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Longitudinal Study on the In Vitro Immune Response to *Plasmodium falciparum* in Sudan

JOHN A. VANDE WAA,[1] JAMES B. JENSEN,[1]* MUSTAFA A. S. AKOOD,[2] AND RIAD BAYOUMI[3]

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101,[1] and Malaria Control Division, Ministry of Health,[2] and Department of Biochemistry, Faculty of Medicine, University of Khartoum,[3] Democratic Republic of Sudan

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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 threefold, in wet-season sera as compared with dry-season sera. Merozoite invasion inhibition activity was moderate and did not change seasonally. Merozoite invasion inhibition was, however, correlated to parasite-specific immunoglobulin G titers and total serum immunoglobulin G concentrations. These results confirm earlier studies which demonstrate two antimalarial activities in Sudanese sera and provide evidence that intraerythrocytic parasite retardation activity plays a role in antimalarial immunity.

Acquired immunity to malaria in the human host is well documented but not well understood (20, 22). Evidence is available suggesting that both humoral (5, 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 and Jensen (23), provides a means of analyzing serum antimalarial components and mechanisms of this immunity. Data from in vitro inhibition assays substantially improve our understanding of malaria immunology, heretofore based on clinical observations (17).

It was previously shown that both purified immunoglobulin G (IgG) and a non-immunoglobulin serum component, found in persons living in malarious endemic areas of Sudan, inhibited *P. falciparum* in vitro (11). The latter component has been termed crisis form factor (CFF), since it retards intraerythrocytic parasite development generating typical crisis forms in vitro (10). Thus, sera from malarious regions in Sudan demonstrate two distinct antimalarial activities, merozoite invasion inhibition, which has been demonstrated to be antibody dependent (4), and intraerythrocytic parasite retardation, an activity suspected to be mediated through non-antibody mechanisms or components (11). 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 (DS) and malarious wet season (WS), 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 to 66 years old, and residents of the Blue Nile Province, Sudan. In this area, malaria is hyperendemic but unstable. Malaria incidence during DS, particularly in early June, is almost undetectable except in young children in which the incidence is ca. 1 to 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 *Plasmodium vivax* and *Plasmodium malariae*. Samples were collected from Sennar army camp and the local villages of Sheihk Talha, Um Shoka, Wad Hashim, and Ismail, all within 80 km 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 DS, June, 1982, and the second sample after the peak of transmission in October or November, 1982. A third sample was obtained from 19 subjects during the following DS, June, 1983. Blood samples were taken by venipuncture in siliconized vacutainers (Becton-Dickenson), allowed to clot, refrigerated overnight at 4°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- to 12,000-molecular-weight-cutoff membranes (Spectropor), first in 0.015 M phosphate-buffered saline, pH 7.2, for two 1:100 dilutions and finally for a 1:100 dilution in RPMI 1640 medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES) and 0.21% sodium bicarbonate; dialysate was changed at 24-h intervals. After dialysis, the paired sera were filter sterilized through 0.45-μm pore membranes (Schleicher & Schuell) and heat inactivated at 56°C for 30 min before testing for antiparasitic activity.

Parasite strain. The paired sera were all tested for antipar-

* Corresponding author.

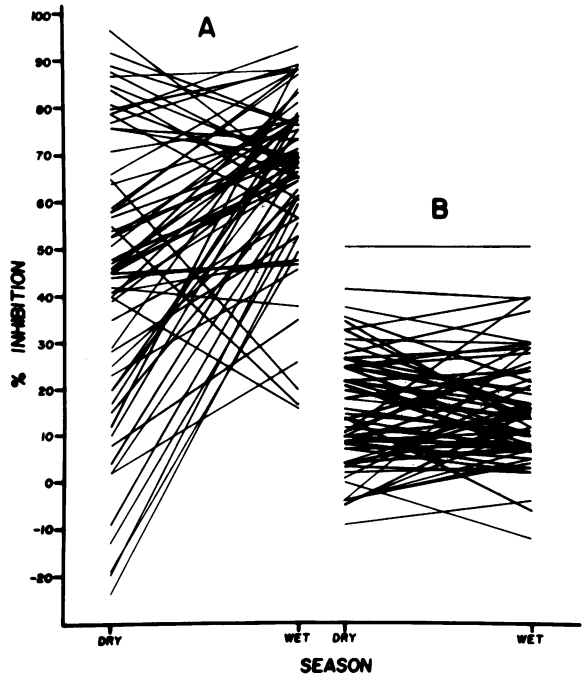

FIG. 1. Antimalarial activities of paired serum samples collected in DS and WS on *P. falciparum* in vitro from 62 individuals. (A) Inhibition of intraerythrocytic parasite development (crisis form activity) as determined by the reduction of [^{3}H]hypoxanthine incorporation into synchronized parasites grown in the presence of 25% immune serum as compared with 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 three experiments (standard deviation ± 10% inhibition).

asitic activity against the FCMSU-1/Sudan strain, isolated in the area of study and transported to Michigan State University (A. A. Divo, J. A. Vande Waa, and J. B. Jensen, manuscript in preparation). Stock cultures were maintained in O+ erythrocytes by the candle jar method (14).

