Membrane-Associated Antigens of Blood Stages of *Plasmodium brasilianum*, a Quartan Malaria Parasite

ALAN H. COCHRANE,1,2* YOSHITSUGU MATSUMOTO,2 KAMAL K. KAMBOJ,1 MARILYN MARACIC,1 RUTH S. NUSSENZWEIG,1 AND MASAMICHI AIKAWA2

Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York 10016,1 and Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 441062

Received 24 March 1988/ Accepted 16 May 1988

The localization of *Plasmodium brasilianum*-derived antigens in short and long clefts within the cytoplasm of infected erythrocytes and in association with knobs of the host cell membrane was demonstrated by immunoelectron microscopy with monoclonal antibodies. Our results document that malaria-induced short and long clefts, previously distinguishable only by morphology, differ also in antigenic composition. Another parasite-derived antigen was found to be associated with the parasitophorous vacuole space in schizonts. In segmenters, this antigen was present in large amounts between merozoites and in the cytoplasm of infected cells. These antigens were characterized by biosynthetic labeling and gel electrophoresis.

*Plasmodium brasilianum* is a quartan malaria parasite of New World monkeys. A close evolutionary relationship between *P. brasilianum* and the human malaria parasite *P. malariae* is suggested by analogies in the morphology and course of development of the erythrocytic and exoerythrocytic stages of these two parasites in primate hosts (5). Immunological data on the immunodominant circumsporozoite protein of these parasites (6, 7) and recent amino acid sequence data on this protein (14) strongly support this relationship. In fact, it has been proposed that *P. brasilianum* is an anthropospecies and represents a strain of *P. malariae* which became adapted to New World monkeys sometime after the early 1600s (5).

Ultrastructural studies have shown that growth of *P. brasilianum* and *P. malariae* in host erythrocytes induces peculiar morphologic changes, including formation of cytoplasmic clefts and membrane-associated knobs (2, 20). Knobs are electron-dense cone-shaped structures, the convex surface of which is bounded by the erythrocyte membrane. They first occur on erythrocytes infected with young trophozoites and increase in number as the parasite matures. Cytoplasmic clefts are membrane-bounded structures that are diverse in size and shape. Short clefts are slitlike, whereas most long clefts are slightly curved or undulated and sometimes appear to originate from the parasitophorous vacuole (PV) membrane. Circular clefts form oblong loops which enclose host cell cytoplasm. The present study is the initial characterization of the antigenic composition of these structures induced by *P. brasilianum*.

We produced a series of monoclonal antibodies (MAbs) to blood stages of *P. brasilianum* of Colombian origin. Some of these antibodies were used in the present investigation to localize the corresponding antigens by immunoelectron microscopy. Parasite-derived antigens were detected in clefts and knobs, and some of these antigens were identified by biosynthetic labeling and Western blotting (immunoblotting). Our results demonstrate for the first time that plasmodium-induced short and long clefts differ in antigenic composition. We also describe an antigen which, in segmenters, accumulates in large quantities between merozoites and in the host erythrocyte cytoplasm and discuss its possible function.

**MATERIALS AND METHODS**

The parasite. *P. brasilianum* (Colombian strain) was originally isolated from an infected squirrel monkey (*Saimiri sciureus*) and kept at the Division of Parasitic Diseases, Centers for Disease Control, Atlanta, Ga. Since 1984, the parasite has also been maintained in our laboratory at New York University School of Medicine by passage in splenectomized *S. sciureus*, mainly of Bolivian origin.

Isolation of parasitized erythrocytes. Infected erythrocytes (IRBC) from squirrel monkeys were collected into vials containing EDTA when parasitemia reached 5 to 7%. Platelets were removed by passage over glass beads (19), and leukocytes were removed by passage through a CF11 cellulose column (10). Parasites of mixed developmental stages (mainly trophozoites and schizonts) were concentrated by density gradient centrifugation in Percoll (4).

