORD SERA
% INHIBITION
80
60
40
20
0
% INHIBITION
MATERNAL SERA
0
20
40
60
80

however, there was a significant negative correlation between IgG IFA and CFF activity, a relationship which have we have found similar populations (10,17). Unfortunately, we were unable to obtain sera from non pregnant adults to determine if this high MII with high CFF is unique to this pregnant population or characteristic of the general population in this area. This apparent deviation from our previous findings requires further investigation.

As expected from the maternal sera the cord sera were similarly characterized by moderate IgG antimalarial antibody titers and moderate to high merozoite invasion inhibition. In contrast to the maternal sera, there was no detectable antimalarial IgM antibody, and virtually no CFF activity. There was a significant positive correlation between IgG IFA and merozoite invasion inhibition which was not seen in the maternal sera. There are many possible differences between maternal and cord sera, the lack of CFF activity and IgM may contribute to the improved predictive value of IgG IFA for merozoite invasion inhibition in ways to be determined.

The correlation between maternal and paired cord sera antimalarial components and activities can be an indication of maternal-newborn passive transfer. All IgG antibodies measured were nearly identical between maternal and paired cord sera. Merozoite invasion inhibition was also highly correlated but somewhat less than the IgG antibodies. These observations are consistent with numerous previous studies which demonstrated the passive transfer of IgG and

antibody mediated antimalarial activities (6,7). Although the CFF activity was high in this gravid adult population, there was no significant correlation between maternal and cord sera levels. These data suggest that in this population CFF activity probably does not cross the placental barrier and consequently does not contribute to the passive protection of the newborn. Why this factor does not cross the placenta may be revealed by further characterization of this serum factor. Interestingly, several cord sera were found to contain some CFF activity as reported previously (9) and whether or not these sera are indicating a prenatal response by the neonate needs to be further examined.

Our results confirm previous studies that maternal antibodies, IgG, are passively transfered to the newborn (3,6,7). We have demonstrated for the first time that antibody-mediated antimalarial activities meassured in vitro are are similarly transfered. In this adult population the predominant antimalarial activity in vitro was CFF and in contrast to the antibody response, this activity was not found in the paired cord sera and did not appear to cross the placental barrier. Consequently, in the absence of any endogenous production of CFF activity by the newborn, the passive protection provided is likely confined to a limited antibody mediated antimalarial mechanisms. We would anticipate that in areas holoendemic for falciparum malaria, where antibody-mediated responses are the predominant antimalarial activity and the passive

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