Merozoite reinvasion inhibition. Parasite cultures grown to 10 to 15% parasitemia were synchronized to 6- to 8-h age differential by a combination of the sorbitol lysis (15) and gelatin flotation methods (9) as previously described (12). At the time of segmentation and merozoite release, the gelatin-concentrated, schizont-infected cells were diluted to 25% parasitemia with freshly washed O+ erythrocytes and were immediately dispensed into 96-well microtiter plates (Linbro). Each well contained 1.5 μl of cells and 100 μl of RPMI 1640 containing 10% (vol/vol) pooled nonimmune human serum (RP-10) and 25% (vol/vol) dialyzed 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 with 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 to 3% parasitemia with fresh O+ erythrocytes and dispensed into 96-well microtiter plates. Each well contained 2 μl of cells, 200 μl of 25% immune sera in RP-10 and 2 μCi of [^{3}H]hypoxanthine (10 Ci/mmol; New England Nuclear). These plates were incubated in a candle jar at 37°C for 40 h and were then harvested onto fiber glass filters with a Bellco Microharvester. The incorporation of [^{3}H]hypoxanthine into parasite nucleic acids was measured by liquid scintillation spectrometry as described previously (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 with development in dialyzed nonimmune serum (10).

Serum immunoglobulins. Indirect fluorescent antibody (IFA) titers were determined with trophozoite and schizont stage parasites of the FCMSU-1/Sudan strain by 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 with Endoplate Immunoglobulin Test Kits (Kallestad Laboratories). Ring diameters were measured by the endpoint method described by Mancini, Carbonara, and Heremans (16).

RESULTS

Intracellular parasite retardation, merozoite invasion inhibition, and parasite-specific and nonspecific antibody responses were compared for paired DS and WS serum samples from 62 individuals.

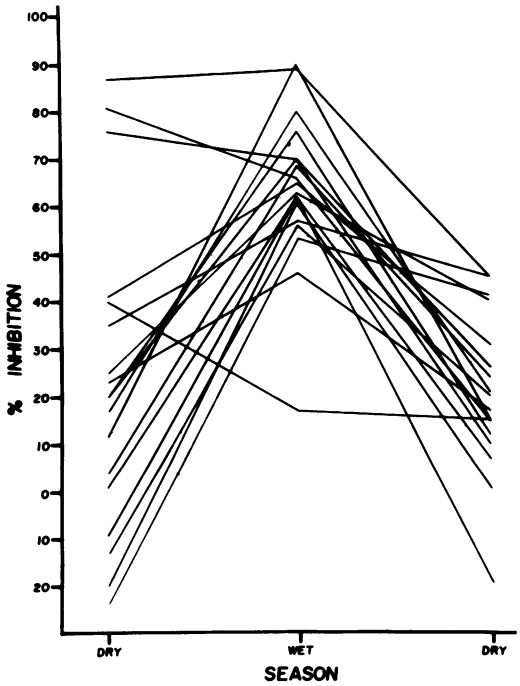

FIG. 2. Inhibition of intraerythrocytic parasite development of serum samples longitudinally collected from 19 individuals during the DS, 1982, WS, 1982, and DS, 1983. All data points from each serum represent mean inhibition values from three experiments (standard deviation ± 10% inhibition).

TABLE 1. Immunoglobulin concentrations and antimalarial activities in serum samples collected from the same individuals during DS and WS, 1982

Expt	DS			WS			Significance[a]
	n	Mean	Range	*n*	Mean	Range	
IgG IFA[b]	62	688	20–2,560	62	1,152	20–5,120	NS
IgM IFA[b]	17	56	0–320	17	72	0–320	NS
IgA IFA[b]	17	7	0–80	17	15	0–80	NS
IgG (mg/dl)[c]	62	1,360	941–2,275	62	1,411	988–2,357	NS
IgM (mg/dl)[c]	62	124	34–362	62	129	34–314	NS
IgA (mg/dl)[c]	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] Student's *t* test used to determine levels of significance; NS, not significant ($P > 0.05$).
[b] Reciprocal endpoint titers.
[c] Serum immunoglobulin concentrations as determined by radial immunodiffusion.

