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Comparison of immunity to malaria in Sudan and Indonesia: Crisis-form versus merozoite-invasion inhibition

(malaria/*in vitro* assay/immunology/splenomegaly/antibody)

JAMES B. JENSEN*, STEPHEN L. HOFFMAN†, MICHAEL T. BOLAND*, MUSTAFA A. S. AKOOD‡, LARRY W. LAUGHLIN†, LILIANA KURNIAWAN§, AND HARIJANI A. MARWOTO§

*Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824; †United States Navy Medical Research Unit #2, Jakarta Detachment, Jakarta, Indonesia; ‡Malaria Control Division, Ministry of Health, Khartoum, Sudan; and §National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia

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ABSTRACT **Immunity to falciparum malaria was compared in two populations from malarious areas of southern Sudan and Flores, Indonesia. In Sudan, splenomegaly in adults was rare and anti-plasmodium indirect fluorescent antibody (IFA) titers were low to moderate, 1:1,280 being the modal titer. Sudanese serum was profoundly inhibitory to cultured *Plasmodium falciparum*, reducing incorporation of radiolabeled hypoxanthine by 63–93% and severely retarding intraerythrocytic parasite development, resulting in moribund crisis-form parasites and virtually no healthy schizonts. In Flores, 64% of the serum donors had splenomegaly ≥ Hackett spleen grade 4 or 5, and the modal IFA titer was 1:10,240. Sera from Indonesia did not retard intraerythrocytic parasite development, but inhibited merozoite erythrocyte invasion 22–87%. Anti-merozoite activity did not correlate with IFA titers. The differences in principal modes of anti-parasitic activity suggest that immunity to malaria in Sudan is based on cell-mediated immune mechanisms associated with crisis forms, merozoite neutralization being of secondary importance. In contrast, malaria immunity in Flores appears to be principally based on anti-merozoite antibody, which does not cause crisis forms and allows for development of reduced numbers of healthy schizonts. This less efficient mechanism may lead to a continuous low-grade parasitemia, which could explain the high specific malaria antibody titers and adult splenomegaly in Flores as compared to Sudan. This latter approach to immunity, being less efficient than the former, apparently results in chronic malaria infections with associated high Ig titers and splenomegaly.**

Recent studies on human immune responses to falciparum malaria in Sudan have shown that serum from adults who live in malarious areas and are clinically immune to malaria contains a factor, or factors, that retard intraerythrocytic parasite development in cultured *Plasmodium falciparum*, leading to abnormal parasites known as crisis forms (1). Sera containing high titers of this factor can inhibit metabolic activity of the cultured parasites by more than 90% over a single 48-hr developmental cycle, as determined by scintillation spectrometry of the incorporation of [^{3}H]hypoxanthine ([^{3}H]Hyp) into parasite nucleic acids; schizogony, if it occurs in the presence of immune serum, rarely results in the production of invasive merozoites (2). Further studies correlating medical histories with regard to clinical malaria and the parasite-inhibition properties of corresponding serum samples have shown that serum factors responsible for induction of crisis-form parasites *in vitro* are not associated with immunoglobulins G or M but (*i*) are strongly associated with clinical immunity to malaria, (*ii*) are found in the umbilical cord blood of neonates born to immune mothers, (*iii*) are highly associated with malaria endemicity, and (*iv*) thus appear to be part of the acquired immunity to falciparum malaria (3). We have coined the term crisis-form factor (CFF) for convenience; although we do not yet know the source of this substance, it is not human leukocyte α-interferon but may be some other mononuclear cell secretory product. We have recently repeated the studies conducted in Sudan (i.e., correlation of clinical observations with regard to malaria with serologic and *in vitro* parasite inhibitory activity) in a malarious area of Flores, Indonesia. We report here remarkable differences in the nature of the immune response and clinical sequelae associated with malaria in Flores as compared to areas of southern Sudan with similar malaria biology.

