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Transfer of a Dense Granule Protein of *Plasmodium falciparum* to the Membrane of Ring Stages and Isolation of Dense Granules

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A 14-kDa protein was localized to the dense granules of *Plasmodium falciparum* by immunoelectron microscopy with monoclonal antibody 1H1. The protein was present in dense granules in late-stage schizonts and free merozoites. After invasion, the protein was localized exclusively on the membrane of the newly invaded ring. The protein is referred to as RIMA, for ring membrane antigen. The 14-kDa protein was synthesized late in schizogony as determined by immunofluorescence microscopy and immunoblotting. At the late schizont stage it was distributed diffusely throughout the intracellular schizont. Only at the segmenter stage was the protein localized in defined spots that correspond to dense granules. Dense granules were isolated from schizont-infected erythrocytes by subcellular fractionation on a sucrose gradient. Fractions containing the 14-kDa protein were detected by immunoblotting with monoclonal antibody 1H1. The 14-kDa protein was first detected in vesicles at the late (8-nucleus) schizont stage. Mature dense granules sedimented with a peak density of 1.17 g/ml, which is similar to the density of rhoptries isolated by the same procedure.

Rhoptries, micronemes, and dense granules represent the apical organelles of Apicomplexa parasites. They are present in the invasive stage of all genera and are considered to play a role in host cell invasion. Contents of the rhoptry appear to be secreted from the organelle into the host membrane and parasitophorous-vacuole membrane during invasion (12). In contrast, dense granules are released from the invading merozoite after it invades the new host cell by a process that resembles exocytosis (5, 14).

Up to now, only one protein in the dense granules of *Plasmodium falciparum* has been identified. This is RESA, which is transported to the erythrocyte membrane after invasion (5). Here, we report on a second protein in the dense granules of *P. falciparum* merozoites. This protein of 14 kDa is defined by a monoclonal antibody (MAb), 1H1, that was produced from mice immunized with purified ring-stage organisms (ring stages) of *P. falciparum* (16). MAb 1H1 served to characterize early extracellular differentiation of merozoites into young rings, since its distribution, as determined by immunofluorescence assay, changed from being sharply localized at one end of the merozoite to being generally distributed on the entire ring. Immunoelectron microscopy confirms these observations and reveals that the antigen moves from the dense granules to the plasma membrane of the ring. Furthermore, we monitored the time of appearance of this protein in late schizonts both by immunofluorescence assay and by isolating the dense granules. In view of the conspicuous localization of the protein on the plasma membrane of the ring stage, we suggest the name RIMA, for ring membrane antigen.

MATERIALS AND METHODS

Parasites. Stock cultures of the HB-3 clone (4) were kept by the continuous-flow method (15) with RPMI medium (9.6 g of RPMI salts, 2.0 g of $NaHCO_3$, 5.9 g of HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 66 mg of gentamicin) supplemented with 10% human serum. They were synchronized by plasma gel separation (11) followed by a 3-h period of invasion and finally by sorbitol treatment (10) to leave only 0- to 3-h-old rings. The still-synchronous cultures were used 3 to 5 days later. Schizonts were concentrated by layering a 25% infected-erythrocyte suspension over an equal volume of 60% Percoll and centrifuging at 1,500 × *g* for 20 min. The brown layer thus obtained was washed once in complete culture medium and then either fixed for immunoelectron microscopy (as described below) or resuspended by being pipetted in culture medium at a 3% (vol/vol) concentration and incubated in a candle jar at 37°C for 75 min. Merozoites released during this time were separated from the bulk of the schizonts by 10-min centrifugation at 200 × *g*. The sedimented schizonts were washed once with RPMI and then fixed. The supernatant, which contained free merozoites, was centrifuged for 10 min at 1,500 × *g* to pellet the merozoites. A small drop of RPMI was added to this pellet, and then a fixative (described below) was added.

Ring stages were prepared by mixing 0.2 ml of packed schizonts with 0.6 ml of 50% human erythrocytes in culture medium. This mixture was resuspended in complete culture medium to a total volume of 40 ml and placed in four petri dishes (10 ml per dish). After overnight incubation in a candle jar, the material was pooled and the cells were washed once in RPMI and then fixed. A count from a stained slide of the material showed 180 rings and 1 schizont to 200 erythrocytes.