Data describing the degree of intracellular parasite retardation and merozoite invasion inhibition in individual immune sera are presented in Fig. 1. The mean difference in parasite developmental retardation from DS to WS (39 versus 66.5%, respectively) was highly significant ($P < 0.001$). Intracellular retardation profoundly increased from DS to WS in nearly 55% of the subjects. In this high responding group, the mean intracellular parasite retardation activity increased nearly threefold, 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; standard deviation ± 10% inhibition); most of these had elevated inhibitory serum at both sample times. In less than 10% of the subjects, parasite development retardation activity decreased from DS to WS. The data shown were obtained in experiments with [^{3}]hypoxanthine incorporation as a measure of parasite maturation; the retardation of growth was confirmed morphologically in Giemsa-stained thin films.

In contrast to the significant increase in intracellular retardation activity seen in 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 merozoite invasion inhibition. A third serum sample was obtained from 19 of the same individuals during the following DS, June, 1983. Antiparasite activities of these DS-WS-DS samples were tested together in the same microtiter plate to minimize interexperiment variability. These data, shown in Fig. 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 DS (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 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 IFA titers. As expected, there was a significant ($P < 0.001$) correlation between serum IgG IFA titers and merozoite invasion inhibition. However, when these data are presented graphically (Fig. 3), it is clear that the large individual variation prevents the use of IgG IFA 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 IFA titers and IgM IFA titers (0.43), IgG IFA titers and total serum IgG concentration (0.31), and total serum IgG concentration and total serum IgA concentration (0.35). In addition, significant correlations existed between IgM IFA titers and total serum IgG concentration (0.62) and between IgM IFA titers and total serum IgM concentration (0.40).

To assess exposure of our population to other parasitoses, we surveyed the villages for urinary and intestinal protozoa and helminths. The actual incidence of these parasitoses was not determined at the time of our survey; however, the relative frequencies of each parasite could be determined,

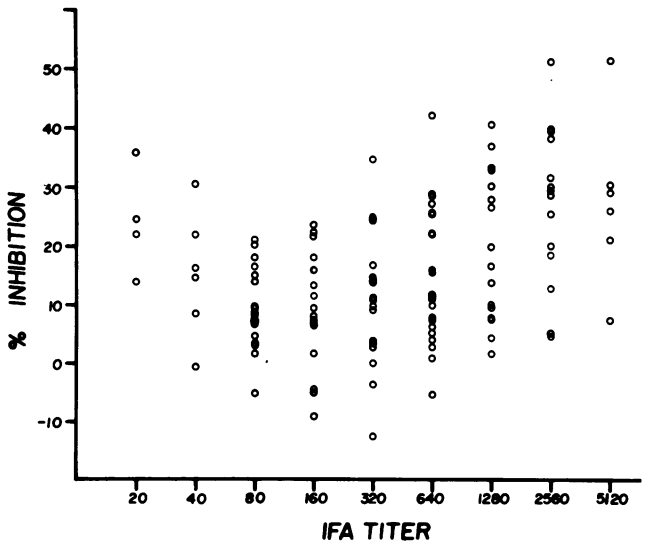

FIG. 3. Distribution pattern of in vitro merozoite invasion inhibition as related to falciparum parasite-specific IgG IFA titer. Although correlation coefficient (Table 2; $P < 0.001$) between IgG IFA titers and antimerozoite activity was positive, the distribution demonstrated that one could not accurately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

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)

Expt	IgG IFA	IgM IFA	IgA IFA	IgG level	IgM level	IgA level	Merozoite inhibition[a]	CFF activity[b]
IgG IFA	—[c]	0.43[d]	0.03	0.31[d]	0.20	0.07	0.45[d]	0.23[e]
IgM IFA	—	—	0.02	0.62[d]	0.49[d]	0.15	0.25	−0.10
IgA IFA	—	—	—	0.04	−0.10	−0.09	0.01	0.03
IgG (mg/dl)	—	—	—	—	−0.04	0.35[d]	0.21[e]	0.03
IgM (mg/dl)	—	—	—	—	—	−0.06	0.09	0.03
IgA (mg/cl)	—	—	—	—	—	—	0.14	0.07
Merozoite inhibition[a]	—	—	—	—	—	—	—	0.06

[a] Merozoite invasion inhibition activity.
[b] Intraerythrocytic parasite development inhibition.
[c] —, No data.
[d] $P < 0.001$ (Student's t test).
[e] $P < 0.05$.

