Blood Stage-Induced *Plasmodium brasilianum* Infection in the Squirrel Monkey Induces Antibodies Which React with the Circumsporozoite Protein

ALAN H. COCHRANE* and MARILYN MARACIC

Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York 10016

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A blood stage-induced *P. brasilianum* infection in a naive squirrel monkey induced antibodies which reacted with the circumsporozoite protein of the parasite. Titers increased with duration of infection and persisted for 3 months after cure. In an immunoblot, these antibodies detected two polypeptides with molecular weights identical to those of the circumsporozoite protein and its precursor.

The surface membrane of plasmodial sporozoites is covered by an antigen designated the circumsporozoite (CS) protein. The CS proteins have been considered to be differentiation antigens and to induce a stage-specific protective immune response. The repeat region of CS proteins is immunodominant and has formed the basis of subunit vaccines for human malaria vaccine trials (reviewed in reference 9).

We recently documented the presence of the CS protein of various plasmodia, including *P. brasilianum*, in the micronemes of blood-stage merozoites (3). That study also showed that a blood stage-induced *P. berghei* infection in mice generates antibodies which react with the corresponding sporozoites. We now present evidence that a blood stage-induced *P. brasilianum* infection in a squirrel monkey induces the formation of antibodies which react with *P. brasilianum* sporozoites and their CS repeats.

An intact laboratory-born juvenile squirrel monkey (*Saimiri sciureus*) was inoculated with frozen blood stages of *P. brasilianum* (Colombian). We used a laboratory-born animal because a large proportion of *S. sciureus* caught in the wild have naturally acquired *P. brasilianum* infection(s) and antibodies which could interfere with our results. Blood samples were obtained prior to inoculation and at 2-week intervals for determination of parasitemia and serology. The animal was treated with halofantrine 40 weeks after the onset of parasitemia. Blood smears were prepared and serum samples were obtained for three additional months after cure by drug treatment. No parasites were detected during this period.

The tetrapeptide (NAAG), representing the CS repeat of *P. malariae*/*P. brasilianum* (7), was used as antigen in an enzyme-linked immunosorbent assay (ELISA) (14) to detect anti-CS repeat antibodies. Peptide-coated microtiter plates were sequentially incubated with the monkey serum and affinity-purified rabbit immunoglobulin to squirrel monkey immunoglobulin G (1 µg/ml). Bound antibody was detected by incubation with peroxidase-conjugated anti-rabbit immunoglobulin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) and ABTS (2,2-azino-di[3-ethylbenzthiazoline sulfonate]).

Indirect immunofluorescence with glutaraldehyde-fixed sporozoites (8) and the CS precipitation reaction (13) were also used to detect anti-CS antibodies in the squirrel monkey serum.

For Western immunoblot, sporozoites were placed directly into sodium dodecyl sulfate (SDS) sample buffer and subjected to electrophoresis on a 10% acrylamide gel (2). This analysis was performed with a 1:50 dilution of monkey serum raised against blood stages of *P. brasilianum* and a purified monoclonal antibody (MAb) that reacted with the CS protein of *P. brasilianum* (10 µg/ml) as a control. Antibodies reactive with sporozoites, absent prior to the inoculation of the animal, occurred as early as 8 weeks postinoculation and were detected by both indirect immunofluorescence and ELISA (Fig. 1). With increasing duration of parasitemia the antibody titers increased, reaching a maximum of 1:5,120 approximately 5 months after initiation of parasitemia. These antibodies persisted in the absence of antigenic stimulation by blood-stage parasites. Three months after the halofantrine treatment, the antisporeozoite titer was still 1:640.

Sera collected approximately 4 and 5 months after initiation of parasitemia were assayed for CS precipitation reactivity. When incubated with viable *P. brasilianum* sporozoites, both serum samples had a CSP titer of 16.

In a Western immunoblot of an extract of *P. brasilianum* sporozoites, the monkey serum detected two polypeptides with molecular masses of 56 and 66 kDa (Fig. 2). These molecular masses correspond exactly to those of the *P. brasilianum* CS protein and its precursor(s).

We recently demonstrated the presence of a CS-like protein in the micronemes of blood stage merozoites of *P. brasilianum* and other plasmodia by using immunogold electron microscopy and antisporeozoite MAb. This protein was detected in rodent, simian, and human plasmodia and had the same isoelectric point and electrophoretic mobility in SDS-polyacrylamide gel electrophoresis as the corresponding CS protein (3).

We cannot exclude the possibility that the anti-CS antibodies induced by the *P. brasilianum* blood stages might have been generated by an unrelated antigen with an epitope similar to that contained in the tetrapeptide (NAAG). However, in Western immunoblotting, MAb as well as polyclonal antibodies raised against sporozoites of *P. brasilianum* detected only the CS polypeptides in extracts of blood stages.

* Corresponding author.
Earlier studies documented the presence of an antigen in blood stages of *P. falciparum* which shares a single tetrapeptide amino acid sequence with the repeat region of the CS protein of this species (4, 6). This *P. falciparum* antigen, however, does not appear to contribute to the humoral antisporozoite response in malaria endemic areas (14).

Alternatively, the antibodies generated by the *P. brasilianum* blood infection might possibly result from polyclonal activation of B cells (11). We consider this unlikely for several reasons. The monkey immune serum did not react with sporozoites of other plasmodial species, with the exception of a small degree of reactivity with sporozoites of *P. cynomolgi* (NIH or Mulligan strain). We had previously observed that several MAbs raised against *P. brasilianum* sporozoites reacted with sporozoites of this *P. cynomolgi* strain (1). The CS repeat of this strain is somewhat similar to that of *P. brasilianum*, consisting of the sequence (NAAG), (5). Furthermore, antibodies induced by polyclonal B-cell activation decline rapidly after treatment of a malaria infection (11). In contrast, the squirrel monkey antibodies persisted at significant levels for at least 3 months after treatment.

These observations raise the issue of what role the merozoite CS proteins of human malaria might play in endemic areas in inducing antibodies that react with the corresponding sporozoites.

In considering the relevance of these findings to studies in endemic areas, some differences between the *P. brasilianum* model and the human malaria-causing organisms *P. falciparum* and *P. vivax* should be pointed out. The rapid occurrence of antibodies reactive with *P. brasilianum* sporozoites during the course of a *P. brasilianum* infection is in sharp contrast with the age-dependent antibody response to the CS proteins of *P. falciparum* and *P. vivax* (8, 12). Little or no antisporozone antibody is detectable in the sera of young children experiencing relatively high levels of parasitemia. The early occurrence of anti-CS antibodies in the squirrel monkey might be explained by our observation that erythrocytic merozoites of *P. brasilianum* are relatively rich in CS protein compared with merozoites of other plasmodial species (3). In addition, the squirrel monkey was laboratory born and never exposed to parasitic infections which could suppress the antibody response to the CS protein. In the *P. berghei* model, parasitemia suppresses the antibody responses to the CS protein (10). Although some of these findings suggest that the blood-stage CS proteins of *P. falciparum* and *P. vivax* elicit little or no antibody response, this requires further investigation.

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