**MAb production.** BALB/c mice were immunized by three biweekly injections of a total of 1.5 × 10⁶ IRBC containing parasites of various stages, administered intramuscularly, subcutaneously, and intraperitoneally in complete Freund adjuvant. At 3 days postinfection, 5.0 × 10⁷ parasites were injected intravenously. The fusion protocol and culture conditions for growth of the hybridomas were as previously described (18). Hybrid supernatants were screened for the presence of anti-parasite antibody with air-dried and acetone-fixed IRBC as the antigen in an indirect immunofluorescence assay. MAbs were purified by passage of mouse ascitic fluids through an Affi-Gel–protein A column (Bio-Rad Laboratories, Richmond, Calif.) and isotype by immunodiffusion with antisera from Litton Bioetics, Kensington, Md.

**Indirect immunofluorescence.** *P. brasilianum* IRBC were placed in multislot glass slides or used to prepare thin films, dried, and maintained over a desiccant under vacuum. Before use, they were fixed in chilled acetone. After sequential incubation with MAbs and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin, the preparations were mounted in phosphate-buffered saline (PBS)–glycerol-containing p-phenylenediamine to reduce bleaching (12).

In some experiments, we also used wet preparations of...
IRBC which were incubated with ascites at a final dilution of 1:10 in PBS containing 1% bovine serum albumin (BSA), washed, and incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin. They were mounted in PBS (17) or PBS containing 0.5% Formalin (3).

Immuemector microscopy. IRBC were fixed for 15 min at 4°C in 0.1% glutaraldehyde-1.0% paraformaldehyde-0.05 M phosphate buffer-4% sucrose. They were washed three times with 0.05 M phosphate buffer, dehydrated in a graded series of ethanol at −20°C, and embedded in LR White resin (Polysciences Inc., Warrington, Pa.) by previously described methodology (22). The resin was polymerized at 37°C for 5 days. Thin sections were cut with a diamond knife and mounted on nickel grids.

Sections were etched by treatment with a saturated aqueous solution of sodium metaperiodate for 1 h at 37°C and then washed and incubated with 0.1 M PBS containing 5% nonfat dry milk and 0.01% Tween 20 (PBS-Milk-Tween) for 30 min. Grids were then transferred to a solution of MAb diluted at 100 μg/ml in PBS-Milk-Tween and incubated for 1 h at room temperature. Control sections were incubated with the same dilution of an unrelated MAb. The grids were rinsed in 0.1 M PBS containing 0.01% Tween 20 (PBS-BSA-Tween) and incubated in a drop of rabbit anti-mouse immunoglobulin G (IgG) or IgM (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) diluted at 1:100 in PBS-Milk-Tween for 1 h at room temperature. After being washed in PBS-BSA-Tween, the grids were transferred to a drop of goat anti-rabbit IgG conjugated with gold particles (15-nm diameter) (Janssen Pharmaceutica, Piscataway, N.J.) diluted at 1:20 with PBS-Milk-Tween. After 1 h of incubation at room temperature, the grids were rinsed in PBS-BSA-Tween followed by distilled water. The sections were dried and stained with 2% uranyl acetate in 50% methanol and examined by a JEOL 100 CX electron microscope.

Culture of the parasite. P. brasilianum blood stages were maintained in short-term culture in squirrel monkey erythrocytes at an 8% hematocrit with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% human O-positive serum, 30 mM HEPEs (N-2-hydroxyethylpiperezine-N′-2-ethanesulfonic acid), and 1 μg of gentamicin per ml. The cultures were kept at 37°C in flasks with a gas mixture of 90% N2-5% CO2-5% O2.

Biosynthetic labeling. RPMI 1640 medium deficient in the amino acid to be incorporated was prepared with a Select-Amine kit (GIBCO). Before being labeled IRBC were washed once in the deficient medium. Parasites were cultured, as described above, for 18 to 24 h in the presence of 50 μCi of [3H]histidine (75 to 105 Ci/mmol), [3H]leucine (140 Ci/mmol), [3H]lactate (40 to 60 Ci/mmol), or [35S]methionine (800 Ci/mmol) (Du Pont-NEN Research Products, Boston, Mass.) per ml.

