SURFACE ANTIGENS OF MALARIA MEROZOITES

A High Molecular Weight Precursor is Processed to an 83,000 Mol Wt Form Expressed on the Surface of Plasmodium falciparum Merozoites

BY ROBERT R. FREEMAN AND ANTHONY A. HOLDER

From the Department of Molecular Biology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, England

Immunity against blood stage Plasmodium falciparum malaria can be passively transferred with immune gamma globulin (1), and protective antibodies appear to act against the merozoite form of the parasite (2). For vaccine development, therefore, it is important to identify and characterize the surface antigens of merozoites against which protective antibodies may be directed. We have reported the identification of a 195,000 mol wt protein antigen of P. falciparum that is synthesized in schizonts and processed through a series of discrete fragments during the last few hours of the parasite's intraerythrocytic growth cycle (3). The major processing product detected after merozoite differentiation was an 83,000 mol wt fragment, and it was suggested that this fragment of the 195,000 mol wt precursor may be expressed on the merozoite surface. More recently, we have demonstrated a serological cross-reaction between the 195,000 mol wt P. falciparum antigen and a 230,000 mol wt antigen of P. yoelii that can be used in purified form to immunize mice against P. yoelii malaria (4). The 195,000 mol wt P. falciparum protein, and its form on the merozoite surface, are therefore of considerable interest for malaria vaccine development.

We have now analyzed the surface antigens of naturally released merozoites harvested from P. falciparum cultures. The results show that the 83,000 mol wt fragment of the 195,000 mol wt precursor is expressed on the surface of merozoites, and that it is a major merozoite surface antigen immunoprecipitated by human immune serum.

Materials and Methods

Media and Reagents. RPMI 1640 tissue-culture medium was obtained from Flow Laboratories Ltd., Ayrshire, Scotland and methionine-free RPMI 1640 was purchased from Gibco Laboratories, Grand Island, NY. d-Sorbitol and d-glucose were supplied by BDH Ltd., Poole, England. Sodium deoxycholate, trypsin (type XI), lactoperoxidase, phenylmethyl sulfonyl fluoride (PMSF), and tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Sigma Chemical Co., St. Louis, MO. The radiolabeled compounds L-[35S]methionine (sp act, 800–1,200 Ci/mmol) and [125I]NaI (17 Ci/mg iodine) were obtained from Amersham International Ltd., Amersham, England.

The mouse-mouse IgG1 monoclonal antibody 89.1 was obtained in serum from BALB/c mice carrying the hybridoma WIC 89.1 as ascites tumors. The production of

1 Abbreviations used in this paper: PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, tosyl-L-lysine chloromethyl ketone.
hybridoma WIC 89.1 and the characterization of antibody 89.1 have been described in detail elsewhere (3). Antibody 89.1 is specific for a 195,000 mol wt \textit{P. falciparum} schizont protein, and also for 153,000, 150,000, 110,000, and 83,000 mol wt processing fragments derived from the 195,000 mol wt precursor.

A pool of human serum was made from samples of serum from 14 \textit{P. falciparum}-immune donors resident in The Gambia. The samples were supplied by Dr. B. M. Greenwood, Medical Research Council Laboratories, The Gambia.

\textit{Collection of P. falciparum Merozoites.} The West African Wellcome strain of \textit{P. falciparum} was cultured in plastic tissue-culture flasks as previously described (3). In these experiments, a 5% hematocrit was used and the flasks, each containing 24 ml of cell suspension, were shaken at 50 rpm in an orbital incubator (model Mk X; L. H. Engineering Co. Ltd., Bells Hill, Stoke Poges, Buckinghamshire, England). The suspension cultures were centrifuged each day to enable the supernatant medium (RPMI 1640 with 10% human serum) to be changed. Parasite development was synchronized using sorbitol (5).

For merozoite collection, cultures at 20% parasitemia were sorbitol treated to give a parasitemia of 18% ring-forms. After 3 h further incubation, the cells were washed in digestion buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM glucose, 1 mM CaCl$_2$, 1 mM MgCl$_2$) and resuspended at 10% hematocrit in digestion buffer to which trypsin was added to 1 mg/ml (6). After 60 min at 37°C the cells were washed in culture medium and resuspended at 5% hematocrit. The cultures were then returned to the incubator. Next morning the medium was changed as usual; 32 h after the sorbitol treatment, when schizont maturation and merozoite release was occurring, the cultures were transferred to sterile 50-ml centrifuge tubes and centrifuged at 550 g for 5 min. The supernatant was replaced with 20 ml per tube of fresh medium. The cells were replaced in new flasks that were then gassed, sealed, and returned to the incubator. After 60 min the cultures were centrifuged at 550 g for 5 min, then the supernatants were centrifuged at 3,000 g for 10 min. The pelleted merozoites were then processed as required. Each flask yielded \(~5 \times 10^8\) merozoites, assessed by counting in a bacterial cell-counting chamber under phase-contrast optics.