MATERIALS AND METHODS

Thirty-three serum samples, collected from 14- to 45-yr-old villagers from a village on Flores, Indonesia, where malaria is hyperendemic by parasitemia and holoendemic by spleen rate, and where tropical splenomegaly syndrome (TSS) is present in >20% of the adult population, were tested according to methods as described (2). These procedures provide information on metabolic and morphologic inhibition induced by test sera. The ability of the test sera to inhibit cultures of *P. falciparum* were compared to indirect fluorescent antibody (IFA) titers to *P. falciparum* schizont antigens and correlated with the donors' Hackett spleen grade (4). Data from these assays and examinations were compared to data collected from >50 villagers of a similar age group living in the southern Sudanese province of Bahr El Ghazal, El Buheyrat, and Eastern Equatoria, where the prevalence of malaria is similar to that in Flores. Briefly, synchronous parasite cultures (FCR_{3TC} strain) having a 6-hr-age differential were tested against a 1:4 dilution of serum dialyzed 1:1,000,000 against RPMI 1640 medium to remove possible contaminating drugs and to equilibrate all sera with the medium used in the parasite cultures. Merozoite-blocking responses were measured by initiating the *in vitro* tests with segmenting schizonts and comparing successful erythrocyte invasion and ring formation of parasites cultured in the presence of immune sera with nonimmune control sera. Intraerythrocytic parasite development retardation was determined by culturing *P. falciparum* ring-stage parasites for 36 hr in the presence of the test sera and monitoring parasite development (*i*) metabolically by determining the degree of [^{3}H]Hyp incorporation into parasite nucleic acids or (*ii*) morphologically by scoring parasite maturation on Giemsa-stained thin films. Control sera used in these experiments were collected in the field study areas from nonimmune research or graduate assistants or from pools of nonimmune

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Abbreviations: CFF, crisis-form factor; IFA, indirect fluorescent antibody; Hyp, hypoxanthine.

Table 1. Representative data from 50 adult serum samples collected in southern Sudan

Serum	1/IFA	% [^{3}H]Hyp incorporation*		% parasite stage distribution†				% of control parasitemia‡
		48 hr	36 hr	Ab	R	T	Sc	
Nonimmune	0	100	100	3	3	62	32	100
S-82-607	0	17	37	38	14	48	0	99
S-82-912	640	5	13	27	33	40	0	93
S-82-921	320	23	31	24	27	69	3	94
S-82-922	320	2	7	27	9	50	4	84
S-82-924	640	16	24	13	33	54	0	72
S-82-1000	80	8	15	14	16	70	0	100

Splenomegaly in adults was rare (spleen score = 0 for serum donors in Table 1), IFA titers to falciparum schizont antigens were low to moderate, and merozoite-invasion inhibition was low. Significant, however, was the marked reduction of [^{3}H]Hyp incorporation into parasite nucleic acids when synchronous parasites were exposed to immune serum for 36 hr (ring to schizont) or 48 hr (schizont to schizont).

*Percentage of [^{3}H]Hyp incorporation into parasite nucleic acids *in vitro* for 48 hr (schizont to schizont) or 36 hr (ring to schizont).

†Percentage distribution of parasite stages after 44 hr when cultures were initiated as synchronous schizonts; Ab, abnormal crisis forms; R, rings; T, trophozoites; and Sc, schizonts.

‡An index of merozoite-invasion inhibition.

blood donors in the United States, supplied by the American Red Cross. Control sera were dialyzed together with the immune sera used in any test to minimize differences between nutritional status of the immune and nonimmune sera.

RESULTS

The inhibitory properties of the Sudanese sera were essentially like those we have reported before (1–3), except that they were somewhat more inhibitory than sera collected from the hyperendemic central provinces. Representative data are summarized in Table 1. These sera had low to moderate IFA titers against *P. falciparum* schizont antigens, ranging between 1:80 and 1:2,560, with a modal value of 1:1,280. Merozoite-invasion inhibition was minimal in most of the African sera because the number of new ring-stage parasites formed after schizont segmentation and merozoite release was always within 72%, and usually within 90%, of the number of rings formed in the presence of nonimmune control sera. This general lack of merozoite neutralization notwithstanding, the Sudanese sera were profoundly inhibitory to developing intraerythrocytic parasites, severely retarding development of synchronous parasite cultures so that few parasites matured beyond the late-ring or early-trophozoite stage. Incorporation of [^{3}H]Hyp into parasite nucleic acids was inhibited 63–93% over a 36-hr period, when newly invaded ring-stage parasites were exposed to 1:4 dilution of immune sera in culture medium containing [^{3}H]Hyp (Table 1). Physical examination of the serum donors tested

Table 2. Representative data from 33 adult serum samples collected in Flores, Indonesia