primarily among the children. The comparative frequencies of the intestinal protozoa were as follows: *Entamoeba coli* > *Giardia lamblia* > *Entamoeba histolytica* > *Chilomastix mesnili* > *Endolimax nana*. The comparative frequencies of urinary or intestinal helminths found were as follows: *Schistosoma haematobium* > *Enterobius vermicularis* > *Hymenolepis nana* > *Taenia saginata* > *S. mansoni* (rare). The frequencies of all of these parasites varied extensively from village to village, especially *S. haematobium*. In one village, more than 85% of the children were passing *S. haematobium* eggs, whereas 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 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 did 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 DS to WS, intracellular parasite retardation activity increased significantly ($P < 0.001$), whereas merozoite invasion inhibition did not change. During the following DS, 1983, intracellular parasite retardation activity, hereafter called crisis form activity, returned to levels corresponding to the previous DS, 1982. This seasonal rise and fall in serum concentrations of CFF 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 data).

Some of the sera did not change seasonally, but maintained reasonably high concentrations of CFF in DS and WS. We do not yet know the actual kinetics, or serum half-life, of this activity, so we can only speculate as to how long CFF remains active in the serum. These individuals could possibly have been exposed to malaria during DS or, alternatively, could harbor subclinical malaria infections, but the latter possibility is less likely in this population since splenomegaly is rare and IgG IFA titers are uniformly low (13). Sera from a few individuals decreased in crisis form activity during WS, which could be explained by a malaria infection that occurred during DS but not in the WS.

In this population, crisis form activity was correlated with IgG IFA titers. However, since schizont-specific IFA titers are more of an indicator of the degree of exposure, or recent exposure, to falciparum infections (18) rather than a reliable index of protection (13), we postulate that the correlation between IgG IFA titers and crisis form activity results from parasite exposure which induced the production of CFF.

The seasonal, and apparently parasite-specific, induction of crisis form activity indicates that CFF may be an integral part of the immune response to malaria in the individuals studied. The ability to produce or maintain high concentrations of CFF and the resultant suppression of malaria infections requires further characterization. However, Jensen et al. (11) have recently demonstrated an association between serum crisis form activity and clinical immunity. Although our data suggest a parasite-specific dependence for the production of CFF, the triggering or induction mechanisms are not known. Nonetheless, the degree of malaria exposure that our subjects experience, primarily during WS, is sufficient to induce highly inhibitory concentrations of CFF when necessary. Since crisis form activity was correlated to IgG IFA titers, which in turn reflect the degree of exposure to malaria infectious, crisis form activity would predictably be greater in holoendemic areas of Africa.

The source of CFF is not known. However, some investigators working with animal models have suggested that CFF results 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 of 40% or more. These results are similar to those reported previously in which only 2 of 12 sera collected from holoendemic southern Sudan had appreciable antimerozoite activity (11). Furthermore, they support the observations of Phillips et al. (19), who found only 2 of 15 Gambian sera to be 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 of more than 80%, but these sera had 8 to 15 times more parasite-specific antibody and no CFF activity (13). Our assay for antimerozoite activity is based on assessing new ring formation and does not determine whether 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 WS, and the degree of such exposure is reflected in the relatively low IgG IFA titers and, subsequently, low merozoite invasion inhibition activity. Presumably, greater antigenic stimulation in areas of higher malaria incidence would produce higher parasite-specific IgG IFA 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 WS, 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 IFA titers, as would be expected from an antibody-mediated activity. However, the use of whole trophozoites and schizonts as antigens in the IFA 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 IFA titers that did not appreciably inhibit merozoite invasion. Clearly, a purer antigen preparation would be required to improve the predictive value of IgG IFA titers for merozoite invasion inhibition. It is also important to note that there was no correlation between merozoite inhibition and CFF 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 various degrees, is a common characteristic of people living in malarious regions (17), and the population examined in this study was no exception. These moderately elevated serum IgG concentrations were also correlated with parasite-specific IgG IFA titers, and this in turn was probably responsible for the merozoite invasion inhibition observed.

No correlation was found between parasite-specific IgM and IgA IFA titers and merozoite invasion inhibition. The reliability of this data is, however, limited since there were so few individuals who demonstrated IgM or IgA IFA titers, all of which were comparatively low. A larger or younger population with a greater range of IgM or IgA IFA titers may have provided more definitive results. Interestingly, the one individual who had the highest antimerozoite activity not only had a high IgG IFA titer, as expected, but also had a uniquely high IgM IFA titer. Whether this was due to both immunoglobulin classes remains to be determined. Other statistically significant correlations existed between the class-specific IFA 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 CFF is positively correlated with exposure to falciparum malaria and, thus, may play a significant role in the acquired immunity to malaria in Sudan.

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