\textit{[^35S]Methionine Biosynthetic Labeling.} Two 24-ml cultures were treated with sorbitol and then trypsin as described above. At 27 h after the sorbitol treatment, the cultures were pulse labeled with 60 $\mu$Ci/ml $[^{35}$S]methionine in methionine-free medium for 60 min as described previously (3). After the labeling period, the cells in one flask were washed in digestion buffer and frozen as a pellet at \(-80^\circ\text{C}\). The cells in the other flask were washed in culture medium, then made to 24 ml with culture medium, and returned to the incubator. 5 h later, released merozoites were collected as described above, washed in 1 ml digestion buffer, and pelleted by centrifugation at 10,000 g for 1 min. The pellet was frozen at \(-80^\circ\text{C}\).

\textit{Lactoperoxidase-catalyzed Iodination.} The merozoites collected from one 24-ml culture (5 \times 10$^8$) were resuspended in 10 ml of labeling buffer (80 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 10 mM glucose, 20 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, pH 7.4) and centrifuged at 3,000 g for 10 mins. The merozoites were resuspended in 0.1 ml of labeling buffer and surface labeled with $[^{125}$I] by the lactoperoxidase method as described by Johnson et al. (7). The labeled merozoites were washed twice in labeling buffer and the merozoite pellet was frozen at \(-80^\circ\text{C}\).

\textit{Cell Solubilization, Immunoprecipitation, and Electrophoresis.} The frozen cell pellets were thawed into buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA, 5 mM 1,10-phenanthroline, 1 mM PMSF, 0.1 mM TLCK, and 0.5% (wt/vol) sodium deoxycholate, and extracted on ice for 10 min. Insoluble material was removed by centrifugation at 100,000 g for 45 min at 4°C. Immunoprecipitations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% slab gels, and detection of labeled bands by fluorography were performed as described previously (3). Molecular weight markers were human spectrin heterodimer (240,000 and 220,000 mol wt), $\beta$-galactosidase (116,000 mol wt), phosphorylase b (95,000 mol wt), bovine serum albumin (68,000 mol wt), and aldolase (39,000 mol wt).

\textit{Western Transfer Analysis.} Samples of solubilized \textit{P. falciparum} schizont and merozoite
proteins were subjected to SDS-PAGE, then transferred electrophoretically to a nitrocellulose sheet (8). The sheet was washed, probed with monoclonal antibody 89.1, developed with $[^{125}\text{I}]$-labeled rabbit anti-mouse IgG, and autoradiographed as described elsewhere (4).

**Results**

$[^{35}\text{S}]$Methionine-labeled Schizont and Merozoite Proteins. Most of the proteins synthesized in schizonts (Fig. 1, lane 1) were retained in merozoites released 5 h after the labeling period (lane 5), with some notable exceptions. Major schizont proteins of 235,000, 205,000, 195,000, 185,000, 153,000, and 78,000 mol wt were absent from merozoites, and must have been either excluded, degraded, or modified during merozoite formation.

$[^{35}\text{S}]$Methionine-labeled Schizont and Merozoite Antigens Recognized by Immune Serum. Many proteins synthesized in schizonts were not immunoprecipitated by human immune serum (Fig. 1, lane 2). Thus, schizont proteins of 230,000, 120,000, 95,000, 93,000, 78,000, 75,000, 55,000, 50,000, and 45,000 mol wt do not appear to induce significant antibody titers in immune individuals. Neither were these proteins precipitated from extracts of free merozoites by immune serum (lane 6). In merozoites, the range of antigens synthesized 5 h before release and recognized by immune serum was limited to polypeptides of 150,000, 140,000, 108,000, 83,000, and 41,000 mol wt. Minor bands at 88,000, 65,000, 39,000, and 33,000 mol wt were also detected.

$[^{35}\text{S}]$Methionine-labeled Schizont and Merozoite Antigens Recognized by Monoclonal Antibody 89.1. During the 60-min pulse labeling of schizonts with $[^{35}\text{S}]$methionine, the 195,000 mol wt protein immunoprecipitated by monoclonal antibody 89.1 was synthesized abundantly and only partially processed into its specific fragments (Fig. 1, lane 3). The bulk of the protein was precipitated in nonprocessed form and as the 153,000 mol wt fragment. However, in merozoites...
released after a 5-h chase with cold methionine, the only labeled polypeptide precipitated by antibody 89.1 was the 83,000 mol wt fragment (lane 7).

Western Transfer Analysis of Schizont and Merozoite Antigens Recognized by Monoclonal Antibody 89.1. Pulse labeling with \(^{35}\)S)methionine had been performed 5 h before merozoite release, and it remained possible that some 195,000 mol wt protein, synthesized after the pulse-labeling period, may have been associated with merozoites, but not detected amongst the labeled polypeptides immunoprecipitated by antibody 89.1. Western transfer analysis was used to check this possibility. Total unlabeled schizont and merozoite proteins were transferred to nitrocellulose and probed with antibody 89.1. In schizonts, the 195,000 mol wt precursor and its processing fragments were detected, whereas in merozoites the only polypeptide present recognized by antibody 89.1 was the 83,000 mol wt fragment (Fig. 2).