For cell fractionation studies, synchronized cultures of *P. falciparum* K1 were obtained by repeated treatments of cells with 5% sorbitol 3 to 4 h after rings were detected (10). Cells were harvested at the 4-, 8-, and 16-nucleus stages as determined by staining cultures with ethidium bromide. At each stage, approximately 70% of the schizonts were at the same stage (i.e., number of nuclei).

Immunoelectron microscopy. Preliminary experiments showed that the RIMA antigen could not withstand 0.1% glutaraldehyde or prolonged fixation. In order to stain the

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antigen and still retain reasonable morphology, we have used a 1/2-h fixation in 2% formaldehyde with 0.05% glutaraldehyde in a 0.1 M cacodylate buffer. The pellet was infused with 2.3 M sucrose in phosphate-buffered saline (PBS) and frozen in liquid nitrogen. Ultrathin frozen sections were prepared by the method of Tokuyasu (13). Rainbow-colored sections were cut with a glass knife on a Reichert-Jung RC-4E cryoultramicrotome and collected on Formvar-carbon-coated nickel grids. The sections were incubated for 2 h in MAb 1H1 ascitic fluid diluted 1:10 in TBS (10 mM Tris, 0.15 M NaCl, 0.1% bovine serum albumin [pH 8.2]). They were then washed and treated for 1 h with goat anti-mouse immunoglobulin G conjugated to 10-nm-diameter gold particles (Amersham Life Sciences). Samples were processed further according to the method of Griffiths et al. (8).

Immunofluorescence microscopy. Thin smears of cultures at different stages of schizogony were fixed in acetone for 5 min at 4°C, air dried, and incubated with MAb 1H1 (ascites fluid, diluted 1:10) for 30 min at room temperature. The slides were washed in PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G for 30 min at room temperature. The slides were washed and stained with ethidium bromide (100 μg/ml) for 30 s and mounted with 50% glycerol in PBS. Cells were viewed with a Nikon epifluorescence microscope.

Subcellular fractionation. Synchronous cultures of *P. falciparum* were collected at different stages of schizogony, equivalent to the 4-, 8-, and 16-nucleus stages. The average number of nuclei in the population was determined by examining 100 cells stained with ethidium bromide. Schizont-infected erythrocytes were concentrated from the culture by centrifugation on 60% Percoll at 1,500 × *g* for 20 min. Cells were disrupted by nitrogen cavitation and fractionated in a linear 0.4 to 1.6 M sucrose gradient by centrifugation at 130,000 × *g* for 2.5 h (7). Fractions (0.5 ml) were collected, and densities were determined by refractometry.

Immunoblotting. Fractions from the gradient were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with MAb 1H1. Fractions were electrophoresed on 10% polyacrylamide gels, transferred to nitrocellulose, and probed with hybridoma supernatant of MAb 1H1 and secondary antibody, ^{125}I-goat anti-mouse antibody (10^6 cpm/ml) (12).

RESULTS

Localization of RIMA by immunoelectron microscopy to the dense granules of merozoites and the plasma membrane of the ring. In preparations of schizonts, only very late stages with well-developed merozoites showed reactivity with MAb 1H1. In all cases, this was restricted to the dense granules (Fig. 1). In free merozoites prepared by incubation of schizonts for 1 h under culture conditions, RIMA was detected in the lamellar organelle as well as in the dense granules (Fig. 2). In 12- to 14-h-old rings, the protein was very abundant in the plasma membrane (Fig. 3). It was not present in the ring cytoplasm and was notably absent from the parasitophorous vacuole and membrane and from the erythrocyte membrane.

