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Serum Inhibition of Merozoite Dispersal from *Plasmodium falciparum* Schizonts: Indicator of Immune Status

THEODORE J. GREEN,†* MARTHA MORHARDT, ROBERT G. BRACKETT, AND RICHARD L. JACOBS

Clinical Immunology, Pharmaceutical Research Division, Warner-Lambert Company, Detroit, Michigan 48232

Specific immune serum was found to inhibit the dispersal of the merozoites from mature schizonts, thus interfering with their subsequent reinvasion of new host erythrocytes. This phenomenon is viewed as a protective mechanism against malaria which can be measured in vitro and reflects the immune status of the donor.

Malaria, a severe hemotropic disease of man caused by intraerythrocytic protozoan parasites of the genus *Plasmodium*, is undergoing a worldwide resurgence after widespread efforts for eradication or control. The reasons for this resurgence are complex, but include the development of resistance by the parasites to chemotherapeutic agents, resistance of the anopheline vectors to various insecticides, and numerous political and socioeconomic factors. The development of an effective malaria vaccine is eagerly sought as an adjunct to existing control methods for malaria. A major impediment to the development of malaria vaccines has been the inability of investigators to accurately assess the immune status of individuals by serological methods. Perhaps this is because resistance to malaria most probably involves not only serum factors, but possibly cell-mediated resistance factors as well. Nevertheless, in vitro tests of serum inhibition of reinvasion by *Plasmodium falciparum* (the most lethal malarial parasite of humans) have shown some promise as indicators of the immune status of individual serum donors (3, 12, 14). We present here a new method for the evaluation of the standard type of growth and reinvasion inhibition test. This method is based upon immune serum-mediated inhibition of dispersal of merozoites from schizonts in synchronous cultures. We have termed the nondispersed schizonts "inhibited schizonts" and used their abundance in the evaluation of in vitro parasite reinvasion inhibition tests.

MATERIALS AND METHODS

Parasite cultures. Our method of stationary culture in candle jars is patterned after that described by Trager and Jensen (15) and will not be presented in detail here. Single-stage synchrony of the parasitized erythrocytes was achieved by pretreatment with sorbitol by the method of Lambros and Vanderberg (9), which spares ring-form trophozoites but destroys older stages. The washed, parasitized cells, now containing only ring-form trophozoites, were then adjusted to a hematocrit of 3% in complete RPMI 1640 medium at a parasitemia of 1 ± 0.5%. The cultures were placed in either 35-mm plastic petri dishes at 1.5-ml volumes or, to conserve serum, in 17-mm wells in multiwell plastic plates at 0.5-ml volumes. All tests were performed in duplicate.

Sera. *Aotus* monkeys which had recovered from induced *P. falciparum* infection were used as the immune serum donors. Seronegative *Aotus* monkeys with no history of malaria infection were used as the nonimmune controls. *Aotus* monkey sera used in the test were included in the RPMI 1640 culture medium at concentrations of 10 or 50%. All sera were heat inactivated at 56°C for 30 min and absorbed with human erythrocytes before incorporation into the medium.

Microscopic examination. Parasitemia and the numbers of inhibited schizonts in the cultures were determined by direct light microscopic examination of methanol-fixed and Giemsa-stained blood films taken at 24-h intervals at the time of medium replacement. In the data presented, the rate of parasitemia is the total number of ring-form trophozoites present in 10,000 erythrocytes, and the number of inhibited schizonts is the number of these forms present per 10,000 erythrocytes.

In vivo challenge. Five *Aotus* monkeys of karyotypes II and III (10) that had previously recovered from infections with *P. falciparum* (FCR-3/FMG) (8) were inoculated intravenously with 10^6 homologous live parasites in *Aotus* erythrocytes (karyotype IV) (Table 1). None of the test animals had previous exposure to karyotype IV erythrocytes. The animals were then examined daily by Giemsa-stained thick and thin blood smears for evidence of parasitemia. Sera taken before inoculation with live parasites were tested in the in vitro parasite inhibition test described herein.

† Present address: Veterinary Microbiology, College of Veterinary Medicine, University of Missouri-Columbia, Columbia, MO 65211.