Sequential detergent extraction of labeled IRBC. Parasite extracts were prepared by essentially following previously described procedures (11). After being washed labeled IRBC were suspended in PBS containing 1% Nonidet P-40 (NP40; Particle Data Laboratories, Elmhurst, Ill.) and the following protease inhibitors at the designated concentrations: 25 μg of leupeptin per ml, 25 μg of antipain per ml, 2.2 trypsin inhibitory units (TIU) of aprotinin per ml, 1 mM chymostatin, 1 mM pepstatin, 2 mM EDTA, 0.2 mM N-tosyl-L-phenylalanine chloromethyl ketone, 0.2 mM N-acetyl-p-tosyl-L-lysine chloromethyl ketone, and 2 mM phenylmethylsulfonyl fluoride. The preparations were incubated at 4°C for 30 min and centrifuged at 12,000 × g for 20 min, and the superna-
tant, i.e., the NP40-soluble fraction (extract), was removed and kept at −20°C. The pellet was extracted in 2% sodium dodecyl sulfate (SDS) for 30 min at room temperature. The preparation was centrifuged as described above, and the supernatant, i.e., the SDS-soluble fraction (extract), was removed and kept at −20°C.

Immunoprecipitation. For immunoprecipitation, we used mouse ascitic fluids at a final concentration of 1:50 incubated with labeled parasite extracts by previously described procedures (11). NP40 extracts were used directly without further dilution. SDS extracts were diluted by addition of NP40 to final concentrations of 1.5% NP40 and 0.2% SDS. Protein A-Sepharose (Pharmacia, Piscataway, N.J.) or anti-mouse IgG and IgM coupled to Sepharose and agarose, respectively (Zymed Laboratories, San Francisco, Calif.), were used to bind immune complexes. Bound antigens were eluted with SDS-sample reducing buffer at 100°C for 5 min.

SDS-polyacrylamide gel electrophoresis. Labeled antigens were slab electrophoresed with either 7.5 to 12.5% or 10 to 20% gradient acrylamide gels (13). The gels were impregnated with En3Hance (Du Pont-NEN) and fluorographed at −70°C for visualization of radiolabeled antigens.

Western blot analysis. Washed IRBC, concentrated by Percoll centrifugation and containing parasites of various stages, were placed directly into SDS-sample buffer. After being boiled for 5 min and centrifuged at 12,000 × g for 10 min, extracts were slab electrophoresed with 10% linear or 7.5 to 12.5% gradient acrylamide gels. Western blot analysis was performed as previously described (8, 21).

RESULTS

Properties of a short-cleft antigen. By indirect immunofluorescence with air-dried IRBC, MAbs 3A4.F10 (IgG2a) and 3H4.D10 (IgG2a) detected an antigen(s) which appeared early in parasite development before the appearance of refractile malaria pigment. The immunofluorescence pattern consisted of irregular-size small dots distributed in random fashion over the entire IRBC and persisted through the segmenter stage (Fig. 1A). With maturation of the parasite, there was an increase in the intensity of fluorescence. These MAbs did not react with wet preparations of IRBC, indicating internal localization of their target epitopes.

When the same two MAbs were used for immunoelectron microscopy, gold particles, corresponding to bound IgG, appeared over short clefts located in the cytoplasm of erythrocytes infected with asexual stages as well as gametocytes (Fig. 1B and C). These gold particles were not associated with long clefts, reflecting the exquisite specificity of binding of these MAb to a short-cleft-associated antigen. In some specimens, we also observed accumulations of gold particles over electron-dense material in the IRBC cytoplasm (Fig. 1D). There was no significant labeling of the parasites or the PV membrane or space.

Both MAbs (3A4.F10 and 3H4.D10) recognized an antigen with an apparent molecular mass of 38 kilodaltons (kDa) as determined by Western blotting of IRBC extract (Fig. 1E).

