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Association Between Human Serum-Induced Crisis Forms in Cultured *Plasmodium falciparum* and Clinical Immunity to Malaria in Sudan†

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Clinical histories with regard to falciparum malaria were collected from adults living in holo-, hyper-, and hypoendemic areas of Sudan and matched to serum samples which were assayed for antiparasitic activity in cultures of *Plasmodium falciparum*. The adult population of the endemic areas could be divided into three groups based on oral histories: those who never experience falciparum malaria; those with a childhood history of malaria, who experience only mild occasional malaria as adults; and those who suffer serious recurring malaria symptoms. In vitro parasite inhibition was greatest with sera from individuals with no clinical histories of malaria, and generally, more inhibition was noted in sera from holoendemic versus hyperendemic areas. Serum from hypoendemic urban Khartoum was not inhibitory. There was no relationship between serum indirect fluorescent antibody titers and parasite inhibition, but there was strong association between clinical immunity and intraerythrocytic parasite inhibition resulting in "crisis" forms. Purified immunoglobulin G was not strongly associated with crisis forms, which were consistently associated with fractions of immune serum remaining after immunoglobulin removal. Thus, it appears that clinical immunity to malaria in Sudan is based on nonantibody serum factors, possibly associated with cell-mediated immunity. Human leukocyte alpha-interferon had no inhibitory effects on cultured *P. falciparum*. Some umbilical cord sera were profoundly inhibitory, producing crisis forms, whereas others were not inhibitory, suggesting that factors that induce crisis forms may play a role in protecting neonates from falciparum malaria.

It is generally accepted that acquired immunity to the blood stages of malaria is based on humoral and cell-mediated mechanisms. The cell-mediated elements have not been well defined (27), but it has been demonstrated in vitro that immunoglobulin G (IgG) neutralizes the invasive properties of the extraerythrocytic merozoite, and that such merozoite blocking antibodies play a role in protective immunity (7). Studies which demonstrated that purified IgG from adults living in malarious areas of Africa offered a degree of protection when passively transferred to young children infected with falciparum malaria (8) have provided the foundation for present malaria blood-stage vaccine development programs. The development of monoclonal antibodies against merozoites of several *Plasmodium* spp. that have proven to be inhibitory to merozoite invasion in parasite cultures (9), or protective when passively transferred to appropriate host animals (10), has further underscored the importance of antimerozoite antibodies in protective immune responses in malaria.

The development of continuous cultivation of *Plasmodium falciparum* by Trager and Jensen (32) has provided the basis for development of in vitro techniques for direct analysis of humoral components of the immune system on the growth and development of this human pathogen. Such studies have been reported involving serum or purified immunoglobulins from the owl monkey, *Aotus trivirgatus*, made immune to falciparum malaria by repeated cycles of infection and drug cure (3, 4, 28). In some of these studies, the degree of merozoite invasion inhibition correlated roughly with the ability of the monkeys to withstand an infective challenge (13). The effects of human "immune" sera on parasite development in vitro have been reported, but the number of serum samples examined

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has been small, and no correlation between functional immunity and serum inhibitory activity was reported (2, 25, 29). Furthermore, the inhibitory properties of these "immune" sera were determined by assessing reductions in total parasite numbers after different periods in vitro, as compared with control cultures; no attempt was made to determine the nature of the inhibition by using synchronous cultures. This point is raised because we have previously reported that serum from adults living in malarious areas of Sudan had at least two different inhibitory properties: merozoite invasion inhibition and intraerythrocytic parasite development retardation resulting in crisis forms of *P. falciparum* (18). In a subsequent report, we demonstrated that intraerythrocytic development inhibition could be readily monitored by measuring the incorporation of radiolabeled [^{3}H]hypoxanthine (^{3}H-Hx) into parasite nucleic acids (19).

In the present study, we have attempted to examine the association between clinical immunity to falciparum malaria and in vitro inhibition of parasite growth and development. Several hundred serum samples were collected from adults living in malarious areas of the Democratic Republic of Sudan. With most of these samples, we obtained detailed oral histories with regard to malaria symptoms. We found little or no association between antiparasitic antibodies—demonstrable in the serum samples by indirect fluorescent antibody (IFA) techniques—and clinical immunity to malaria in the human population. However, we observed a strong relationship between clinical immunity and the ability of serum from apparently immune individuals to induce intraerythrocytic retardation of parasite development, a phenomenon not associated with antibody.