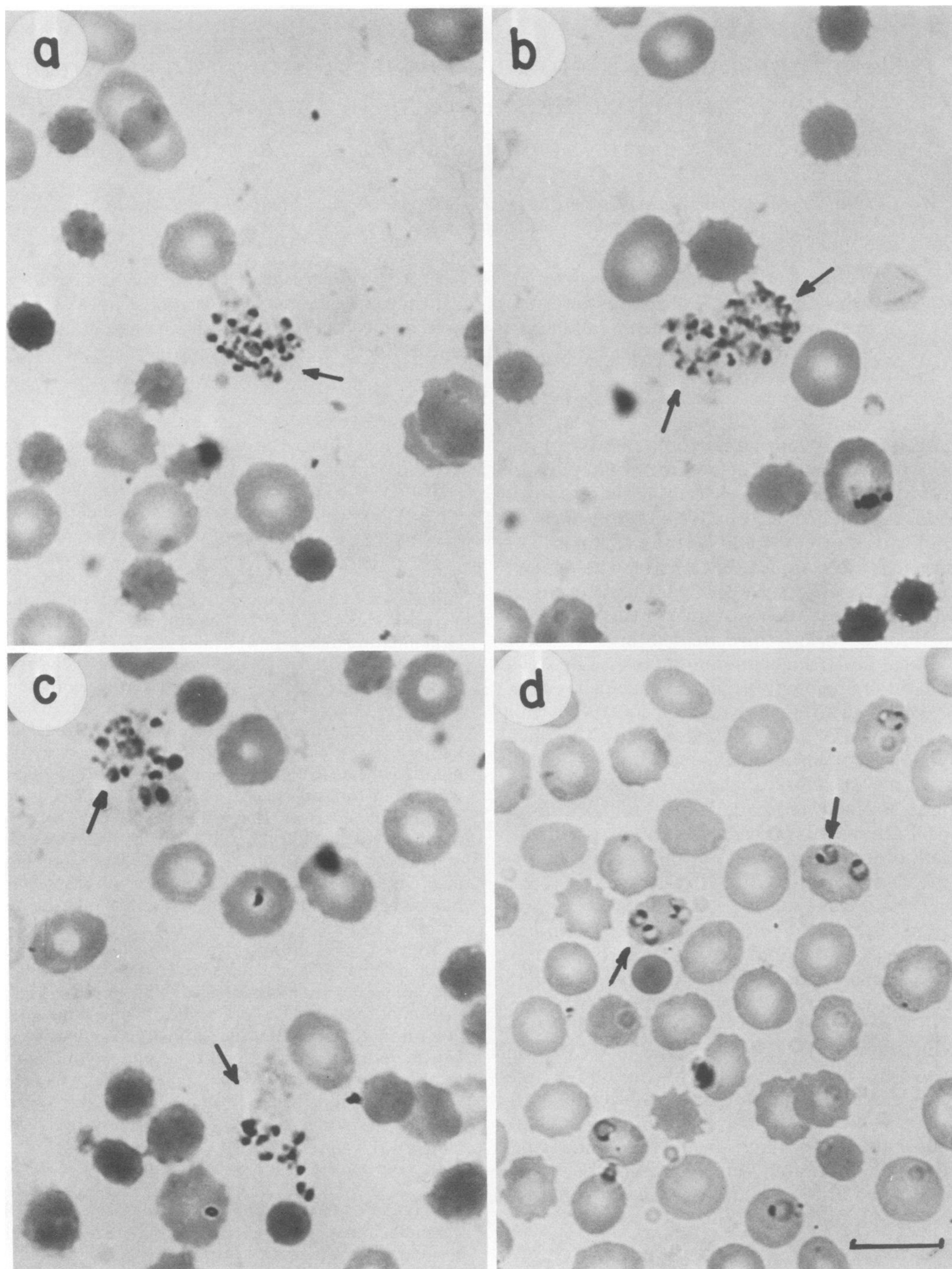

FIG. 1. *Photomicrographs of Giemsa-stained blood films of P. falciparum FCR-3 (African strain) infected human erythrocytes from 48-h cultures. RPMI 1640 culture medium containing 50% Aotus monkey serum immune to P. falciparum FCR-3 was used in a, b, and c; note inhibited schizonts (arrows). RPMI 1640 culture medium containing 50% normal nonimmune Aotus monkey serum served as a control (d). Note the examples of normal ring-form trophozoites (arrows). Bar, 10 μm.*

RESULTS

Photomicrographs of 48-h cultures. Figures 1a, b, and c are photomicrographs of typical inhibited schizonts (arrows) from 48-h cultures containing RPMI 1640 culture medium plus 50% *Aotus* monkey serum immune to *P. falciparum* FCR-3/FMG. Figure 1d shows the ring-form trophozoites (arrows) which predominate in comparable control cultures containing RPMI 1640 plus 50% nonimmune *Aotus* monkey serum.

