

Initial extracellular forms of *Plasmodium falciparum*: Their ultrastructure and their definition with monoclonal antibodies

(malaria/parasitophorous vacuole membrane)

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ABSTRACT Merozoites of the erythrocytic stage of the human malaria parasite *Plasmodium falciparum*, when placed under appropriate conditions in a culture medium with erythrocyte extract, differentiate into early trophic forms. These forms have much the same ultrastructure as rings of the same age that have developed intracellularly and have then been freed from their host cells by immune lysis. However, these forms differ in two respects: the extracellular forms have only their single plasma membrane, whereas the forms freed from host cells have, in addition, a surrounding parasitophorous vacuole membrane; the forms that develop extracellularly have fewer ribosomes. Five monoclonal antibodies against the ring stage have been prepared and characterized. Their pattern of immunofluorescence localization differs in merozoites as compared with rings, but their pattern is identical in rings developed extracellularly and those developed intracellularly. These results and the observations on fine structure demonstrate biochemical and morphological differentiation in the extracellular forms.

We would like to know the nature of the dependence of intracellular parasitic protozoa on their living host cell. One approach to the problem is to attempt to replace the host cell with an environment in which the parasite can develop extracellularly. In experiments of this type with the erythrocytic stage of the human malaria parasite *Plasmodium falciparum*, we have found that merozoites placed under appropriate conditions in a medium with erythrocyte extract will differentiate into early trophic forms (1). These trophic forms take up the fluorescent dye rhodamine 123, showing the existence of a potential across their plasma membrane and indicating their viability.

Here we describe the ultrastructure of these forms and compare it with that of young rings that have developed intracellularly and have then been freed from their host cells. Furthermore we show that the forms that differentiate extracellularly react with certain recently developed monoclonal antibodies (mAbs) in the same way as do intracellular rings.

MATERIALS AND METHODS

The preparation of merozoite suspensions and of rings freed from their host cells by immune lysis have been described in detail, as have the methods for the overnight cultures and their assessment (1).

For electron microscopy a sample of the culture was placed in a microcentrifuge tube, sedimented, and processed as described (2).

Production of mAbs to Rings. The methods of Zola (3) were applied. Female BALB/c mice were immunized with $20 \times$

10^6 rings of *P. falciparum* (clone A-2 of FCR-3/Gambia) prepared by immune lysis (1) and washed and suspended in RP medium [standard *P. falciparum* culture medium without serum (1)]. After 3 or 6 weeks and again at 3 months, 6 months, and 9 months, the mice were boosted with an additional 20×10^6 rings. Serum antibody showed specificity to erythrocytes and rings after 9 months, and the fusion was done at this time. Immunized mouse spleen cells were fused with mouse myeloma cell line X63-Ag 8.653 (a nonsecreting cell line) (4). The hybridomas were plated out into eight 96-well tissue culture plates. Culture supernatants from growing hybridomas were screened by indirect immunofluorescence. Thin smears of acetone-fixed ring or multistage-infected erythrocytes were incubated with hybridoma supernatant for 1 hr at room temperature in a humidified chamber, washed in phosphate-buffered saline, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel Laboratories) diluted 1:50 in phosphate-buffered saline for 45 min. Parasite nuclei were stained with ethidium bromide (10 mg/ml) for 30 sec. Slides were mounted with 5% (wt/vol) 1-propyl gallate in glycerol to retard fading (5). Positive cells were cloned by limiting dilution (3). Ascites were prepared by inoculating female BALB/c mice with 2.5×10^6 cloned hybridoma cells 1 to 2 weeks after they had been injected i.p. with 0.5 ml of pristane (Sigma). Ascitic fluid was collected 7–21 days later, allowed to clot, and centrifuged; the supernatant was stored at -20°C . Antibody isotypes were determined by the Ouchterlony method (6) by using purified goat anti-mouse IgM and IgG (Cappel Laboratories).