MATERIALS AND METHODS

Study areas and clinical histories. The southern provinces of the Sudan are holoendemic for falciparum malaria, despite some fluctuations in parasite transmission rates from wet and dry seasons. Serum samples for this study were collected from 18- to 60-year-old adults living in villages within 50 miles of Wau, Bahr El Ghazal Province. In these villages, primary health care in general, and antimalarial therapy in particular, were minimal to nonexistent. Villages hyperendemic for falciparum malaria, within a 25-mile radius of Sennar, Blue Nile Province in central Sudan, have some outpatient public health clinics at which chloroquine is dispensed when available. The actual availability of chloroquine in these villages was difficult to assess because some people maintain private stores of the drug. The supplies of chloroquine in the clinics are sporadic, and availability is the exception, rather than the rule. Transmission of malaria during the 9-month dry season is maintained by *Anopheles funestus*, a vector that breeds in slow-moving fresh water such as irrigation canals and swampy areas along the banks of the Blue Nile. Parasite slide positivity rates in school-aged children during the dry season are usually in the 1 to 5% range. After the peak of the rains, *A. funestus* is displaced by *Anopheles gambiae arabiense*, which breeds in standing rain pools and is an unusually effective, anthropophilic vector of falciparum malaria. Thus, the onset of the rains brings a marked change in the prevalence of falciparum malaria, with parasite slide positivity rates often exceeding 50% in adults and children. Sera, with corresponding clinical histories with regard to malaria, were collected from adults in villages of Blue Nile Province. Since children are generally less immune to malaria than adults, they were excluded from this study. Sera were more readily obtainable from males than from females in Moslem Central Sudan; thus, more males were sampled from this region.

The capital city of Khartoum is hypoendemic for falciparum malaria, with rare infections occurring chiefly in outlying areas where the fields are irrigated from the White and Blue Nile Rivers. Serum samples were collected from adults in all three areas described above and were matched to oral histories with regard to experience with malaria. The interviewers attempted to determine clinical histories of malaria-like episodes by inquiring about the most recent symptoms of febrile illness, with special reference to intensity and frequency of headaches, joint pains, intermittence of fevers, whether the symptoms abated with chloroquine or without treatment, how often the individual experienced malaria-like symptoms, where he or she lived as a child, and whether siblings suffered from malaria.

Serum preparation. In most cases, serum was drawn in 20-ml siliconized Vacutainers and clotted at 4°C, and the formed elements were removed by centrifugation. We also obtained 35 samples of umbilical cord serum from neonates born at the Sennar Civil Hospital. The mothers were interviewed concerning malaria-like symptoms they may have experienced during pregnancy. The carefully labeled glass vials containing the sera were frozen at −20°C until they were transported to the United States in small picnic coolers packed with frozen blue-ice gel. Despite the 60 h in transit from our laboratories in Sudan to East Lansing, Mich., the samples always arrived frozen. Details of the processing of serum samples and the in vitro assay for parasite inhibition have been reported elsewhere (19). Briefly, all serum samples were thawed, heat inactivated at 56°C for 30 min, and dialyzed at 1:100,000 against medium RPMI 1640 to remove any contaminating antimalarial drugs and to equilibrate nutritionally the test serum with the medium used to culture *P. falciparum*.

Parasites of strain FCR_{3TC} (20) were grown in human O-positive erythrocytes, using the candle-jar technique (21), and were synchronized to a 6-h age differential by using a modification of the sorbitol technique (22). They were cultured to the late schizont stage before being concentrated by the gelatin flotation method (17) and subcultured to 0.5% parasitemia in freshly washed O-positive erythrocytes. Cultures thus prepared were dispensed to 96-well flat-bottom microculture plates. The dialyzed test sera were diluted 1:2,

1:4, 1:8, and 1:16 with RPMI 1640 containing 5% pooled nonimmune serum and 40 μg of gentamicin sulfate per ml. Each serum concentration was tested for total inhibition (merozoite invasion inhibition and growth retardation) by exposing the synchronized schizonts to the test serum for 48 h, and for retardation of intraerythrocytic parasite development from the ring to the schizont stage by allowing schizont maturation and subsequent merozoite invasion to occur in nonimmune serum and then culturing these parasites in various concentrations of test sera. For each serum dilution, a series of wells was labeled with ^{3}H-Hx for the final 24 h of culture and then was harvested onto glass fiber filter paper strips, using a Bellco Microharvester. Incorporation of purine label into parasite nucleic acids was determined by scintillation spectrometry, using a Beckman LS 7500 liquid scintillation counter. Identical sets of wells were prepared as Giemsa-stained thin films for visual assessment of serum-induced parasite inhibition.