Stage-dependent synthesis of RIMA. Schizonts were stained with MAb 1H1 to detect the 14-kDa protein and with ethidium bromide to reveal the number of nuclei at different stages of schizogony. Little or no fluorescence was detected in most cells at the 4-nucleus stage (data not shown). The fluorescence pattern of schizonts at the 8-nucleus stage was diffuse and not localized (Fig. 4a). Punctate or polar fluorescence was detected only at the 16-nucleus segmenter stage (Fig. 4c). Figure 4b shows two late-stage schizonts from cultures at the 16-nucleus stage. The schizont on the right appears to be undergoing division from 8 to 16 nuclei and is characterized by small amounts of diffuse fluorescence. The schizont on the left is at the 16-nucleus stage and is highly fluorescent. Also shown in this micrograph is a merozoite which illustrates the polar nature of the immunofluorescence assay pattern in relation to the nucleus.

Cell fractionation of dense granules. Schizonts at the 4-, 8-, and 16-nucleus stages were fractionated on a 0.4 to 1.6 M sucrose gradient, and fractions were collected and immunoblotted with MAb 1H1 (Fig. 5). At the 4-nucleus stage, the RIMA was barely detectable, consistent with the fluorescence studies. The low level of reactivity seen at this time point was in fractions with densities of 1.12 to 1.13 g/ml, which could represent the presence of newly synthesized RIMA in a low-density vesicle. At the 8-nucleus stage, RIMA was detected in fractions having a density of 1.12 to 1.19 g/ml. At the 16-nucleus stage, RIMA was concentrated in three fractions with densities of 1.16 to 1.18 g/ml. The diffusion of protein between lanes may be due to the low molecular weight of this protein and its ability to diffuse through the acrylamide gel. The lower density of vesicles containing RIMA at the 4- and 8-nucleus stages may reflect the fact that the granules are initially formed as smaller, less dense vesicles.

DISCUSSION

The finding that a protein localized to the dense granules of merozoites subsequently appears on the plasma membrane of the ring suggests that the composition and functions of dense granules may be even more complex than previously supposed. Early work by Bannister and colleagues (3) showed that in invading merozoites of *Plasmodium knowlesi*, the dense granules moved to the periphery of the merozoite and discharged their contents into the newly formed parasitophorous vacuole. This has been confirmed by immunoelectron microscopy (14). Exocytosis of the dense granules into the secondary parasitophorous vacuole has also been demonstrated for *Toxoplasma gondii* (1) and for *Sarcocystis muris* (6). In *T. gondii*, four different proteins in the dense granules were identified. All were exocytosed and found in the vacuolar network, but one of them also was present on the parasitophorous-vacuole membrane (6). In *P. falciparum*, RESA, a protein of 155 kDa, has been found in the dense granules of merozoites and subsequently in the plasma membrane of the newly invaded erythrocyte (2, 5). Similarly, a rhoptry protein of 110 kDa appears on the erythrocyte plasma membrane (12). In all of these examples, the proteins are presumably released by exocytosis into the parasitophorous vacuole and then transported somehow to their final destinations. With RIMA, the situation must be rather different, since this protein is not exocytosed from the parasite cell but is instead transported to the ring plasma membrane. In no instance is the function of any of these proteins known. For those that become localized on the plasma membrane of the parasite or of the host erythrocyte, it seems reasonable to hypothesize that they play a role in transport across these membranes. It is difficult to speculate how one dense granule protein, such as RESA, can be transported to the plasma membrane and another, such as RIMA, is transported only to the ring plasma membrane.

The virtual disappearance of RIMA in late trophozoites and early schizonts suggests a special role for the protein in