Immunoblotting of Parasite Proteins. Ring- and schizont-infected erythrocytes were prepared as described (1). Parasite-infected erythrocytes were lysed in cold 10 mM Tris, pH 8.0, containing protease inhibitors (5 mM iodoacetamide/2 mM phenylmethylsulfonyl fluoride/20 mM aprotinin) and centrifuged at $11,000 \times g$ at 4°C . The pellet was extracted in an equal volume of 1% Nonidet P-40/0.1% SDS. The extract was then solubilized by boiling in an equal volume of electrophoresis sample buffer and separated by SDS/PAGE on 5–15% polyacrylamide gradient gel in the Laemmli system under either reducing or nonreducing conditions (7). SDS/PAGE gels were transferred electrophoretically to nitrocellulose (8), blocked in 1% gelatin in Tris-saline buffer (10 mM Tris/150 mM NaCl, pH 7.4) for 1 hr at 37°C , and incubated with hybridoma culture supernatant or ascitic fluid for 2 hr at room temperature. After being washed the antibody bound to the protein was detected by ^{125}I -labeled goat anti-mouse immunoglobulin (1×10^6 cpm/ml) (New England Nuclear). The washed and dried nitrocellulose (9) was exposed to Kodak X-Omat AR-5 film.

RESULTS AND DISCUSSION

Comparative Ultrastructure of Young Rings That Have Developed Extracellularly or Intracellularly. Rings developed

Abbreviation: mAb, monoclonal antibody.

extracellularly differ from merozoites (Fig. 1A) in that they lack the outer coat, the rhoptries, and the several layers of membrane material found directly beneath the plasma membrane of the merozoite. The rings formed extracellularly from merozoites (Fig. 1B, C, E, and F) have much the same

structures as those that have developed intracellularly and have then been freed from their host cells by immune lysis (Fig. 1D). There are two main differences: (i) The extracellular rings have fewer ribosomes in keeping with the known fact that the extracellular conditions are less favorable than