\[^{125}\text{I}]\) Surface-labeled Merozoite Proteins. The SDS-PAGE profile of the surface proteins of \(P. falciparum\) merozoites labeled by lactoperoxidase-catalyzed iodonation is shown in Fig. 3, lane 1. The molecular weights of the labeled merozoite surface proteins were estimated to be 160,000, 105,000, 83,000, 73,000, 70,000, 65,000, 48,000, 42,000, 41,000, 38,500, and 37,000 mol wt. The 83,000 mol wt polypeptide was a major labeled species.

\[^{125}\text{I}]\) Surface-labeled Merozoite Antigens Recognized by Immune Serum and Monoclonal Antibody 89.1. Human immune serum precipitated surface-labeled polypeptides of 88,000, 83,000, 65,000, 48,000, 42,000, 41,000, and 35,000 mol wt (Fig. 3, lane 2). Of these seven surface antigens, the 83,000 and 42,000 mol wt species were apparently the most abundant. Monoclonal antibody 89.1 immunoprecipitated only the 83,000 mol wt species (lane 3).

Discussion

The method we have used for obtaining naturally released merozoites from \(P. falciparum\) cultures derives from the recent demonstration by Breuer, Gins-
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FIGURE 3. SDS-PAGE analysis of total [125I] surface-labeled *P. falciparum* merozoite proteins (lane 1), and those immunoprecipitated by human immune serum (lane 2), monoclonal antibody 89.1 (lane 3), and normal mouse serum (lane 4). Positions of the molecular weight markers are indicated.

burg, and Cabantchik (6) that trypsin treatment of erythrocytes does not interfere with intracellular parasite development or merozoite release, while it does render erythrocytes refractory to invasion. Trypsin treatment of synchronized cultures enables released merozoites to be harvested with much higher yields than has been otherwise possible (9–11). Using this procedure, the culture medium is changed four times between the trypsin treatment and merozoite release, so the released merozoites are not exposed to trypsin digestion. The merozoites obtained are mostly intact and free from contamination with schizonts and erythrocytes, as assessed by light and electron microscopy (not shown).

The biosynthesis of high molecular weight proteins in schizonts of *P. falciparum* has been noted by several groups (3, 12, 13), and we have used a monoclonal antibody to demonstrate that a 195,000 mol wt schizont protein is specifically processed into smaller fragments as schizonts mature (3). The results of the pulse-chase labeling experiment in the present study indicate that several other schizont proteins may be similarly processed as merozoites develop.

Only five major [35S]methionine-labeled polypeptides were immunoprecipitated from merozoite extracts by human immune serum. One of these was the 83,000 mol wt fragment recognized by monoclonal antibody 89.1 and derived from the 195,000 mol wt precursor protein synthesized in schizonts. None of the intermediate fragments (3) was associated with merozoites, and thus it can be concluded that the 83,000 mol wt antigen is an end product of processing of the 195,000 mol wt precursor, and that it is a major component of free merozoites. This conclusion is supported by Western transfer analysis of schizont and merozoite antigens.

The profile of lactoperoxidase-iodinated merozoite proteins of *P. falciparum* was similar to that found for merozoite surface proteins of *P. knowlesi* (7), and the results of Miller et al. (14) indicate extensive serological cross-reaction between the merozoite surface antigens of these two malaria parasite species. Human malaria-immune sera reacted with all *P. knowlesi* merozoite surface
proteins, including proteins of 150,000 and 105,000 mol wt (14). However, in
the present study, human immune serum did not immunoprecipitate the 160,000
or the 105,000 mol wt P. falciparum merozoite surface proteins; an 88,000 mol
wt species was the largest surface antigen precipitated. The 83,000 mol wt
polypeptide recognized by antibody 89.1 was clearly one of the major merozoite
surface antigens precipitated by human immune serum.

Further study will be required in order to elucidate the function of the 83,000
mol wt polypeptide, the mechanism of its processing and attachment at the
merozoite surface, and its role in protective immunity against P. falciparum
malaria. It has been established, however, that it cross-reacts serologically with a
known protective antigen of a rodent malaria parasite, P. yoelii (4). Thus, the
immunochemical characteristics of the 83,000 mol wt merozoite surface antigen
indicate that it may be an important candidate for use in immunization against
the blood stage of P. falciparum malaria.

Summary

A technique was developed for obtaining high yields of naturally released
Plasmodium falciparum merozoites from synchronous cultures of parasitized eryth-
rocytes. The cultured erythrocytes were treated with trypsin to prevent reinvad-
sion (6), and the released merozoites that accumulated extracellularly were
harvested by differential centrifugation. The total biosynthetically labeled pro-
teins of schizonts and merozoites, and those immunoprecipitated by human
immune serum were analyzed and compared. The surface antigens of free
merozoites, labeled by lactoperoxidase-catalyzed iodination, were also described.
A monoclonal antibody, specific for a 195,000 mol wt schizont protein, and
processing fragments derived from it (3) were used in immunoprecipitation and
Western transfer analyses to determine which of the processing fragments are
associated with merozoites and which of them are located on the merozoite
surface. It was found that processing of the 195,000 mol wt precursor down to
an 83,000 mol wt fragment is complete in free merozoites, and that this fragment
is expressed as one of the major surface antigens of P. falciparum merozoites.

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