Parasite reinvasion. Figure 2 compares the relative inhibition of parasite reinvasion in cultures containing RPMI 1640 culture medium plus 10 or 50% immune *Aotus* monkey serum with that of control cultures containing RPMI 1640 culture medium plus 10 or 50% nonimmune *Aotus* monkey serum. Each data pair consists of the starting parasitemia and the final 48-h parasitemia after reinvasion. Parasites were presynchronized by the sorbitol method so that the data represented ring-form trophozoites for both sampling times. All data points are averages of duplicate experiments. The data were derived by determining the total numbers of parasites per 10,000 erythrocytes. In no case was inhibition of reinvasion complete.

Schizont inhibition. Figure 3 presents data based upon the relative abundance of inhibited schizonts after 48 h of culture in the presence of immune versus nonimmune *Aotus* monkey sera at either 10 or 50% concentration in the culture medium. Each data point is the mean of duplicate experiments and was derived by determining the percentage of ring-form trophozoites which were arrested upon maturity as inhibited schizonts, using the following formula: % inhibition = [(number of inhibited schizonts)/(10,000 cells on day 2)]/[(number of ring-form trophozoites)/(10,000 cells on day 0)] $\times$ 100. Inhibited schizonts failed to disperse their merozoites and therefore were still present in 48-h cultures, when merozoites from noninhibited schizonts had successfully reinvaded and differentiated to ring-form trophozoites.

TABLE 1. *History of P. falciparum infection in Aotus monkeys*

Monkey	Karyotype[a]	Date infected	No. of recrudescences (date of most recent occurrence)
B3	III	Dec. 1977	2 (Apr. 1978)
B5	III	Mar. 1978	3 (Sep. 1978)
B9	III	Nov. 1977[b]	5 (Jun. 1978)
B20	II	Feb. 1979	1 (Apr. 1979)
B21	III	Apr. 1978	None

[a] See Ma et al. (10).
[b] Splenectomized.

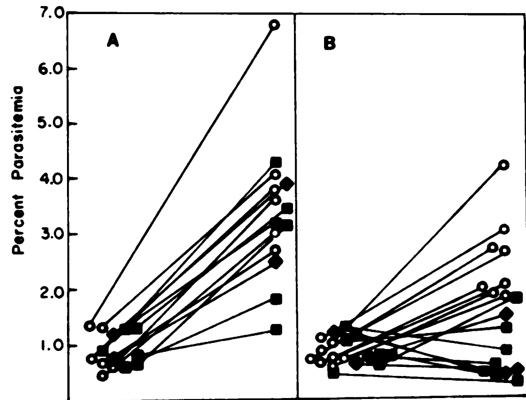

FIG. 2. *Reinvasion test data. Parasitemias are of P. falciparum FCR-3 (African strain) grown for 48 h in in vitro candle jar cultures in RPMI 1640 culture medium containing 10 (A) or 50% (B) Aotus monkey serum. Each data pair consists of the starting parasitemia and the final 48-h parasitemia after reinvasion. Parasites were presynchronized by the sorbitol method so that the data represent predominantly ring-form trophozoites for both sampling times. The sera used were nonimmune Aotus (○), P. falciparum FCR-3 (African) immune Aotus (■), and P. falciparum FCR-1/FVO (Asian) immune Aotus (◆). All data points are averages of duplicate experiments. The data were derived by determining the total number of parasites present per 10,000 erythrocytes on Giemsa-stained blood films from the cultures.*

One asexual reproductive cycle of *P. falciparum* takes 48 h. As each inhibited schizont is a mature parasite developed from an individual ring-form trophozoite of the same generation, the degree of inhibition by immune serum can be calculated without consideration of the reinvasion efficiency of merozoites or the average number of merozoites per schizont. By assuming that any schizont prevented from dispersing its merozoites constitutes a unit of protection, the test can be applied to the problem of serological evaluation of immune status. From examination of the data in Fig. 3, it is apparent that the number of inhibited schizonts in the cultures is related to the immune serum concentration in the culture. The data presented in Fig. 2 and 3 were obtained from the same sets of specimen slides and represent the same experiments.

