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Monoclonal Antibodies Produced against Sporozoites of the Human Parasite *Plasmodium malariae* Abolish Infectivity of Sporozoites of the Simian Parasite *Plasmodium brasilianum*

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We have used a sporozoite neutralization assay to define the biological relevance of the cross-reactivity of two monoclonal antibodies, raised against sporozoites of the human parasite *Plasmodium malariae* (Uganda 1/CDC), with sporozoites of the simian parasite *Plasmodium brasilianum* (Colombian). In vitro incubation of each of these two monoclonal antibodies with sporozoites of *P. brasilianum* totally abolished the infectivity of these parasites for *Saimiri sciureus*. Using Western blot analysis and one of the *P. malariae* monoclonal antibodies, we identified two sporozoite proteins characteristic of the Colombian isolate of *P. brasilianum* with apparent molecular weights of 56,000 and 66,000. The same monoclonal antibody identified two proteins in an extract of the Peruvian isolate of *P. brasilianum* with apparent molecular weights of 59,000 and 69,000.

Malaria sporozoites, when inoculated into recipient hosts, induce formation of antisporozoite antibodies which react with the membrane of the sporozoite, as shown by the circumsporozoite (CS) precipitation reaction (20), by indirect immunofluorescence (14), and by ultrastructural studies (1, 3, 10). This humoral response, which is stage and species specific, plays a major role in protection against sporozoite-induced malaria infection (5).

Using monoclonal antibodies (moABs) produced against sporozoites of rodent (21), simian (7), and human malaria parasites (15), we have shown that the target antigen for the protective response is a membrane-associated protein which uniformly and densely covers the surface of sporozoites. These antigens, which have been designated CS proteins, arise from intracellular precursor polypeptides (7, 22). The antisporozoite immune response is in large part directed against an immunodominant region of the CS protein containing a repetitive epitope (23). CS proteins, like the immunity they induce, are stage specific, and in all studies to date on the human malaria parasites *Plasmodium vivax* and *P. falciparum*, they have been found to be strictly species specific, but not strain specific (15).

Recently we showed that moAB produced against sporozoites of the human malaria parasite *Plasmodium malariae* recognize sporozoites of the New World simian parasite *Plasmodium brasilianum* in a two-site immunoradiometric assay (4), indicating a close similarity or identity of epitopes of the CS proteins of these two species. In this present report, we have defined the biological relevance of this cross-reactivity by demonstrating loss of infectivity of *P. brasilianum* sporozoites after their in vitro incubation with *P. malariae* antisporozoite moAB. We have also compared the electrophoretic mobility of the CS proteins of two isolates of *P. brasilianum*.

MATERIALS AND METHODS

Sporozoites. With previously described methodology (19), sporozoites of the Uganda 1/CDC isolate of *P. malariae* (8)

were recovered from the salivary glands and midguts of *Anopheles freeborni* mosquitoes which had been membrane fed (17) 15 to 21 days earlier on blood of infected owl monkeys (*Aotus trivirgatus*). Salivary gland sporozoites of both the Colombian and Peruvian isolates of *P. brasilianum* were recovered from *A. freeborni* mosquitoes which had fed 13 to 18 days earlier on infected squirrel monkeys (*Saimiri sciureus*).

Immunization of mice and production of hybridomas. Production of hybridomas and purification of antisporozoite moABs were as previously described (12, 16). Adult BALB/c mice were immunized by multiple intravenous inoculations of a total of 1.2×10^5 viable midgut and salivary gland sporozoites of *P. malariae* or 3.0×10^5 salivary gland sporozoites of *P. brasilianum*. Two *P. malariae* antisporozoite moABs (6B10, an immunoglobulin G1 [IgG1], and 4E8, an IgM) were purified from mouse ascites by salt precipitation and molecular sieve chromatography.