Serum fractions. Twelve sera that were both highly inhibitory and had relatively high antiparasitic antibody titers, as determined by IFA techniques involving cultured *P. falciparum* schizonts, were selected for fractionation to purify the IgG portion from the remaining serum, which was subsequently dialyzed to remove all $(NH_4)_2SO_4$ and equilibrate it with RPMI 1640. The IgG was first precipitated from the serum by using 35% (vol/vol) saturated $(NH_4)_2SO_4$. The precipitated immunoglobulins were washed twice in 40% $(NH_4)_2SO_4$, dissolved in phosphate-buffered saline, reprecipitated in 35% saturated $(NH_4)_2SO_4$, redissolved in phosphate-buffered saline, and dialyzed against 0.02 M K_2HPO_4 (pH 8.0) buffer to remove the $(NH_4)_2SO_4$ and to prepare the immunoglobulins for further purification on DEAE Affi-Gel Blue (Bio-Rad Laboratories). Since many African sera are hypergammaglobulinemic due to multiple parasitoses, the purified IgGs were adjusted to a final concentration in RPMI 1640, using IFA titration against schizonts so that the final solutions contained at least as much specific antimalarial antibodies as the original sera. The purified IgG portion and the serum components that remained in solution after the 35% $(NH_4)_2SO_4$ precipitation were tested in the parasite inhibition assay described above. Other serum samples that were markedly inhibitory were passed through a column containing Sepharose CL-4B conjugated with *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals) according to standard protocols. The serum, minus the IgG portion, was collected as one fraction, and the eluted IgG was collected as a second fraction. After dialysis against RPMI 1640, these fractions were tested for inhibitory activity, with samples of the intact sera as control.

Since the major inhibitory fraction of the serum was not IgG, we tested human leukocyte alpha-interferon (a gift from Kenneth Swartz, Michigan State University) and interferon obtained from Sigma Chemical Co. Interferon was tested in our parasite inhibition protocol outlined above at 10, 100, and 1,000 U/ml.

Titers of antiplasmodial immunoglobulins were determined for all sera assayed for antiparasitic activity by IFA, using goat antihuman immunoglobulin antibodies conjugated with fluorescein (Cappel Laboratories) according to standard procedures with schizonts from cultured parasites (15).

RESULTS

Oral histories concerning experience with malaria taken from villagers living in the southern provinces were not satisfactory. This was due principally to difficulty in translating our clinical inquiries into local dialects without asking overtly leading questions, and to a general lack of understanding of malaria as a distinct medical entity by the rural indigenous population. Such was not the case for our efforts to collect oral clinical histories from persons in the Blue Nile Province of central Sudan. Despite the obvious difficulty in separating malaria from other febrile diseases without microscopic confirmation, most of the villagers in this area were reasonably articulate with regard to classical malaria symptomatology, and we felt confident that most of their oral histories were a good indication of past experiences with the disease. Based on the results of our interviews, we found that this population could be divided into three general categories; the majority reported numerous episodes of malaria-like symptoms as children, fewer episodes as adolescents, and only occasional mild symptoms as adults. Approximately 5% reported never having experienced the symptoms of malaria—no fever, chills, headaches, etc.; and a smaller portion reported that they suffered numerous serious episodes of malaria-like illness, most of which required chloroquine therapy. Individuals from Khartoum rarely experienced malaria, although some reported having had the symptoms as children. There were no differences in clinical histories between males and females, except during pregnancy, when females became more susceptible to malaria.

We assayed nearly 200 sera obtained from the holoendemic southern and hyperendemic central regions of the Sudan, and sera from residents of Khartoum, for in vitro parasite development retardation and merozoite invasion inhibition. We also assayed for antiplasmodium antibodies by IFA techniques, using cultured falciparum schizonts. The data from these assays are summarized in Table 1. Those individuals who reported that they could not recall ever experiencing malaria symptoms had IFA titers $\leq$ 1:20, with over half having no antiplasmodium antibodies demonstrable by IFA. As a group, however, these sera were the most inhibitory, with 1:4 and 1:8 dilutions being 90 and 80% inhibitory, respectively, over 48 h, as determined by incorporation of ^{3}H-Hx into parasite nucleic acids. These sera were also profoundly inhibitory to intraerythrocytic parasite development: ring-stage parasites cultured for 24 to 30 h in 1:4 or 1:8 dilutions of serum were inhibited by 79 and 75%, respectively. Visual examination of Giemsa-stained thin films revealed that parasites

TABLE 1. Summary of clinical histories, antiplasmodium IFA titers, and percent inhibition of parasite metabolism as determined by incorporation of radiolabeled ^{3}H-Hx into parasite nucleic acids when synchronized cultures of *P. falciparum* were cultured for 24 h (late-ring to schizont) or 48 h (schizont to schizont) in immune sera diluted 1:4 or 1:8[a]

Malaria history	n[b]	IFA titer[c]	Serum dilution	% Inhibition	
				24 h[d]	48 h[e]
No malaria	10	1:20	1:4	79	90
			1:8	75	80
Occasional malaria	62	1:320	1:4	50	71
			1:8	40	64
Southern sera[f]	97	1:1,280	1:4	66	82
			1:8	45	64
KTM sera[g]	8	1:40	1:4	0	20
			1:8	0	0

[a] All sera were collected from 18- to 60-year-old adults, the majority being 20 to 35 years old. No differences between males and females were noted with regard to clinical histories or inhibitory responses.