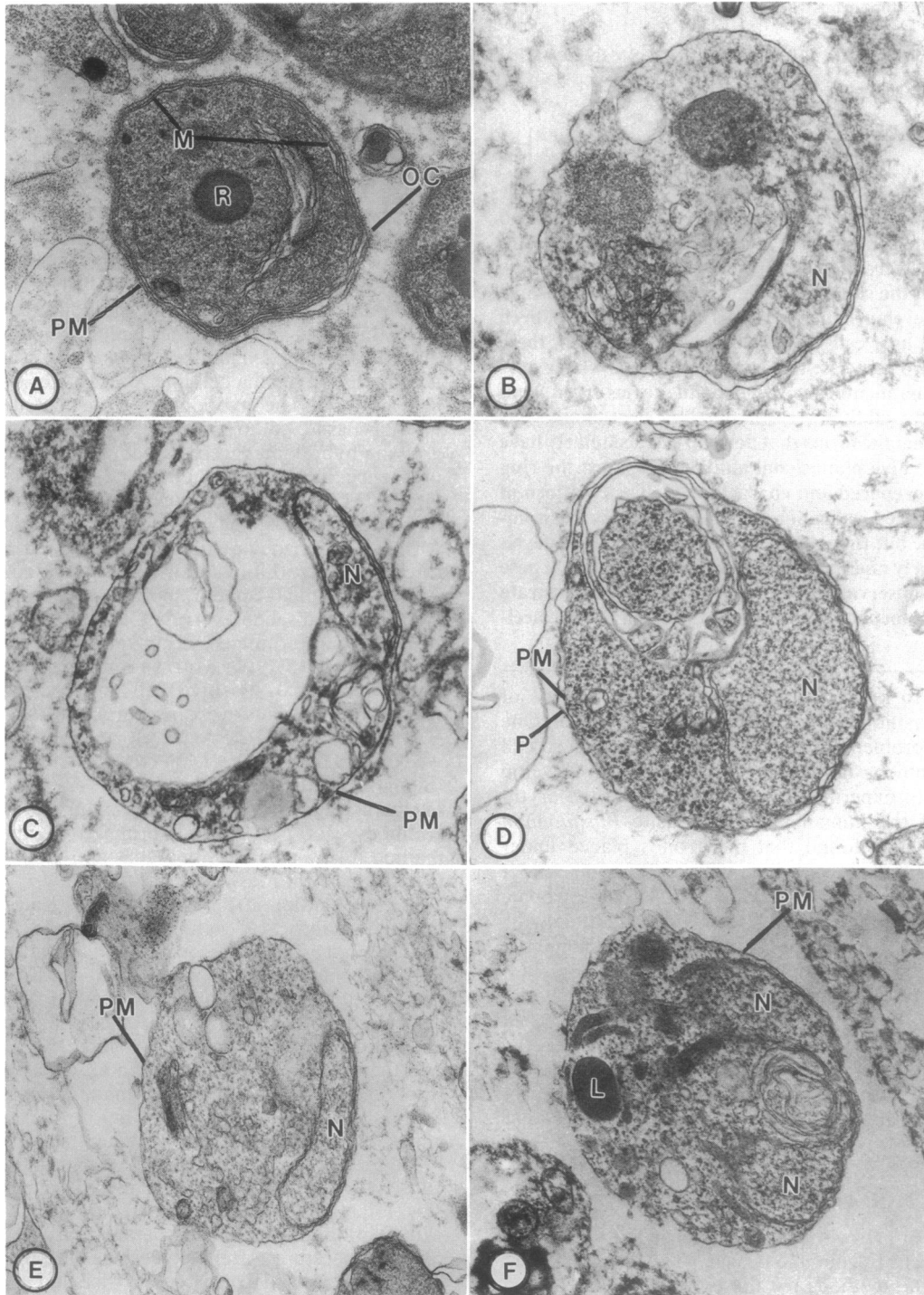


FIG. 1. Transmission electron micrographs of extracellular forms of *P. falciparum*. ($\times 30,000$.) (A) Newly released merozoite from the type of preparation used to inoculate the different experimental media. A rhoptry (R) can be seen in the healthy granular cytoplasm containing many ribosomes. Note the layers of membranous material (M) beneath the plasma membrane (PM) of the cell and the outer coat (OC). (B and C) Rings that have developed extracellularly overnight from merozoites inoculated into erythrocyte extract prepared by freeze-thawing (1). Note that the parasites are surrounded by only a single plasma membrane (PM). N, nucleus. (D) A ring that had developed overnight intracellularly and was then freed from its host cell with antiserum and complement (1). Note that this parasite is surrounded by two membranes, its own plasma membrane (PM) and the outer parasitophorous membrane (P). N, nucleus. (E and F) Rings that have developed extracellularly overnight from merozoites inoculated into erythrocyte extract prepared by sonication (1). The parasites are surrounded by only a single plasma membrane (PM). Their cytoplasm looks healthier, containing more ribosomes than those cultured in frozen-thawed extract (B and C). N, nucleus; L, lipid inclusion.

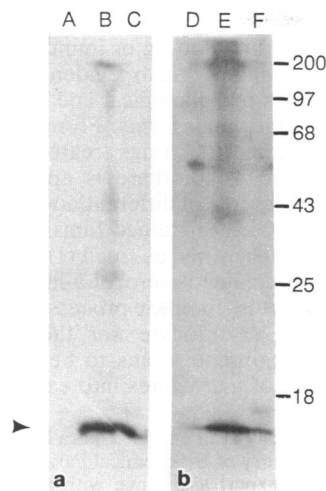


FIG. 2. Immunoblot with mAb 1H1 under reducing (a) and nonreducing (b) conditions. Ring- and schizont-infected erythrocytes were extracted in 1% Nonidet P-40/0.1% SDS. Lanes: A and D, uninfected erythrocytes; B and E, ring-infected erythrocytes; C and F, schizont-infected erythrocytes. Arrowhead marks the 14-kDa parasite antigen detected in rings and schizonts by mAb 1H1. Positions of molecular mass markers (in kDa) are indicated at right.

those within the intact host cell. The forms that developed in sonicated erythrocyte extract (Fig. 1 E and F) had more ribosomes and a generally better ultrastructural appearance

than those in frozen-thawed extract (Fig. 1 B and C). This result is consistent with the larger proportions of extracellular forms that developed in sonicated than in frozen-thawed extracts (1). (ii) The extracellular forms have only their single plasma membrane, whereas the forms lysed out after intracellular development are surrounded by a second, closely apposed membrane, the parasitophorous membrane (Fig. 1D). This is the membrane formed when the host erythrocyte membrane invaginates to receive the entering merozoite (10). We note that immune lysis carried out by the methods used here yields *P. falciparum* parasites freed from the erythrocyte plasma membrane, just as it does for *Plasmodium lophurae* (11). It is also of interest that immune lysis, like N₂ cavitation (12) or saponin lysis (13), yields parasites enclosed in the parasitophorous membrane.