Serum no.	Spleen score	1/IFA	% [^{3}H]Hyp incorporation*		% parasite stage distribution†			% of control parasitemias‡
			48 hr	36 hr	R	T	Sc	
Nonimmune, United States	0	0	100	100	0	36	64	100
Nonimmune, Flores	0	0	100	100	28§	9	62	100
1125¶	0	320	44	57	5	52	43	50
1603¶	1	1,280	53	53	3	70	27	38
1729	0	5,120	90	94	20§	16	64	22
4060	0	10,240	92	114	0	42	58	52
1105	1	2,560	70	82	0	30	70	48
2202	1	10,240	80	90	0	34	66	72
2203	1	5,120	70	73	0	32	68	35
1053	4	10,240	92	115	1	38	61	78
1574	4	2,560	53	87	11§	19	70	68
2412	4	5,120	78	100	3	34	63	55
4080	4	5,120	77	96	1	47	52	65
7138	4	20,480	4	92	11§	14	74	33
1820	5	10,240	107	100	0	32	68	63
4058	5	10,240	23	88	3	30	67	34
7111	5	10,240	21	97	0	23	77	13

Splenomegaly, a common feature, and high IFA titers against falciparum schizont antigens were not firmly associated with merozoite-invasion inhibition, which ranged from low to high. Significant, especially in comparison to Sudanese serum data represented in Table 1, was the general absence (except for serums 1125 and 1603) of intraerythrocytic parasite development retardation, i.e., CFF. In contrast, the Indonesian sera generally enhanced [^{3}H]Hyp incorporation and accelerated intracellular parasite development compared with pooled nonimmune sera from the United States.

*Percentage of [^{3}H]Hyp incorporation into parasite nucleic acids *in vitro* for 48 hr (schizont to schizont) or 36 hr (ring to schizont). Flores nonimmune sera incorporated [^{3}H]Hyp at a rate 147% times that of the nonimmune U.S. pool.

†Percentage distribution of parasite stages after 44 hr when cultures were initiated as synchronous schizonts; R, rings; T, trophozoites; Sc, schizonts.

‡An index of merozoite-invasion inhibition.

§New rings, apparently from accelerated parasite development.

¶Only Indonesian serum showing parasite development retardation.

in this study revealed Hackett spleen scores ≥ 2 in only 1% of the population; thus, splenomegaly was rare among Sudanese adults.

In contrast to the Sudanese sera, those collected from Flores had antiplasmodium IFA titers ranging between 1:320 and 1:20,480, with a modal titer of 1:10,240. Not all these sera showed inhibitory activity against cultured *P. falciparum*, and no firm relationship between IFA titer and antiparasitic activity was noted because some sera having Ig titers ≥ 1:20,240 were not inhibitory, whereas others with similar or lower IFA titers showed merozoite-invasion inhibition as high as 87% (Table 2). Nevertheless, no sera were completely inhibitory, and new rings were produced after each schizogonic cycle. Most remarkable, however, was the fact that the Indonesian sera generally lacked intraerythrocytic parasite development retardation—i.e., crisis-form activity. Two exceptions to this observation were sera 1125 and 1603, which showed moderate reductions of 43% and 47%, respectively in [^{3}H]Hyp incorporation into nucleic acids of parasites cultured 36 hr from ring to schizont stages. Giemsa-stained thin films of synchronous cultures containing 1:4 dilutions of these sera showed modest retardation of parasite development, but numerous normal looking schizonts were, nonetheless, seen in these cultures. Despite those two exceptions, and in marked contrast to the Sudanese sera, the Indonesian sera generally enhanced the incorporation of [^{3}H]Hyp into parasite nucleic acids, especially when incorporation data were compared with pooled, nonimmune sera obtained in the United States. Furthermore, many of the cultures grown in the Indonesian sera showed acceleration of parasite maturation so that, in synchronous cultures examined after 44 hr, the controls contained multinucleated schizonts, while some Indonesian serum cultures had already released merozoites and new ring-stages were present. In contrast to the patients from Sudan, who did not have splenomegaly, 64% of the Indonesian serum donors had massive splenomegaly with Hackett spleen grades of 4 or 5.

There were no firm associations between IFA titers, splenic sizes, and *in vitro* parasite inhibition discriminable within the Sudanese and Indonesian groups. IFA titers in Flores sera could not be used to predict anti-merozoite activity because some individuals with IFA titers ≥ 1:10,240 had little measurable inhibition.