[b] Number of sera assayed.

[c] Arithmetic mean.

[d] Intraerythrocytic parasite development retardation, rings to schizonts, exposed for 24 to 30 h to serum.

[e] Total parasite retardation, schizont to schizont, exposed for 48 h to serum.

[f] Malaria histories not included.

[g] Serum from Khartoum city, where malaria is hypoendemic.

cultured in the presence of these sera were severely retarded in their development, appearing shrunken, pyknotic, and karyorrhexic, looking very much like classic "crisis form" parasites (Fig. 1). Careful inquiries satisfied us that these individuals were not on chloroquine or other antimalarial drugs. Furthermore, their sera were equally inhibitory to parasite strains having over 100-fold differences in chloroquine sensitivities. Finally, our dialysis procedure removed all chloroquine from sera known to contain the drug (19).

The majority of individuals in villages in central Sudan reported a childhood history of malaria, with occasional, generally mild episodes of the disease as adults. Serum samples from these persons were reasonably inhibitory to parasite cultures (Table 1), but they were less inhibitory than sera from individuals who never experienced malaria symptoms; their mean IFA titers were also higher—1:320 versus ≤ 1:20. Although the mean inhibition rates for 1:4 and 1:8 serum dilutions were 71 and 64%, respectively, for 48 h, the inhibition of individual sera ranged from greater than 90% to less than 20%, reflecting the diversity in malaria experience recorded in the oral clinical histories. Serum samples collected from residents of Khartoum had a mean IFA titer of ≤ 1:40 and were not particularly inhibitory. Although we were unable to obtain satisfactory oral histories from villagers in the holoendemic southern region of Sudan, their sera were highly inhibitory for parasite cultures. The mean IFA titer of these sera was 1:1,280, and these sera were more inhibitory than most of the sera from the central region (Table 1), except for those samples from individuals who had never experienced malaria.

Generally, in each case, the oral histories with regard to experience with malaria related well to total parasite inhibition, with the exception of sera obtained from those individuals who reported that they suffered numerous recurring episodes of malaria symptoms. Antibody titers, detected by IFA, varied from high to low in these individuals: some were > 1:5,180, whereas others were < 1:40. The inhibition of in vitro parasite development also ranged from highly inhibitory to noninhibitory.

Inhibition of parasite growth and development in the presence of umbilical cord sera varied greatly. Some umbilical cord sera supported in vitro parasite development better than our pooled nonimmune serum controls, whereas others were > 90% inhibitory (Table 2). Although an attempt was made to correlate the malaria histories of mothers during pregnancy with inhibition levels in cord sera, no pattern was discernable.

Because in the most inhibitory sera, little or no IFA antibody to falciparum schizonts could be demonstrated, we purified IgG from a group of 12 sera having both high IFA titers and high parasite inhibition activity. We first precipitated IgG by using 35% saturated $(NH_4)_2SO_4$ and then further purified the immunoglobulins by using ion-exchange chromatography. We also dialyzed the $(NH_4)_2SO_4$ from the soluble portion of the sera, assayed for residual IgG and IgM, and compared the unfractionated sera, purified IgG, and $(NH_4)_2SO_4$-soluble serum portion for parasite inhibitory activity. The results are given in Table 3. Of the 12 sera fractionated, IgG was inhibitory in only 2. Parasite inhibition was found to be associated with the portion of the serum not precipitated by 35% $(NH_4)_2SO_4$, and the inhibitory activity of this fraction was principally of the intraerythrocytic parasite retardation type. Although this "soluble" fraction retained some residual antiparasitic IgG and, in a few cases, IgM, there was no association between inhibition and immunoglobulins, except in sera 1,714 and S-81-113. In the former sample, the IgG was exclusively merozoite blocking in its action, whereas in the latter sample, the action was principally merozoite blocking, with some intraerythrocytic parasite development re-