Extracellular development from merozoites, on the other hand, gives us early rings lacking the parasitophorous membrane. Clearly this membrane is not essential, at least for early development. This membrane also is not required for maintaining a potential across the plasma membrane of these extracellular forms, as shown by their uptake of rhodamine 123 (1). Whether the parasitophorous membrane is essential for the full development of a schizont (or a gametocyte) and what is its role in nature can now, perhaps, be approached experimentally.

Definition of the Extracellular Rings with mAbs. mAbs have been used to demonstrate several erythrocytic stage-specific antigens of malaria parasites (14–16). In an effort to show further the development and the viability of the extracellular

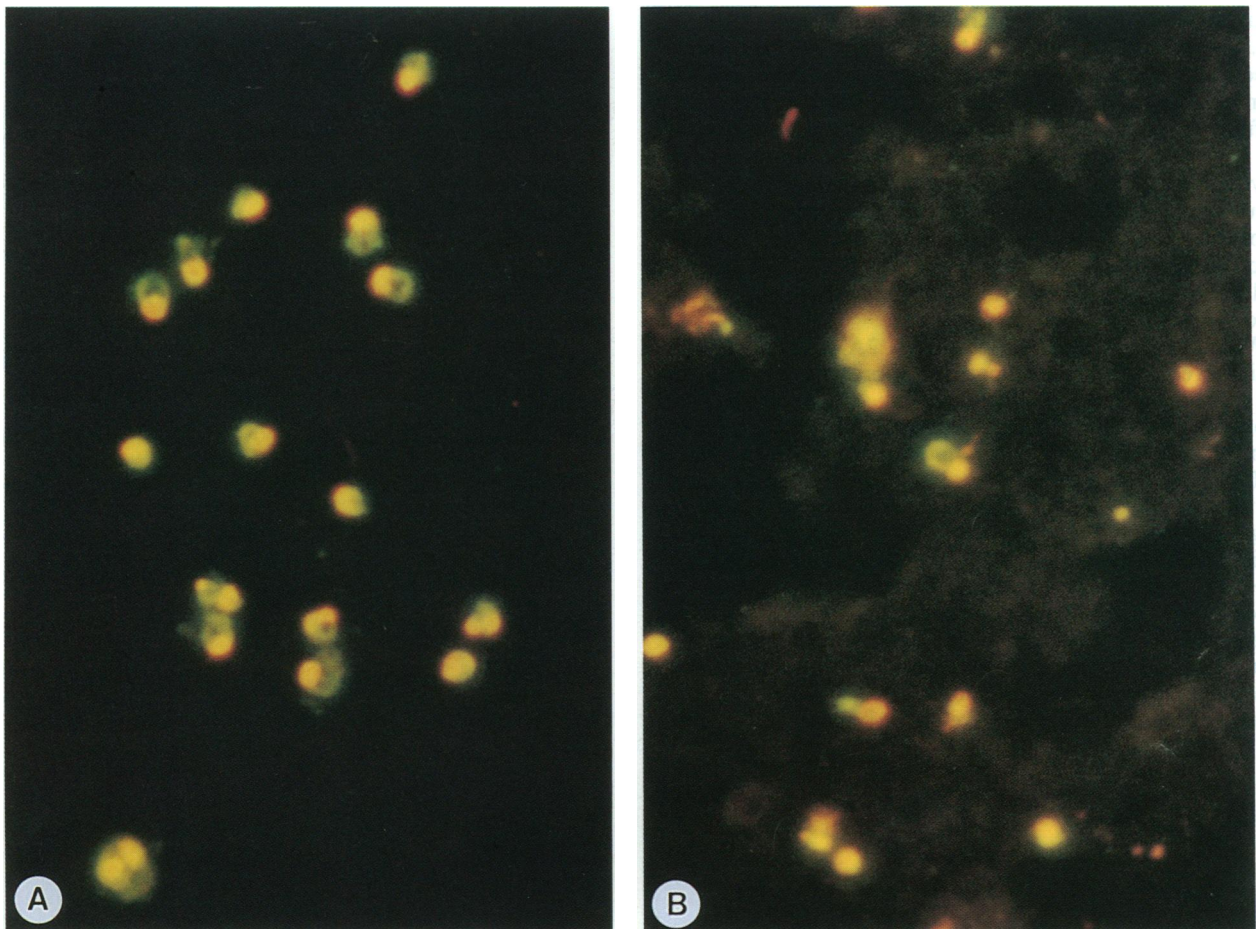


FIG. 3. Immunofluorescence localization of 14-kDa parasite antigen in intracellular (A) and extracellular (B) rings by mAb 1H1. Acetone-fixed smears were incubated with mAb 1H1 and fluorescein isothiocyanate-goat anti-mouse immunoglobulin. Location of parasite nuclei is shown by counterstaining with ethidium bromide (orange). The extracellular rings were grown overnight from merozoites inoculated into erythrocyte extract prepared by sonication.