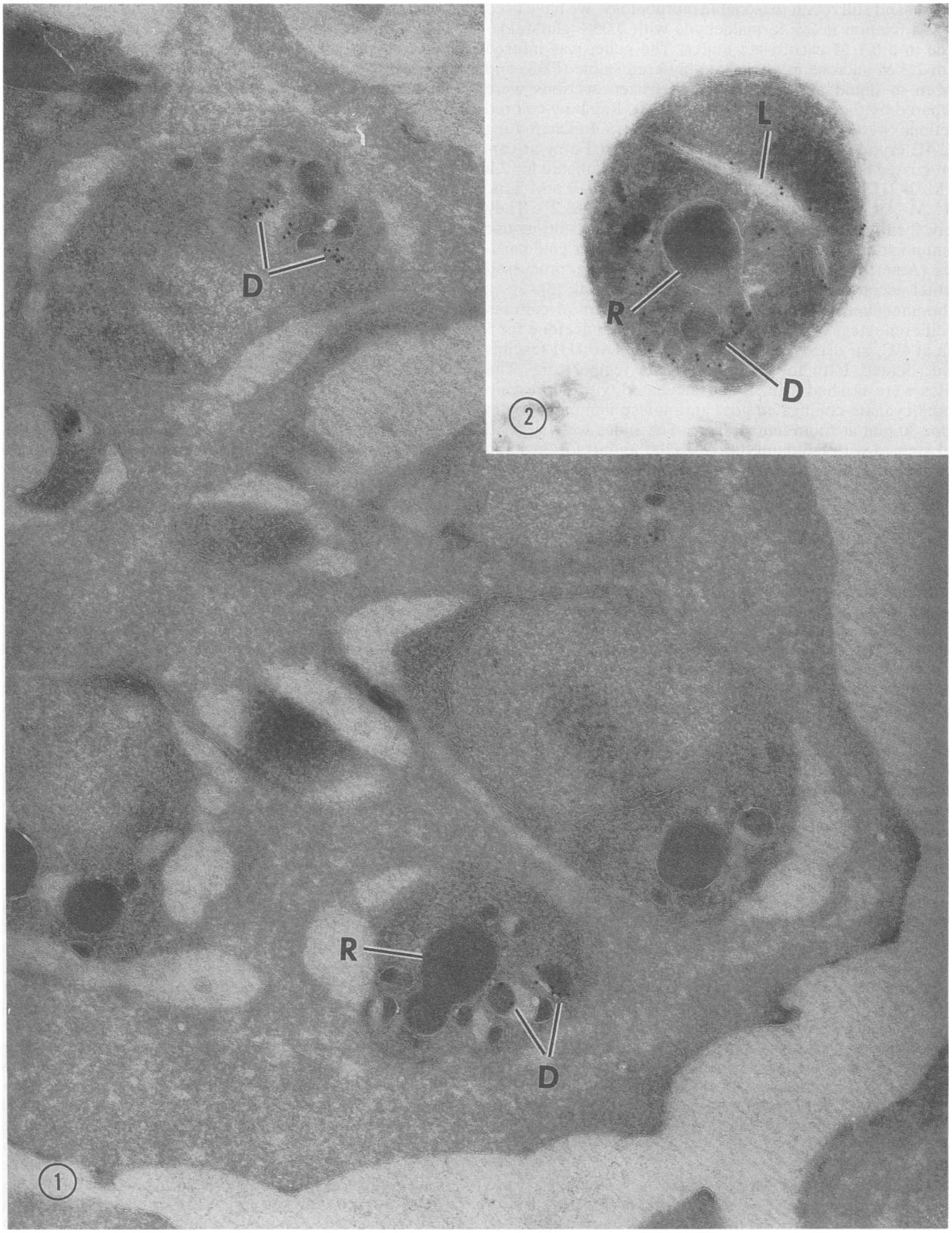

FIG. 1. Section of a very late schizont, showing gold particles over the dense granules (D) of the well-formed merozoites. Note the absence of gold particles from the rhoptry (R). Magnification, ×50,000.

FIG. 2. Section of a free merozoite, showing gold particles at the lamellar body (L) as well as at the dense granules (D). A few gold particles are also seen at the plasma membrane. Note the absence of reaction over the flask-shaped rhoptry (R). Magnification, ×65,000.