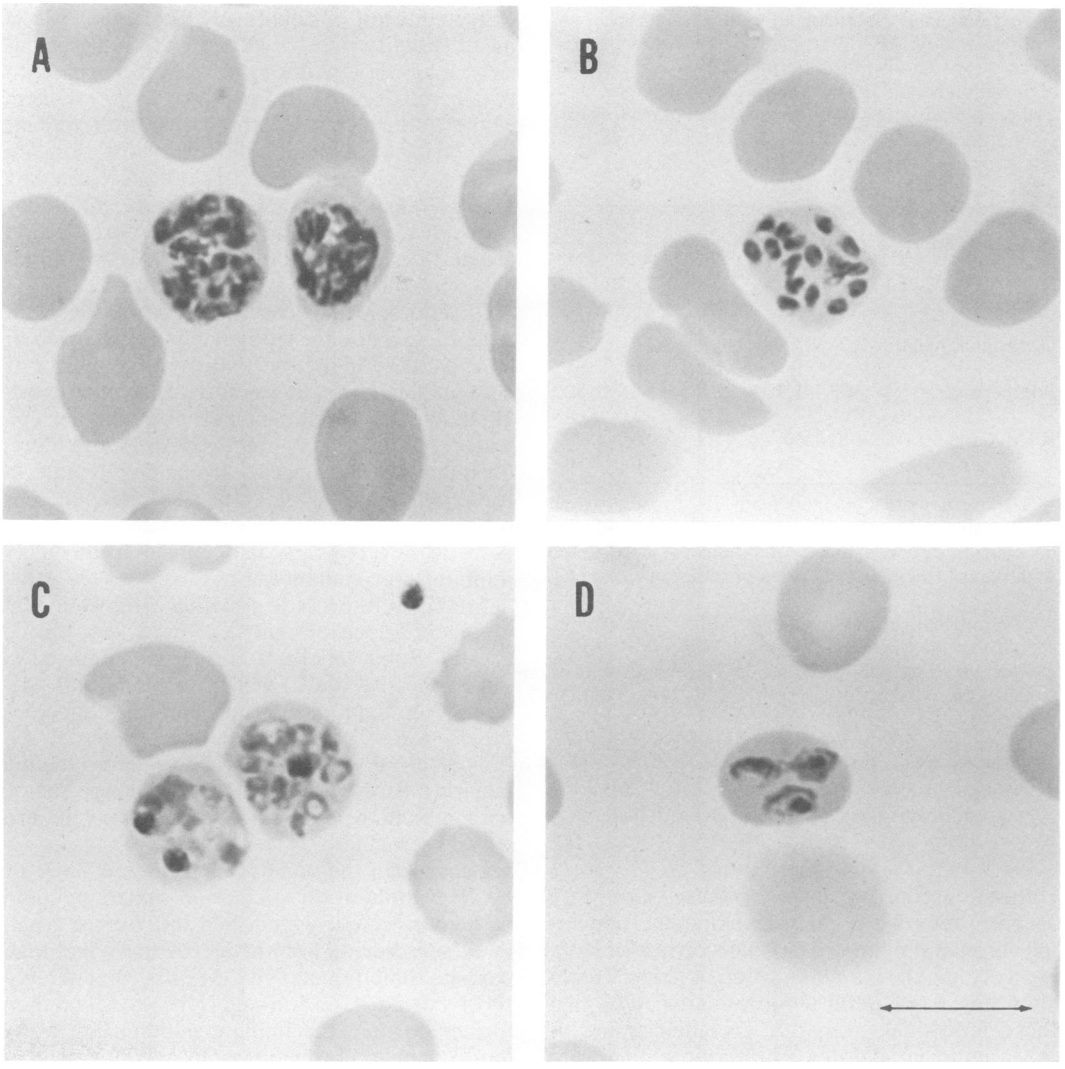

FIG. 1. Photomicrographs of *P. falciparum*-infected erythrocytes cultured in a 1:4 dilution of pooled, nonimmune serum (A and B) or a 1:4 dilution of serum obtained from a Sudanese who had never experienced clinical malaria. In some experiments, the assay was initiated with synchronous cultures containing mature schizonts (A). After 48 h in the presence of immune serum, the parasites were severely retarded in their development (D). In other experiments, young trophozoite-stage parasites were exposed to immune serum for 24 to 30 h; these parasites failed to mature to normal segmenters (B) but became abnormal looking, vacuolated with enlarged, faintly stained merozoites (C). These abnormal parasites had markedly reduced metabolism, as determined by ^{3}H-Hx incorporation, and apparently failed to release invasive merozoites. Bar = 10 μm.

tardation. In further support of our finding that the inhibitory activity of the immune sera was generally not associated with IgG, two inhibitory sera were passed over a column of Sepharose CL-4B containing protein A. This procedure removes all IgG subclasses except for IgG3. Nonetheless, the inhibitory properties of the intact sera were compared with those of the IgG-reduced serum eluted from the column and the IgG washed from the protein A. The results are shown in Table 4. The serum fraction, less the IgG subclasses 1, 2, and 4, retained most of the inhibitory activity. Since the most inhibitory sera examined in this report came from individuals having little or no antiplasmodium IgG discernable by specific IFA, it is unlikely that IgG3, which remained after protein A fractionation, could account for the antiparasite activity.