In vivo challenge. The results of the in vivo challenge and the corresponding in vitro parasite inhibition test values are presented in Table 2.

Several important findings have emerged from this study. First, it is apparent that the ability of immune serum incorporated in the culture medium to interfere with merozoite invasion of erythrocytes is concentration dependent. There

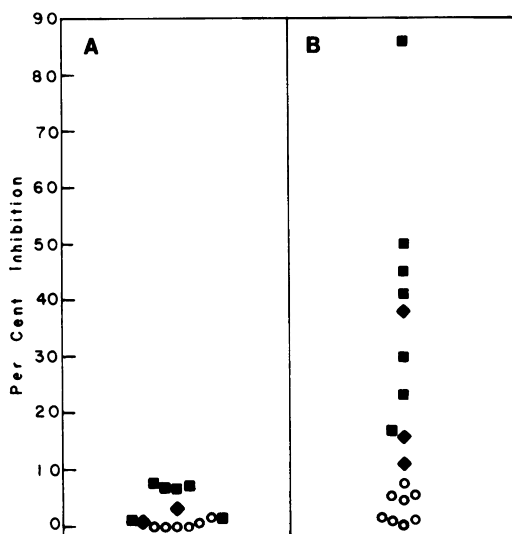

FIG. 3. *Schizont inhibition data. P. falciparum FCR-3 (African strain) parasites were grown for 48 h in in vitro candle jar cultures in RPMI 1640 culture medium containing 10 (A) or 50% (B) Aotus monkey serum. Each data point is the mean of duplicate experiments and was derived by determining the percentage of ring-form trophozoites which were arrested upon maturity as inhibited schizonts, using the following formula: % inhibition = [(number of inhibited schizonts)/(10,000 cells on day 2)]/[(number of ring-form trophozoites)/(10,000 cells on day 0)] × 100. Inhibited schizonts failed to disperse their merozoites and therefore were still present in 48-h cultures, when merozoites which successfully reinvaded had differentiated to ring-form trophozoites. The sera used were nonimmune Aotus (○), P. falciparum FCR-3 (African) immune (■), or P. falciparum FCR-1/FVO (Asian) immune (◆). The data shown in Fig. 2 and 3 were obtained from the same sets of specimen slides and represent the same experiments.*

was no significantly greater inhibition of reinvasion in the presence of 10% immune serum than in the presence of nonimmune serum ($P = 0.166$); however, at a 50% immune serum concentration, there was a significantly greater inhibition of reinvasion in the immune than in the nonimmune groups ($P < 0.001$). Second, interpretation of an individual test on the basis of reinvasion alone could be difficult since parasitemias in some cultures rose somewhat during the 48 h of incubation, whereas others fell, even though reinvasion in medium containing 50% immune serum was less than with nonimmune serum. The data shown in Fig. 2 demonstrate this point. Third, the presence of inhibited schizonts in a 48-h culture is strongly correlated with the concentration of immune serum in the culture. The average percentage of a given generation of parasites arrested as inhibited schizonts in medium containing 10% nonimmune serum was 0.3%, whereas in the same concentration of homologous FCR-3 immune *Aotus* monkey serum, the average value was 5.0% ($P < 0.01$), and in heterologous FCR-1/FVO (8) immune *Aotus* monkey serum, the value was 1.8%. The average percentage of a single parasite generation arrested as inhibited schizonts in medium containing 50% nonimmune serum was 3.2%, whereas in the same concentration of homologous FCR-3 immune serum, the average value was 41.9% ($P < 0.001$), and in heterologous FCR-1/FVO immune *Aotus* monkey serum, the percentage was 21.5% (Fig. 3). Although the ranges of activity of homologous and heterologous sera overlap, there appears to be less effect associated with heterologous serum. It is of considerable interest that antiserum against the FCR-1/FVO Asian strain of *P. falciparum* appears to possess measurable protective activity against the heterolo-

TABLE 2. *Results of the in vitro parasite inhibition test and in vivo Aotus monkey challenge*[a]