Western immunoblot analysis. To identify CS proteins we used Western blot analysis (18) with minor modifications of our previously described methodology for detection of CS proteins (6). Sporozoite extract in sample reducing buffer was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13) and then electroblotted to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.). The membrane was incubated overnight with phosphate-buffered saline (PBS) containing 5% nonfat powdered milk (11) and then incubated for 2 h either with purified *P. malariae* moAB at a concentration of 20 μg/ml in PBS or with mouse immune serum, raised against *P. brasilianum* sporozoites, at a final dilution of 1:50 in the same buffer. Controls were incubated under the same conditions with either a non-cross-reacting moAB (3D11) or an immune serum produced against sporozoites of the rodent parasite *Plasmodium berghei*. All preparations were washed three times with PBS-milk and then incubated for 2 h with 1.0×10^6 cpm of affinity-purified goat anti-mouse ^{125}I-labeled immunoglobulin. After being washed in PBS–0.05% Tween 20, the preparations were autoradiographed at −70°C with X-Omat AR film and an image-intensifying screen.
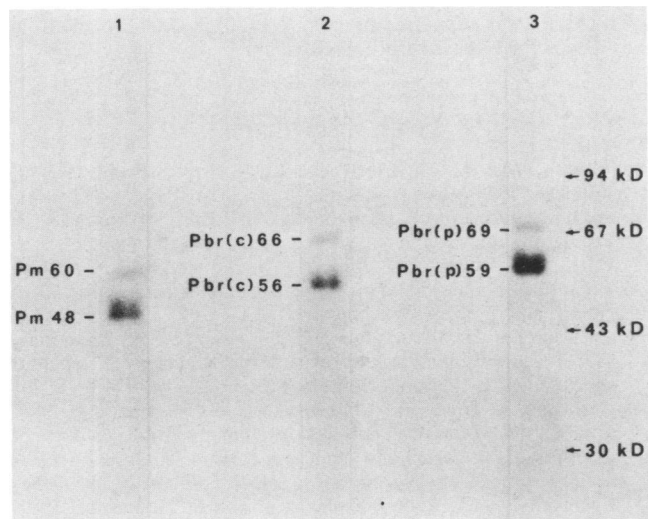
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FIG. 1. Western blot analysis of sporozoite extracts of *P. malariae* and *P. brasilianum* as described in the text. The *P. malariae* antisporozoite moAB 6B10 identified two *P. malariae* (Uganda 1/CDC) sporozoite proteins (lane 1) with apparent molecular weights of 48,000 (Pm48) and 60,000 (Pm60). The same moAB identified two sporozoite proteins, in an extract of the Colombian isolate of *P. brasilianum* (lane 2), with apparent molecular weights of 56,000 [Pbr(c)56] and 66,000 [Pbr(c)66]; and two proteins, in an extract of sporozoites of the Peruvian isolate of *P. brasilianum* (lane 3), with apparent molecular weights of 59,000 [Pbr(p)59] and 69,000 [Pbr(p)69].

Sporozoite neutralization assay. In a first experiment, infective sporozoites of the Colombian isolate of *P. brasilianum* were incubated in vitro, for 60 min at room temperature, in a mixture of both of the purified anti-*P. malariae* sporozoite moABs at a final concentration of 3.5 mg/ml in PBS containing 20% normal monkey serum. In a second experiment, the two purified moABs were assayed separately under the same experimental conditions. Control sporozoites were incubated either with PBS containing 20% normal monkey serum or with an unrelated moAB at a final concentration of 3.5 mg/ml in PBS containing 20% normal monkey serum.

Squirrel monkeys of either Guyanan or Bolivian origin (experiments 1 and 2, respectively) were injected intravenously with 2.5 × 10^4 of the treated sporozoites to test for loss of parasite infectivity. Nine days after sporozoite inoculation, the animals were splenectomized. Beginning on day 14 after sporozoite injection, the animals were examined daily for the presence of parasites by both thick and thin blood films.

RESULTS

Identification of CS proteins. We used Western blot analysis and the *P. malariae* moAB 6B10 to determine the molecular weights of the CS proteins of two isolates of *P. brasilianum* (Fig. 1). This was based on the fact that we had previously shown that moAB 6B10 and our second *P. malariae* moAB, 4E8, identified the same *P. malariae* CS protein and reacted with the same epitope (Cochrane et al., unpublished data).

Immune serum raised in mice against sporozoites of the Colombian isolate of *P. brasilianum* (data not shown) and moAB 6B10 identified two proteins, characteristic of this isolate, with apparent molecular weights of 56,000 [Pbr(c)56]

and 66,000 [Pbr(c)66] (Fig. 1, lane 2). The same immune serum (data not shown) and moAB 6B10 identified two sporozoite proteins in extract of the Peruvian isolate with apparent molecular weights of 59,000 [Pbr(p)59] and 69,000 [Pbr(p)69] (Fig. 1, lane 3). These proteins were not recognized by moAb 3D11 raised against sporozoites of *P. berghei* (data not shown). moAB 6B10, in the same preparation, identified the *P. malariae* (Uganda 1/CDC) CS proteins with molecular weights of 48,000 (Pm48) and 60,000 (Pm60) (Fig. 1, lane 1).

P. malariae moAB induces loss of infectivity of *P. brasilianum* sporozoites. Based on the observations that the *P. malariae* moAB cross-reacts with sporozoites of *P. brasilianum* in a two-site immunoradiometric assay (4) and by Western blotting, we assayed the two *P. malariae* moABs for their ability to abolish infectivity of sporozoites of the Colombian isolate of *P. brasilianum*.