We were fortunate to be able to collect serum samples near the end of both the dry and wet seasons from three individuals who had never experienced malaria symptoms. The data on

TABLE 2. Inhibition of synchronized cultures of *P. falciparum* in 1:4 dilutions of umbilical cord sera obtained from neonates born in hyperendemic Blue Nile Province, Sudan, as determined by incorporation of ^{3}H-Hx into parasite nucleic acid[a]

Serum	IFA titer	% Inhibition by serum diluted 1:4	
		24 h[b]	48 h[c]
S-81-U_1	1:160	61	86
S-81-U_3	1:40	0	0
S-81-U_4	0	0	0
S-81-U_5	1:160	0	0
S-81-U_6	0	0	0
S-81-U_8	1:320	62	83
S-81-U_9	0	82	95
S-81-U_{12}	0	50	60
S-81-U_{14}	1:20	24	52
S-81-U_{16}	1:40	66	75
S-81-U_{17}	0	0	0
S-81-U_{20}	0	64	74
S-81-U_{21}	1:320	86	100
S-81-U_{30}	1:40	32	60
S-81-U_{33}	1:20	16	70
S-81-U_{34}	1:80	47	56

[a] Sera that were inhibitory not only blocked successful establishment of new parasite generation (48 h of inhibition) but also inhibited intraerythrocytic parasite development (24 h of inhibition).

[b] Intraerythrocytic parasite development retardation, ring to schizont, in serum for 24 to 30 h.

[c] Parasite inhibition, schizont to schizont, in serum for 48 h.

these sera are given in Table 5. There was a small but notable decrease in both IFA titers and inhibitory activity near the end of the 9-month dry season, as compared with the sera collected at the peak of malaria transmission (Table 5). Since it was apparent that most of the inhibition noted in our immune sera was not due to antibody, and that its action was principally of the intraerythrocytic parasite retardation type, we tested human leukocyte alpha-interferon as a possible nonantibody serum factor involved in this phenomenon. Even when 1,000 U of interferon per ml was used, no parasite inhibition was noted. These results were consistent with interferon from different sources.

DISCUSSION

Our finding that some adults living in hyperendemic areas of Sudan never experience malaria symptoms was not unexpected, especially in view of the fact that several erythrocyte anomalies, such as sickle-cell trait (1, 11), β-thalassaemia, and glucose-6-phosphate-dehydrogenase deficiency (23, 26) have been shown to correlate with marked reductions in malaria incidence and pathology. However, the finding of a strong relationship between intraerythrocytic parasite development retardation and apparent clinical immunity to malaria is an important new observation. The variation in degree of in vitro parasite inhibition by sera from adults who experience occasional, generally mild malaria was as expected. Those individuals who claimed to suffer from recurring episodes of malaria symptoms generally fell into two groups. Some probably were not suffering from malaria because, despite the physical symptoms, their sera were unusually inhibitory to parasite development and their IFA titers were low. These people probably were suffering from some other febrile illness; the study areas are endemic for typhoid fever, a disease in which physical symptoms cannot readily be distinguished from those of malaria without microscopic confirmation. Some of the chronic malaria sufferers reported that to alleviate their symptoms, they took chloroquine prophylactically. These individuals had low serum IFA titers, and after dialysis to remove the chloroquine, their sera were not inhibitory. Only a few of the malaria sufferers had serum with high IFA titers and moderately low parasite inhibition activity. These individuals evidently had chronic falciparum infections and may have been deficient in those factors that obviously cleared parasites from the blood of the clinically immune persons noted above.

We found no consistent relationship between antiplasmodium IFA titers and immunity to malaria, determined by oral clinical histories, and no correlation between IFA titers and inhibitory properties of the Sudanese sera. These findings are in contrast to those reported by Reese et al. (29); however, they examined only a few serum samples with no corresponding clinical data. We found the IFA antibodies to be more an indication of recent or prevailing malaria infections than an index of clinical immunity. Our most inhibitory sera were obtained from individuals reporting no clinical experiences with malaria. These sera had little or no antiplasmodium IFA titer, suggesting the presence of a potent nonantibody antiparasitic factor. Although most of the sera with moderate to high IFA titers were inhibitory, this observation probably reflects a recent malarial infection that boosted the inhibitory properties of the serum of the donor, rather than a positive correlation between IFA titers and clinical immunity. Therefore, although it is generally true that sera with moderate to high IFA titers are inhibitory, the converse, that sera with low IFA titers have low antiparasite activity, is not supported by our findings. Individuals living in nonmalarious areas of Sudan had low IFA titers, and their sera were not inhibitory to parasite development, but individuals living in hyperendemic areas who never experienced malaria symptoms also had low IFA titers, although

TABLE 3. Inhibitory sera with moderately high antiplasmodium IFA titers were fractionated into DEAE-purified IgG, and those serum components that remained in solution in 35% $(NH_4)_2SO_4$[a]