rings, we attempted to develop mAbs that would be specific to the ring stage. Ten mAbs showing specificity to *P. falciparum* and not to the erythrocyte have been obtained and partly characterized. Among these mAbs, 1H1, 6E6, 2E5, 2A2, and 4A10 are of special interest because they react differently with merozoites than with young intracellular rings. Most significantly, these mAbs react in an identical way with rings formed extracellularly as with intracellular rings, demonstrating biochemical differentiation from merozoites to the extracellular forms.

Immunoblots of mAbs 1H1 and 6E6 show that these two mAbs recognize a parasite antigen of 14-kDa molecular mass in rings and schizonts in both reduced and nonreduced forms (Fig. 2), indicating no involvement of disulfide bonds at the epitope. In rings the antigen is localized by immunofluorescence to the ring-shaped cytoplasm (Fig. 3), whereas in schizonts and free merozoites, the antigen is localized to two small dots within each merozoite (data not shown). This punctate pattern is very distinct from that seen in the ring. Most importantly, extracellular rings (Fig. 3B) present the same fluorescence pattern as intracellular rings (Fig. 3A) and not that of the merozoites, clearly demonstrating the extracellular development of merozoites to early rings. It is interesting to note that not all schizonts or merozoites in a field react with mAbs 1H1 and 6E6. This fact might be due to a subpopulation of parasites that may not have the same recognized antigen or, perhaps, have a different epitope on that antigen. The double-dot fluorescence of the merozoite suggests that the antigen is localized in the rhoptries. Several rhoptry antigens presenting the same fluorescent pattern in schizonts have been demonstrated by using other mAbs (17, 18). Several of these antigens have been shown to be stage-specific, appearing only in late-stage trophozoites and schizonts (19–21), whereas others have been shown to be diffusely present in the cytoplasm of the parasite throughout the asexual cycle, appearing to be packaged during schizogony (22–24). Our 14-kDa protein seems to belong to the latter group. mAbs 1H1 and 6E6 appear identical through immunofluorescence, immunoblotting, and IgG isotype. Immunoelectron-microscopic observations with mAbs 1H1 and 6E6 will be necessary to determine whether they are, indeed, recognizing rhoptry proteins.

With the other three mAbs—2E5, 2A2, and 4A10—the initial screening revealed a positive fluorescence to rings but no reaction to late trophozoites or schizonts (data not shown). This screening had been done with acetone-fixed thin films prepared in advance and stored several weeks at -20°C . When freshly fixed preparations were used, however, all three of these mAbs reacted with both late stages and rings. Further study showed that, indeed, only the ring-stage antigen was recognized on slides stored for 3 weeks or longer at -20°C . Presumably, the mAbs recognize a common epitope on both ring and late-stage antigens, but the latter is less stable at -20°C . Once again, the rings that had developed extracellularly reacted in exactly the same way as intracellular rings, showing positive fluorescence on stored as well as freshly prepared films. We have so far been unsuccessful in

attempts to determine the molecular masses of these three mAbs by either immunoblotting or immunoprecipitation.

The ultrastructural and mAb evidence presented here clearly demonstrates morphological and biochemical differentiation in the extracellular forms. From the study of many preparations of extracellular rings treated with mAbs 1H1, 6E6, 2E5, 2A2, and 4A10, there is apparently a greater amount of initial biochemical differentiation than can be seen morphologically in Giemsa-stained films or electron micrographs; those forms classified as small (1) and many of those that seem to have remained merozoite-like react with these five mAbs. Neither the complex process of invasion of the merozoite into the erythrocyte nor the presence of the parasitophorous membrane seems to be necessary for the initial development of merozoites into early trophic forms.

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