Properties of a long-cleft antigen. By indirect immunofluorescence with air-dried IRBC, MAb 2A12.H5 (IgG1) reacted with an antigen which was present from the early trophozoite to the segmenter stage. In young trophozoites, the fluorescence appeared to define the outline of the growing parasite. In older parasites, the fluorescence increased in intensity and frequently appeared as a scalloped pattern over the entire IRBC (Fig. 2A). In segmenting parasites, the fluorescence appeared mostly to delineate the membrane of
the IRBC. No reactivity of this MAb was observed with wet IRBC preparations.

By immunoelectron microscopy, dense accumulations of gold particles appeared over long clefts in the cytoplasm of erythrocytes infected with asexual as well as sexual stages and also in association with the PV membrane or space or both (Fig. 2B). Very few gold particles were present over the parasite. In some specimens, the long clefts appeared to be almost continuous with both the PV and IRBC membranes. MAb 2A12.H5 also reacted with circular and oval-shaped clefts (Fig. 2C).

An antigen with an apparent molecular mass of 16 kDa was identified by using this MAb to immunoprecipitate NP40-soluble extracts of parasites metabolically labeled with [3H]leucine or [3H]isoleucine (Fig. 2D).

Properties of an antigen associated with the PV space and IRBC cytoplasm. By immunofluorescence with air-dried IRBC, MAb 11A10.D9 (IgG1) reacted with an antigen which first appeared in the early trophozoite stage. The fluorescence appeared as weak spots over the entire IRBC. With further maturation of the parasite, the fluorescence became diffuse and increased in intensity, and in some specimens it was concentrated in a crescent, containing intensely stained bodies, on the IRBC (Fig. 3C). In segmenters, the fluorescence pattern reflected a further change in antigen distribution and appeared to delineate the outline of the individual merozoites (Fig. 4A). MAb 11A10.D9 did not react with wet IRBC preparations by immunofluorescence.

By immunoelectron microscopy with the same MAb, the antigen was detected in young schizonts within the IRBC cytoplasm as discrete aggregates, which did not appear to be membrane bounded (Fig. 3A). In presegmenters, abundant gold particles were intimately associated with the PV space (Fig. 3B and D). Upon segmentation, the gold particles appeared in patches over the PV membrane and between the individual merozoites. In mature segmenters, there was heavy labeling of the gold particles between the merozoites, which were themselves almost totally devoid of gold particles, and also in the IRBC cytoplasm (Fig. 4B).

With MAb 11A10.D9, an antigen with an apparent molecular mass of 120 kDa was detected in Western blots of parasite extracts or upon immunoprecipitation of an NP40-soluble extract of parasites metabolically labeled with either [3H]leucine or [3H]isoleucine (Fig. 4C). Minor bands with
FIG. 2. (A) Immunofluorescent staining pattern of MAb 2A12.H5 with a squirrel monkey erythrocyte infected with a trophozoite of *P. brasiliatum*. (B) Immunoelectron micrograph of MAb 2A12.H5 with an erythrocyte infected with a trophozoite (P). Gold particles appear over a long cleft (LC) in the erythrocyte cytoplasm and the PV space but are absent from short clefts (SC). (C) Immunoelectron micrograph of MAb 2A12.H5 with an erythrocyte infected with a trophozoite (P). Gold particles are concentrated over a circular cleft (CC). (D) Fluorogram of SDS-polyacrylamide gel electrophoretic analysis of isoleucine-labeled NP40 extract of *P. brasiliatum*-IRBC precipitated with MAb 2A12.H5 (lane 1) and control MAb 3D11 (lane 2). MAb 2A12.H5 specifically precipitated an antigen with a molecular mass of 16 kDa. The numbers to the right indicate molecular size in kilodaltons. Bars, 5 μm.
molecular masses of 105, 93, and 53 kDa were also detected by biosynthetic labeling.