Serum	IFA titer				% Inhibition		
	Unfractionated	Fractionated			Unfractionated	Fractionated	
		Purified IgG	$(NH_4)_2SO_4$ soluble:			Purified IgG	$(NH_4)_2SO_4$ soluble
			Immunoglobulins[b]	IgM[c]			
1,663	1:2,560	1:2,560	1:640	1:40	78	0	90
1,664	1:2,560	1:5,120	1:320	0	83	10	95
1,670	1:2,560	1:5,120	1:320	0	74	0	72
1,714	1:1,280	1:2,560	1:640	1:160	79	84	63
1,721	1:1,280	1:2,560	1:320	1:40	92	0	94
1,761	1:2,560	1:2,560	1:640	1:10	72	27	69
1,773	1:1,280	1:5,120	1:320	1:320	83	4	69
1,803	1:1,280	1:5,120	1:320	1:0	62	10	57
1,859	1:1,280	1:5,120	1:640	1:80	90	0	46
1,869	1:640	1:5,120	1:640	1:40	88	10	48
S-81-104	ND[d]	1:5,120	0	0	98	0	92
S-81-113	ND	1:640	1:20	0	89	81	0

[a] Antiplasmodium IFA titers were determined for intact sera, purified IgG fraction, and 35% $(NH_4)_2SO_4$-soluble fraction. Inhibition of synchronous cultures of *P. falciparum* in 1:4 dilutions of each serum preparation over 48 h (schizont to schizont), as determined by incorporation of ^{3}H-Hx into parasite nucleic acids, demonstrated that despite the relatively high immunoglobulin titers, neither the intact serum nor the purified IgG fractions had significant antiparasitic activity. Giemsa-stained thin films from each serum tested revealed that parasite inhibition was accurately reflected in the ^{3}H-Hx incorporation data, i.e., sera 1,714 and S-81-113 were the only sera with appreciable merozite blocking activity (visual data not tabulated).

[b] IFA titers were determined by using goat antihuman IgG, IgA, and IgM conjugated with fluorescein.

[c] IFA titers were determined by using goat antihuman IgM conjugated with fluorescein.

[d] ND, Not done.

their sera were profoundly inhibitory. The picture that emerges from these observations is as follows. Those individuals who live in malarious areas develop resistance to clinical falciparum malaria that is reflected in the inhibitory properties of their sera. The most clinically immune individuals generally have the most inhibitory sera. The degree of inhibition observed in these sera was not consistently associated with serum antiplasmodium IFA titers.

The lack of association between serum IFA titers and parasite inhibition was further underscored by our experiments using purified IgG in parasite cultures. Even when the purified IgG was reconstituted to the original, or twice the original, serum IFA titers, only 2 of 12 sera were appreciably inhibitory to cultured *P. falciparum*. The portion of the serum that remained soluble after the G class immunoglobulins were precipitated with $(NH_4)_2SO_4$ retained the antiplasmodial activity. As was the case with the intact serum, the inhibitory portion of the fractionated serum retarded intraerythrocytic parasite development. Thus, clinical immunity to falciparum malaria in our study was strongly associated with the ability of the subjects' serum to inhibit intraerythrocytic parasite development—a property not firmly correlated with antiparasitic IFA titers, nor, in most cases, with purified IgG or IgM derived from inhibitory serum.

Intraerythrocytic death of *Plasmodium* spp. associated with the immunological resolution of malaria infections was described in detail by Taliaferro and Taliaferro (30), who used the term "crisis form" to describe abnormal and disintegrating parasites. In their classical study on crisis forms, the Taliaferros state that their experiments were undertaken to confirm anecdotal evidence of modification of intraerythrocytic development in avian malaria parasites. Trager and McGhee (33) demonstrated a reduction in merozoites in vivo when chickens infected with *Plasmodium lophurae* were inoculated with plasma from older, resistant chickens. These authors subsequently showed that plasma from malarious birds contained antitumor activity when injected into chickens with breast muscle tumors induced by Rous tumor I virus (34). These reports are of historical interest because they record early observations on crisis forms and possible interrelationships between crisis forms and tumor necrosis factor, to be discussed below. We recently reported that serum from Sudan induces crisis forms in cultured *P. falciparum* (18) and that these crisis forms were relatively inactive metabolically; thus, the degree of parasite inhibition could be monitored by using radiolabeled hypoxanthine (19). We have coined the term "crisis-form factor" (CFF) for convenience in referring to the serum factor

TABLE 4. Inhibition of ^{3}H-Hx incorporation into synchronized culture of *P. falciparum* after 48 h in dilutions of immune sera, before and after IgG removal on a Sepharose-protein A column

Serum	Dilution	% Inhibition[a]		
		Unfractionated	Without IgG	Purified IgG
S-81-4	1:4	66	46	33
	1:8	41	33	5
	1:16	40	22	1
S-81-75	1:4	60	80	20
	1:8	58	80	22
	1:16	43	50	10

[a] Protein A does not bind IgG_3; consequently, this subclass of IgG remained in the serum after passage through the column. See the text.