DISCUSSION

Despite the lack of strong correlations between splenic sizes, IFA titers, and *in vitro* parasite inhibition within the Sudanese and Indonesian groups studied, there were marked differences between the two populations. The Sudanese had much lower anti-plasmodium IFA titers and essentially no adult splenomegaly; although merozoite-invasion-blocking activity was modest, their sera were profoundly inhibitory to intraerythrocytic parasite development, reducing [^{3}H]Hyp incorporation by 63–93% and severely retarding parasite development to produce crisis forms *in vitro*. In contrast, the Indonesian sera had modal anti-plasmodium IFA titers that were 8 times greater than those collected in Sudan, and 72% of the adults whose sera were examined showed splenomegaly; 64% of those with splenomegaly had Hackett grades 4 or 5. Furthermore, except for the two sera with modest CFF activity, the anti-parasitic activity of the Indonesian sera when present was directed against the extracellular merozoite, and not the intraerythrocytic stages.

In a previous study, we demonstrated that clinical immunity to falciparum malaria in Sudan was strongly associated with nonantibody factor(s) in the serum that induced intraerythrocytic retardation of parasite development, resulting in moribund crisis-form parasites. In some clinically immune individuals whose sera were profoundly inhibitory, no antiplasmodium IFA could be demonstrated, and no anti-merozoite activity was seen. The titer of the CFF was correlated with malaria incidence and increased in individuals after the annual rains, when malaria transmission was particularly high (3). Because crisis forms in rodent malaria models have been shown to be associated with activation of cell-mediated immune mechanisms and may result from the action of mononuclear cell secretions [lymphokines or monokines (5–9)], we propose that immunity based primarily on induction of crisis forms, as seen in Sudan, results from the action of secretory products of mononuclear cells. In contrast to the situation in Sudan, clinical immunity to malaria in Flores appears to result from merozoite-invasion-blocking antibodies, crisis forms being generally undemonstrable *in vitro*. Furthermore, Sudanese sera, which are rich in CFF and relatively poor in effective anti-merozoite Igs, are profoundly more inhibitory to the development of healthy schizonts in cultured parasites than are the Indonesian sera, which by comparison have much higher anti-plasmodium antibody titers but essentially no CFF activity. If our *in vitro* data accurately reflect clinical immunity, one might expect the Sudanese to be relatively more immune to malaria than the Indonesians studied because their sera were consistently more inhibitory to cultured parasites. Based on the 8-fold difference in IFA titers and marked splenomegaly seen in the Indonesians, such appears to be the case. In support of this argument, we propose the following hypothesis. If immunity to malaria is based principally on antibody, as it apparently is in Flores, it is possible that in the microenvironment of the capillaries, where merozoites are released in falciparum malaria, the first merozoites released could absorb Ig, reducing the titer below the critical concentration required to abrogate merozoite invasion. This is apparently the case in *Plasmodium berghei* infections in mice, where sequestration of parasitized erythrocytes in bone marrow sinusoids favors merozoite invasion by reducing merozoite–antibody interactions (10). Thus, in the absence of CFF, any successful merozoite invasions would result in a new parasite generation that could be inhibited only upon the release of the subsequent schizont progeny. Such an immune system could lead to chronic infections, with continuous antigenic stimulation resulting in high Ig titers and splenomegaly. On the other hand, immunity based principally on CFF, a possible product of cell-mediated immune mechanisms, with some merozoite-invasion inhibition would be much more efficient because any merozoites that succeeded in escaping the inhibitory activity of the antibody would be retarded in their development, producing noninvasive merozoites. Such were the conclusions of Taliaferro and Taliaferro, who noted the appearance of crisis-form parasites at the time when *Cebus capucinus* monkeys began to immunologically resolve infections with *Plasmodium brasilianum* (11). At its best, immunity based primarily on CFF could result in sterilizing immunity, thus IFA titers and the incidence of malaria-related splenomegaly would be low.