The sporozoite neutralizing activity of both moABs was determined in two experiments (Table 1). In experiment 1, two squirrel monkeys received sporozoites incubated in a mixture of both moABs (6B10 and 4E8) at a concentration of 3.5 mg/ml in PBS containing 20% normal monkey serum. Both animals failed to develop a patent infection up to day 81 after sporozoite inoculation, when the last blood smears were taken. Parasites were detected in the blood of two control animals on day 21 after inoculation. In experiment two, the two moABs were tested individually at a final concentration of 3.5 mg/ml, and both were found to abolish sporozoite infectivity. Blood smears of the experimental animals were negative for parasites up to day 96 after inoculation. Both control animals, injected with sporozoites incubated with an unrelated moAB, developed a parasitemia 36 days postinoculation.

DISCUSSION

A close relationship between the human malaria parasite *P. malariae* and the simian parasite *P. brasilianum* has long been indicated by observations on the development and morphology of the sporogonic stages in mosquitoes as well as on exoerythrocytic and erythrocytic stages in the primate hosts (2). In addition, *P. brasilianum* is readily transmitted under experimental conditions to humans by both mosquito bites (9) and inoculation of parasitized blood cells (2). These features led Coatney et al., in their treatise on the primate malarias, to propose that *P. brasilianum* may actually be a strain of *P. malariae* which became adapted to New World monkeys sometime after the early 1600s (2).

TABLE 1. *P. brasilianum* sporozoites lose infectivity for *S. sciureus* monkeys upon incubation with *P. malariae* antisporozoite moAB

Expt. no.	Animals[a] (no.)	Sporozoite incubation[b]	Prepatent period (days)
1	Experimental (2)	moAB 6B10 + 4E8	−,[c] −
	Control (2)	PBS	21, 21
2	Experimental (2)	moAB 6B10	−, −
	Experimental (2)	moAB 4E8	−, −
	Control (2)	Unrelated moAB	36, 36

[a] Each monkey was injected intravenously with 2.5 × 10^4 sporozoites.
[b] moABs were at a final concentration of 3.5 mg/ml in PBS; all incubation media contained 20% normal monkey serum.
[c] −, Negative.

Our immunological data strongly support this previously recognized relationship between *P. malariae* and *P. brasilianum*. Recently we showed that an moAB, raised against sporozoites of the Uganda 1/CDC isolate of *P. malariae*, reacted with an extract of sporozoites of the Colombian isolate of *P. brasilianum* in a two-site immunoradiometric assay (4). Our current observation that the same purified anti-*P. malariae* moAB and a second anti-*P. malariae* moAB abolish by in vitro incubation the infectivity of sporozoites of *P. brasilianum*, albeit for a small number of squirrel monkeys, defines the biological relevance of this cross-reactivity and most likely indicates the close similarity or identity of the repeated epitopes of the CS proteins of these two parasites. Nucleotide sequencing of the CS protein-encoding genes of *P. malariae* and *P. brasilianum* will ultimately define the precise relationship between the CS proteins of the two parasites.

Previously we demonstrated recognition of a simian malaria (*Plasmodium cynomolgi*) sporozoite antigen by moABs produced against sporozoites of another simian (*P. knowlesi*) parasite by using the CS precipitation reaction and indirect immunofluorescence as well as immunoprecipitation of metabolically labeled sporozoite antigen (7). The cross-reacting antibody titers, in this instance, were considerably lower than the homologous titers. These *P. knowlesi* moABs failed to abolish infectivity of the *P. cynomolgi* sporozoites. In the present study, the immunofluorescent antibody titers of the *P. malariae* moABs were comparable with heat-fixed sporozoites of either *P. malariae* or *P. brasilianum* as the antigen $(1: \geq 50,000)$.

The considerable variation in the prepatent periods of the control animals in the two sporozoite neutralizing experiments may reflect differences in the infectivities of the sporozoites used. Alternatively, it may reflect a difference in the susceptibility of squirrel monkeys from Guyana (karyotype 14-7) and from Bolivia (karyotype 12-6) to sporozoite-induced *P. brasilianum* infections. A large variation in prepatent periods in sporozoite-induced *P. brasilianum* infections in primates has previously been reported (2).

An interesting observation was the difference in the apparent molecular weights of the CS proteins of *P. malariae* and of the Colombian and Peruvian isolates of *P. brasilianum*. A similar observation, involving the apparent conservation of an immunodominant epitope with shifts in molecular weights of CS proteins, has previously been documented for a series of isolates of both *P. vivax* and *P. falciparum* (F. Zavala, A. Masuda, P. H. Graves, V. Nussenzweig, and R. S. Nussenzweig, J. Immunol., in press). It remains to be established whether these phenomena reflect differences in the numbers of repeated epitopes of the CS proteins, or whether their molecular basis lies outside the repetitive immunodominant epitope-encoding region of the CS protein gene.

A close immunological relationship of the immunodominant epitopes of the CS proteins of *P. malariae* and *P. brasilianum* has been established. This feature, combined with the possibility of using squirrel monkeys as an experimental animal susceptible to sporozoite-induced *P. brasilianum* infections, should facilitate development of a synthetic *P. malariae* antisporozoite vaccine.

ACKNOWLEDGMENTS

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