responsible for intracellular parasite development retardation. We are not suggesting that antibody, which interferes with merozoite invasion, is unimportant in acquired resistance to malaria in the population we have studied. Indeed, there was evidence of some merozoite-blocking antibody in our purified IgG fractions. However, the strongest association between clinical immunity and serum inhibition was with CFF, not IgG. Visual examination of Giemsa-stained thin films often revealed that invasion of erythrocytes was markedly reduced when schizonts were incubated in immune serum. However, this reduction in new ring forms after merozoite invasion could not be specifically attributed to antimerozoite antibodies, because the CFF apparently can inhibit the final maturation of the schizonts, reducing the number of viable merozoites released at the time of segmentation. When newly invaded rings were exposed to serum containing CFF, these rings failed to mature through the trophozoite stage (Fig. 1D), and when trophozoites were exposed to CFF, they usually produced degenerate schizonts with reduced numbers of abnormally large merozoites (Fig. 1C). Accordingly, we have noted that the number of new rings formed from synchronous schizonts exposed to immune serum depends, in part, upon how synchronous the culture was, how long the schizonts were exposed to serum containing CFF, and probably the amount of CFF and antimerozoite antibody present in the serum.

Our observations raise important questions about CFF, its source, its mode of action, its physicochemical nature, whether it is unique to the population studied, and whether it is part of the acquired immune response to falciparum malaria. From our experience with CFF, it is apparently not immunoglobulin of the classes G or M. In our previous report, we stated that purified IgG caused retardation of parasite development, but not as profoundly as did the intact serum (18) from which it was derived. These early observations were made on a limited number of samples. In the present study, we found only one sample of purified IgG that, in addition to blocking merozoite invasion, would also retard the intraerythrocytic parasite development. These few exceptions notwithstanding, most samples of purified IgG exerted their antiparasitic action, if any, against the merozoite. These observations are in general agreement with reports on the action of antiplasmodial IgG antibody (7, 24). In a recent report, *Plasmodium knowlesi* partially purified monoclonal antibody, collected from ascites fluid of rats inoculated with hybrid lymphoid cells, contained CFF after $(NH_4)_2SO_4$ precipitation, but this factor was lost after subsequent chromatographic purification (9). These results suggest that CFF was associated with the ascites fluid and not the purified monoclonal antibodies. Extensive studies on factors that induce crisis forms in rodent *Plasmodium* and *Babesia* infections have strongly implied that CFF is a monocyte secretory substance—a monokine, similar or identical to tumor necrosis factor (5, 6, 31). Although we have little information about the nature or origin of CFF, we know that it is not human leukocyte alpha-interferon, that it is found in the umbilical cord blood of some Sudanese neonates, and that it therefore may play a role in passively acquired immunity to malaria in newborns. Its prevalence and concentration in human serum are related to (i) exposure to falciparum malaria and (ii) clinical immunity to this disease in Sudan. If CFF is

TABLE 5. Changes in parasite inhibiting properties of sera drawn during the dry and wet (malarious) seasons of three individuals who never experienced clinical malaria

Serum	Season	IFA titer	Dilution	% Inhibition	
				24 h[a]	48 h[b]
S-80-1	Wet	1:80	1:4	90	100
			1:8	72	98
	Dry	1:20	1:4	59	92
			1:8	26	79
S-81-20	Wet	1:40	1:4	79	99
			1:8	33	96
	Dry	0	1:4	68	98
			1:8	28	85
S-82-607	Wet	1:80	1:4	52	97
			1:8	35	84
	Dry	0	1:4	43	84
			1:8	25	62

[a] Percent inhibition as measured by ^{3}H-Hx incorporation into synchronous *P. falciparum* cultures over 24 h (rings to schizonts).

[b] Percent inhibition as measured by ^{3}H-Hx incorporation into synchronous *P. falciparum* cultures over 48 h (schizont to schizont).

a product of cell-mediated immune mechanisms, as suggested by studies in murine malaria models, we propose the following hypothesis concerning its possible production. Since some evidence exists suggesting that immunity to malaria is based on T cells, and that such immunity may be transferred in rodent malaria models by T cells alone (16), antigen-specific subsets of T cells may respond to *Plasmodium* antigens and, upon activation, may release cytotoxic or monocyte-activating lymphokines. In the former case, CFF may be a T cell lymphokine or, in the latter case, a monokine. In either case, CFF may be a specific or nonspecific lymphoid cell secretory factor, but if CFF is to be part of the acquired immune response to malaria, the trigger must be antigen-specific T lymphocytes. Conversely, since extracts of *P. falciparum* contain potent polyclonal lymphocyte activators (12, 14), the stimulation of lymphokine secretion need not be antigen specific. However, since data from our studies suggest strongly that the presence of CFF is closely associated with acquired clinical immunity to malaria, this latter supposition is less likely. In conclusion, we feel that our results have demonstrated that resistance to falciparum malaria in Sudan is based only in part on immunoglobulins, and that some serum factor, possibly produced as part of the cell-mediated responses, is the principal antiplasmodium component of this resistance.

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