Although the differences in malaria immune responses between the two populations reported here are distinct, we cannot discount the role that epidemiologic parameters may play. For example, parasite ratios by plasmodium species in Flores are 45:45:10 for *P. falciparum*, *P. vivax*, and *P. malariae*, respectively, although there are numerous multiple-species infections. Malaria infections in Sudan due to *P. falciparum* are >95%. On the other hand, the differences in immune responses themselves may greatly influence the epidemiologic picture because in >5,000 blood films that we have examined in Sudan during the past 3 yr, only 2 had notable gametocytemia. Mature falciparum gametocytes were, however, a common finding in Flores. Thus, greater numbers of chronic infections may promote parasite transmission in Indonesia. Another explanation for our observa-

uons suggests a genetic basis for differences in the way these two populations respond to plasmodium parasite antigens. There is strong evidence that Afro-Americans without sickle-cell trait, glucose-6-phosphate dehydrogenase deficiency, or other hemoglobin anomalies are less susceptible to falciparum malaria and develop resistance to challenge that is not parasite-strain specific more readily than do Caucasian Americans (12). Furthermore, Afro-Americans fighting in Vietnam experienced significantly less problems with malaria (13) and had far fewer instances of chloroquine-resistant *P. falciparum* infections than did Euro-Americans (14). These observations, coupled with ours reported here, may suggest a genetic basis for different immune responses to malaria. One possibility is that Sudanese may possess a specific subset of T lymphocytes which, when stimulated by malaria parasite antigens, trigger a cell-mediated cascade resulting in the production of CFF, a different T-cell subset being responsible for B-cell activation that leads to protective anti-merozoite antibody production. The Indonesians, on the other hand, may lack such a T-cell subset responsible for cell-mediated responses; thus, parasite antigenic stimulation produces merozoite-invasion-blocking antibody as the principal immune mechanism. This is one testable hypothesis that may account for the differences we observed; other explanations are also plausible. Because it is apparent that immunity based primarily on CFF and secondarily on anti-merozoite antibody is more efficient at clearing parasite infections than immunity based solely on anti-merozoite antibody, this could explain the low anti-plasmodium antibody titers and lack of splenomegaly in Sudan. In contrast, Indonesians, who possess high anti-plasmodium antibody titers and massive splenomegaly, may have subclinical parasite infections whose constant antigenic stimulation maintains antibody concentrations sufficient to eliminate clinical symptoms but not the parasites—a condition known as premunition immunity.

In summary, we have demonstrated that clinical immunity to falciparum malaria in Flores, Indonesia, is, at least partially, based on merozoite-invasion-blocking antibody, in contrast to the situation in Sudan, where anti-merozoite antibodies apparently play a less important role but where non-antibody factors that retard intraerythrocytic parasite development, leading to crisis-form parasites, are of primary importance. If this latter mechanism is more efficient than the former, it may provide an explanation for why Sudanese have relatively low anti-plasmodium titers and spleen size as compared to Florenese. These findings are likely to have wide ranging implications for efforts to develop an effective vaccine against blood-stages of *P. falciparum*.

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1. Jensen, J. B., Boland, M. T. & Akood, M. A. S. (1982) *Science* **216,** 1230–1232.
2. Jensen, J. B., Boland, M. T., Hayes, M. & Akood, M. A. S. (1982) *Exp. Parasitol.* **64,** 416–424.
3. Jensen, J. B., Boland, M. T., Allan, J. S., Carlin, M., Vandewaa, J. A. & Divo, A. A. (1983) *Infect. Immun.* **41,** 1302–1311.
4. Bruce-Chwatt, L. J. (1980) *Essential Malariology* (William Heinemann Medical Books, London), p. 143.
5. Clark, I. A., Allison, A. C. & Cox, F. E. G. (1976) *Nature (London)* **259,** 309–311.
6. Clark, I. A., Cox, F. E. G. & Allison, A. C. (1977) *Parasitology* **74,** 9–18.
7. Clark, I. A., Virelizer, J. L., Carswell, E. A. & Wood, P. R. (1981) *Infect. Immun.* **32,** 1058–1066.
8. Taverne, J., Dockrell, H. M. & Playfair, H. L. (1981) *Infect. Immun.* **33,** 83–89.
9. Murphy, J. R. (1981) *Infect. Immun.* **33,** 199–211.
10. Wiess, L. (1983) *J. Parasitol.* **69,** 307–318.
11. Taliaferro, W. H. & Taliaferro, L. G. (1944) *J. Infect. Dis.* **75,** 1–32.
12. Pazzaglia, G. & Woodward, W. E. (1982) *Am. J. Trop. Med. Hyg.* **31,** 202–210.
13. Powell, R. D., McNamara, J. V. & Rieckmann, K. H. (1972) *Proc. Helminthol. Soc. Wash.* (Special Issue) **39,** 51–66.
14. Hall, A. P. & Canfield, C. J. (1972) *Proc. Helminthol. Soc. Wash.* (Special Issue) **39,** 66–74.