**Properties of knob-associated antigens.** By indirect immunofluorescence, with air-dried IRBC, four MAbs (1E2.G7 [IgM], 3C5.H12 [IgG1], 6H8.D3 [IgG1], and 11H9.D11 [IgM]) appeared to react with the IRBC membrane. The fluorescence was demonstrable before the appearance of malarial pigment and increased in intensity as the parasites matured to the segmenter stage (Fig. 5A). Wet IRBC preparations did not fluoresce after incubation with these MAbs. Using three of these MAbs (1E2.G7, 6H8.D3, and 11H9.D11) in immunoelectron microscopy, we observed dense accumulations of gold particles under knob protusions of the IRBC membrane (Fig. 5B). Most gold particles were associated with the cytoplasmic face of the IRBC membrane. With these three MAbs, gold particles also appeared...
FIG. 4. (A) Immunofluorescent staining pattern of MAb 11A10.D9 with an erythrocyte infected with a mature segmenter. (B) Immunoelectron micrograph of MAb 11A10.D9 with an erythrocyte infected with a mature segmenter. Gold particles are heavily concentrated between the merozoites (M) and in the host cell cytoplasm. Bar, 0.5 μm. (C) Fluorogram of SDS-polyacrylamide gel electrophoretic analysis of isoleucine-labeled NP40 extract of *P. brasiliianum*-IRBC precipitated with MAb 11A10.D9 (lane 1) and control MAb 3D11 (lane 2). MAb 11A10.D9 specifically precipitated a major antigen with a molecular mass of 120 kDa and minor antigens of 105, 93, and 53 kDa. The numbers to the right indicate molecular size in kilodaltons.
FIG. 5. (A) Immunofluorescent staining pattern of MAb 6H8.D3 with an erythrocyte infected with a trophozoite of *P. brasilianum*. (B) Immunoelectron micrograph of MAb 6H8.D3 with an erythrocyte infected with a trophozoite (P). Gold particles are concentrated over knobs (arrows) in the host cell membrane. (C) Immunoelectron micrograph of MAb 11H9.D11 with an erythrocyte infected with a segmenter. Gold particles are clustered over micronemes (arrowheads) in the budding merozoite (M) and over knobs (arrows) in the membrane of the IRBC. (D) Fluorogram of SDS-polyacrylamide gel electrophoretic analysis of methionine-labeled SDS extract of *P. brasilianum*-IRBC precipitated with MAb 11H9.D11 (lane 1) and control MAb 3D11 (lane 2). MAb 11H9.D11 specifically precipitated three antigens with molecular masses of 18, 16, and 14 kDa. The numbers indicate molecular size in kilodaltons. Bars, 0.5 μm.

to localize over micronemes present in the developing merozoites (Fig. 5C).

MAb 11H9.D11 immunoprecipitated a triplet of low-molecular-mass antigens (14, 16, and 18 kDa) from an SDS-soluble extract of parasites metabolically labeled with [35S]methionine (Fig. 5D). The same-molecular-mass bands appeared weakly after immunoprecipitation of a [3H]isoleucine-labeled extract. Using the other knob-associated MAb's, we failed to detect an antigen by Western blot or immunoprecipitation of labeled parasite extracts.

**DISCUSSION**

In this first study of blood stage immunogens of *P. brasilianum*, we detected several antigens which, during parasite growth and development, are found in association with the membrane and cytoplasm of host erythrocytes. These parasite antigens were found in clefts within the cytoplasm and associated with knobs of the IRBC membrane. In addition, we found a parasite-derived antigen which intimately associates with growing parasites and
which accumulates in large quantities in the IRBC cytoplasm.

The difference in antigenic composition of short and long clefts which we detected has not been previously described for any malaria-causing species. The process by which the different antigens become selectively incorporated into short and long clefts remains to be clarified. The fact that the long-cleft antigen was also intimately associated with the PV, whereas the short-cleft antigen was not, suggests that a different mechanism operates for incorporation of the two antigens into their respective clefts. The role that these antigens play in growth of the intracellular parasites is unknown. We are currently conducting studies to determine whether the long- and short-cleft antigens might be released into culture medium by growing parasites.