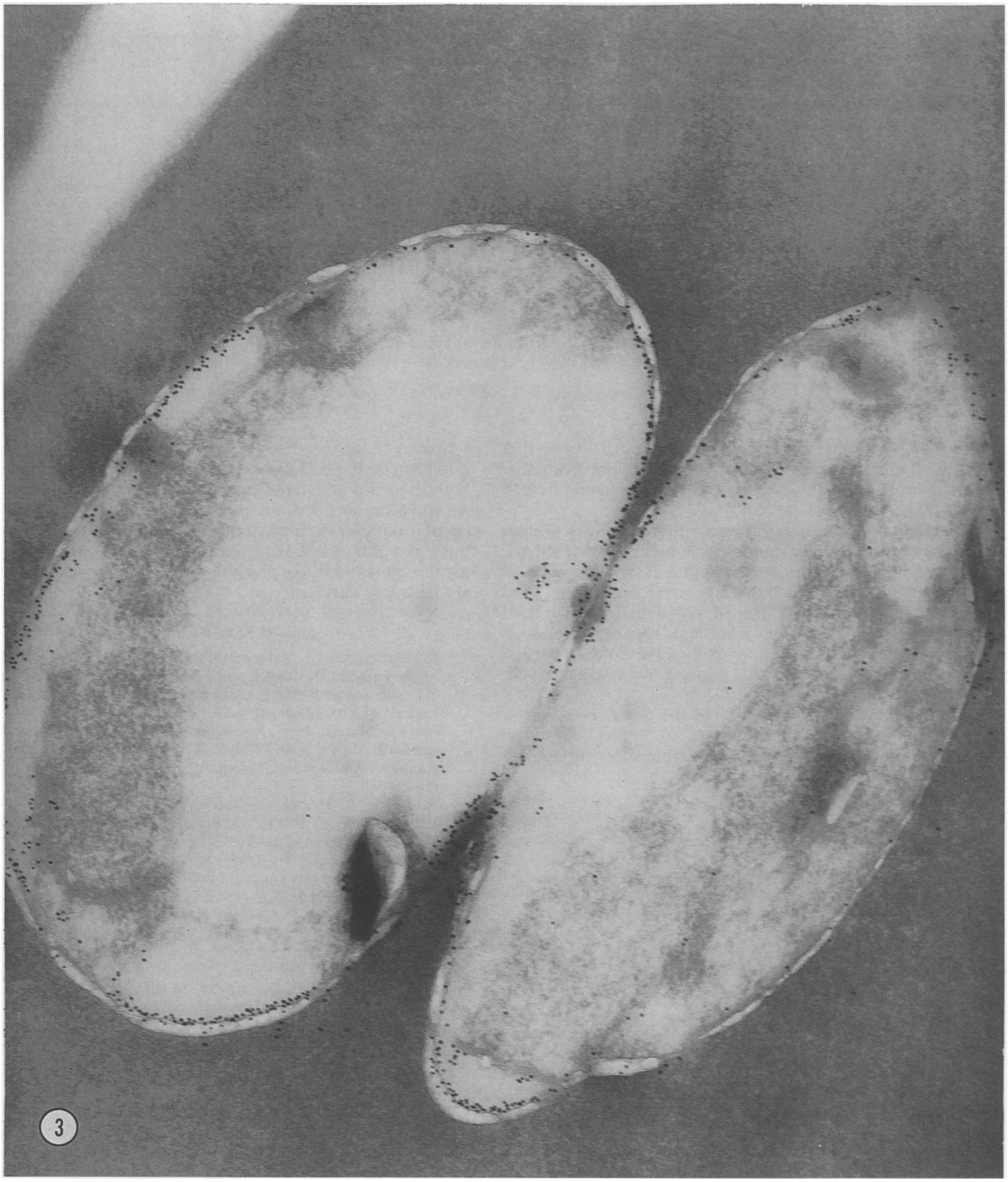

FIG. 3. Section through two adjacent rings, showing abundant gold particles on their plasma membranes. In contrast, very few gold particles can be seen in the immediately adjacent parasitophorous vacuole and none can be seen on the parasitophorous membrane. Magnification, ×50,000.