Monkey	Ring-form trophozoites/10,000 erythrocytes		Inhibited schizonts/10,000 erythrocytes on day 2	% schizont inhibition	Results of in vivo challenge
	Day 0	Day 2			
B3	130 (95)[b]	130 (240)	31 (0.5)	24 (<1)	Rare parasites on days 24 and 25
B5	120 (110)	45 (305)	50 (1.0)	42 (<1)	No parasites found
B9[c]	65 (60)	40 (305)	26 (0.5)	40 (<1)	Parasitemia on days 8–21
B20	65 (100)	60 (390)	32 (2.5)	49 (2.5)	No parasites found
B21	85 (130)	110 (735)	33 (7.0)	39 (5.3)	No parasites found

[a] These in vitro parasite inhibition tests were performed with 50% immune or nonimmune *Aotus* monkey serum. Sera used in these tests were collected during April, May, and June 1979. All animals were challenged on 24 March 1980.

[b] Nonimmune control in vitro data are shown within parentheses.

[c] Monkey B9 was splenectomized and infected during November 1977. There were five subsequent recrudescences, with the last occurring during June 1978. Suppressive drug intervention was required during the initial infection and the last three recrudescences.

gous FCR-3/FMG African strain of parasites in vitro.

DISCUSSION

It has been demonstrated that bivalent antibodies are necessary to prevent reinvasion of malaria parasites in vitro (5), a finding that is consistent with the suggestion that merozoite agglutination is associated with reduced reinvasion (11). Complement seems to be unimportant in the protective immune response to malaria (1, 6, 16), and its addition had no effect on previous test results in this laboratory. Phagocytosis of the agglutinated parasites seems to be the method of their elimination (2, 4, 7). We believe that immune antibody is acting in vitro just before or at the time of schizont rupture by entering the cell and agglutinating the merozoites, thereby interfering with their dispersal for reinvasion. This mechanism requires that specific antimerozoite antibody is present in sufficient concentration to accomplish this action before dispersal can occur. We have demonstrated immune antibody to be associated with inhibited schizonts by the indirect fluorescent antibody technique (unpublished data). If such inhibition occurs in vivo, resulting in diminished numbers of merozoites successfully reinvading new host erythrocytes, then the presence of inhibited schizonts in vitro can be considered a direct indicator of protection. Our work has been done with antibody raised against *P. falciparum* in *Aotus* monkeys. Phillips et al. (13) noted the presence of "increased numbers of abnormal segmenters which appeared to be lysed" in their in vitro reinvasion studies utilizing *P. falciparum* immune human serum. We believe that the abnormal segmenters observed by Phillips et al. (13) in cultures containing immune human serum are similar to the inhibited schizonts we have observed in these studies and that their numbers can be used as a simple estimate of the protective potency of immune serum while providing some insight into the specific immune mechanisms involved.

The *Aotus* monkeys in the in vivo challenge experiment presented herein all had significant serum levels of inhibitory activity in the in vitro parasite inhibition test. At 24%, monkey B3 had the lowest schizont inhibition value, and a subpatent infection after challenge was detected. Protection was nevertheless adequate to prevent a patent parasitemia. Monkey B9, with a schizont inhibition value of 40%, exhibited a patent parasitemia of 14-day duration. Since this monkey was splenectomized, the role of the spleen for the rapid clearance of parasites from the blood in the presence of strong serum inhibitory activity is apparent. Intact monkeys B5, B20, and B21 had in vitro serum inhibitory activity comparable to B9 and did not develop a detectable parasitemia after challenge. Immunity to malaria is known to fade rapidly after radical cure of infection. Chronic subpatent infections (premunition), however, could supply the necessary antigenic stimulation to maintain an adequate state of immunity. Premunition is recognized in malaria endemic areas where the adult population is in balance with its malaria parasites. Since merozoites associated with inhibited schizonts are not lysed, some low level of escape and reinvasion by relatively few merozoites could serve to fine tune the premune state and would be advantageous to both the host and a virulent parasite such as *P. falciparum*. Four of the monkeys in this study had recrudesced from one to four times during the 8 months after their initial infections, with no detectable parasitemias between recrudescences. None had recrudescences detected for 1 year before this in vivo challenge study; however, the possibility remains that premunition may have persisted in these animals throughout that period. This could account for the persistent level of resistance to challenge observed in this study.

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