It has been proposed that Maurer’s clefts function in the transport of membrane-associated knob materials. Electron-dense material, similar in morphology and density to the electron-dense material of knobs, has been described as associated with the PV space and Maurer’s clefts for both P. falciparum (1) and P. malariae (2). However, the present study indicates that cleft and knob antigens of P. brasilianum are immunologically distinct. The possible presence of additional antigens associated with both knobs and clefts cannot be excluded. In this respect, it is possible that the nature of antigens transported via clefts varies with the stage of parasite development and that early in the parasite life cycle knob proteins are principally transported. At a later stage of development, the antigenic composition of the clefts might change. In the current study, most of our ultrastructural observations were made with erythrocytes infected with late trophozoites and more mature stages.

The relationship between the knob proteins of P. brasilianum and those of P. falciparum remains unclear. We have been unable to demonstrate the association of a histidine-rich protein with knobs of P. brasilianum. In fact, only after incorporation of methionine or isoleucine were we able to detect a knob-associated antigen by one of the anti-knob MAb. This MAb recognized low-molecular-mass proteins of 18, 16, and 14 kDa, of which the 16- and 14-kDa moieties may be degradative products. In addition, knob-directed MAb’s of the present study did not react by indirect immunofluorescence with erythrocytes infected with knob-positive P. falciparum.

It has been proposed that P. falciparum sequestration of IRBC, which occurs in the visceral microcirculation and appears to be mediated by knobs, allows the parasite to avoid spleen-dependent immune mechanisms (15, 16). The function of knobs on erythrocytes infected with P. brasilianum is unknown. In P. brasilianum, membrane-associated knobs are present on erythrocytes containing trophozoites and schizonts, as well as gametocytes (20). The fact that all of these developmental stages are found in the peripheral circulation suggests that P. brasilianum-parasitized erythrocytes are not sequestered. However, a precise quantitative study done under controlled conditions in a susceptible host has not been undertaken. In fact, Sterling et al. (20) described P. brasilianum-parasitized cells as being “sequestered in great numbers within the liver” of a naturally infected squirrel monkey.

It has recently been shown that a 126-kDa protein of P. falciparum, localized on the periphery of schizonts, is processed to low-molecular-mass fragments which are proposed to have enzymatic activity and to function in merozoite release (9). In schizonts of P. brasilianum, we detected a 120-kDa antigen over the PV space or membrane. In mature segmenters, this antigen was present in large amounts between the budding merozoites and in the cytoplasm of the IRBC. In view of this subcellular localization, we hypothesize that the 120-kDa antigen of P. brasilianum might be an analog of the 126-kDa protein of P. falciparum. This antigen may therefore be a protease and play a role in the segmentation process or the disruption of the IRBC membrane (or both) to allow release of merozoites. We are currently assaying this antigen for enzymatic activity.

Most of the anti-P. brasilianum MAb’s that we have produced, including those used in the present study, react with blood stages of P. malariae by indirect immunofluorescence (Cochrane et al., unpublished data), indicating extensive sharing of antigens by the two parasites. The most prominent clinical manifestation of both parasites in their hosts is extensive nephropathy due to deposition of immune complexes. It remains to be determined, with the anti-P. brasilianum MAb’s, whether some of the currently described antigens or others might be the etiologic agents of this pathologic change.

ACKNOWLEDGMENTS

We acknowledge the support of the Agency for International Development (DPE-0453-A-00-5012-00 and DPE-0453-A-00-4027-00) and the U.S. Public Health Service (grant AI-10645 from the National Institutes of Health). We also thank the United Nations Development Program/World Bank/World Health Organization Special Programme for Research and Training in Tropical Medicine for their support.

We thank William E. Collins for P. malariae parasites, Kiet Dan Luc for excellent technical assistance, and Michael J. Stewart for many helpful suggestions concerning the manuscript.

LITERATURE CITED


Downloaded from http://iai.asm.org/ on August 27, 2017 by guest