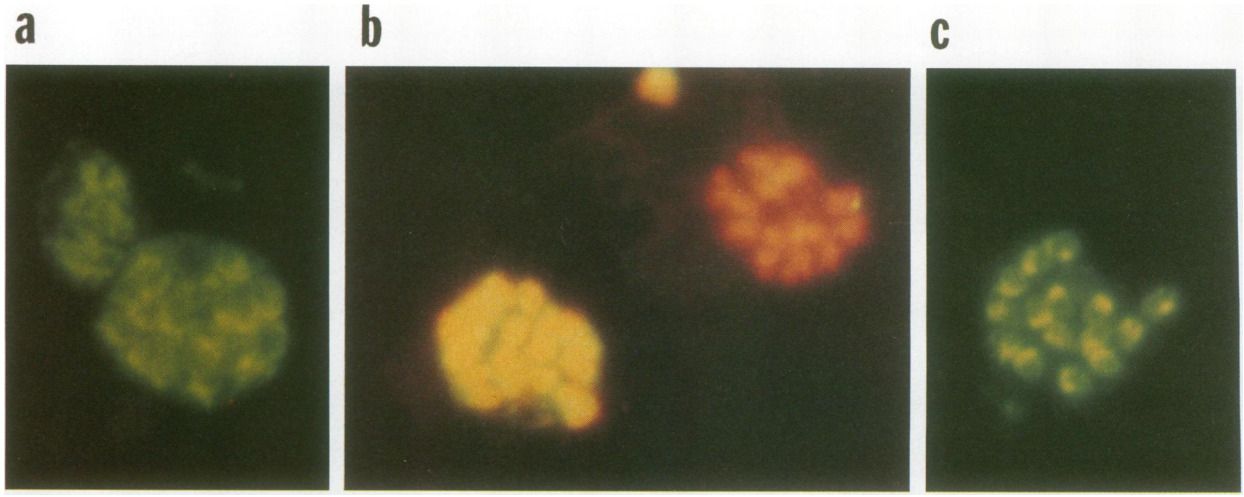

FIG. 4. Immunofluorescent staining of RIMA with MAb 1H1 at the 8-nucleus (a) and 16-nucleus (b and c) stages. Cells in panel b were counterstained with ethidium bromide (orange) to detect nuclei. Cells staining with both fluorescein isothiocyanate and ethidium bromide appear yellow (b).

the parasite newly established in its host cell. RESA and the 110-kDa rhoptry protein also appear to be lost from the plasma membrane of the erythrocyte as the parasite develops (2, 12).

The isolation of dense granules at different stages of late schizogony by subcellular fractionation indicates that RIMA is first present in residues that migrate in sucrose with a density relatively lower than that of mature organelles. This suggests that they are first formed as smaller vesicles that increase in size and density. The small size of vesicles at early stages of development may explain the diffuse fluorescence at this time point. Punctate fluorescence was observed only at the segmenter stage.

It is interesting that the density of the dense granules is similar to that of the mature rhoptry (7, 9). In fact, in studies to isolate rhoptries, it was apparent from electron microscopic examination that dense granules were present in the same fractions (7). Although it appears that the contents of both rhoptries and dense granules are exocytosed during or shortly after invasion, the precise stimulus that triggers this event is not known. Since morphological studies suggest that there is a time lapse between rhoptry extrusion and dense granule exocytosis, the physiological stimuli for these events are probably different.

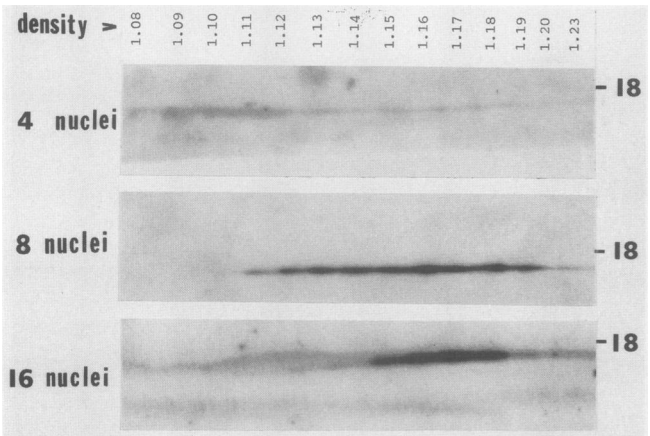

FIG. 5. Subcellular fractionation of schizonts at different stages. Cultures at the 4-, 8-, and 16-nucleus stages were disrupted by nitrogen cavitation and centrifuged on a 0.4 to 1.6 M linear sucrose gradient. The densities of fractions in sucrose (in milligrams per milliliter) were determined by refractometry. Fractions were immunoblotted with MAb 1H1. The molecular mass marker of 18 kDa is shown.

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