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Release of Merozoite Dense Granules during Erythrocyte Invasion by *Plasmodium knowlesi*†

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We used immunoelectron microscopy to study the fate of dense granules during the invasion of erythrocytes by *Plasmodium knowlesi* merozoites. When merozoites entered host cells, dense granules moved to the pellicle, released their contents into the parasitophorous vacuole space, and then moved into fingerlike channels of the vacuole membrane. This is the first report showing that the content of dense granules of *P. knowlesi* is different from the contents of rhoptries and micronemes and is associated with the formation of channels from the parasitophorous vacuole.

Three types of membrane-bound organelles, namely, rhoptries, micronemes, and dense granules, in *Plasmodium knowlesi* merozoites have been described. During invasion of the host, the apical end of the merozoite attaches to the erythrocyte plasma membrane, and junction formation occurs. The parasitophorous vacuole is then formed, and the parasite invades the erythrocytes (2, 3, 6). It has been suggested that the contents of the rhoptries discharge into the erythrocyte membrane during invasion and assist the merozoite invasion process (2, 9, 11).

Bannister et al. described the movement of dense granules (microspheres) to the periphery of *P. knowlesi* merozoites after erythrocyte entry and suggested that release of the dense granule content into the parasitophorous vacuole caused further invagination of the parasitophorous vacuole membrane (3). Nearly identical dense granules in *Sarcocystis muris* (4, 5) and *Toxoplasma gondii* (J. F. Dubremetz, C. Dissous, and E. Ferreira, J. Protozool. **29**:303, 1982) have been described. The dense granules from these coccidial species were shown to be released into the parasitophorous vacuole after invasion of a host cell. The dense granules of *Sarcocystis* merozoites were released through the pellicle lateral to the apical end, and the dense-granule contents appeared to form invaginations of the host membrane of the parasitophorous vacuole (4).

In the present study, we used immunoelectron microscopy with antibodies which react specifically with *P. knowlesi* dense granules to study the fate of dense-granule contents during erythrocyte entry by the merozoite.

Merozoites of *P. knowlesi* Malayan H were prepared from schizont-infected rhesus erythrocytes as described previously (7). Free merozoites were mixed and incubated for 3 min with rhesus erythrocytes in RPMI 1640 medium with 2% heat-activated fetal bovine serum before fixation for electron microscopy.

Antibodies used for immunoelectron microscopy were obtained in the following manner. Filter lifts of induced wild-type lambda gt11 in *Escherichia coli* Y1090 were used to select antibody from anti-*P. knowlesi* hyperimmune monkey serum (8). The rhesus sera used in the experiment were obtained from a monkey which was inoculated seven times with three different isolates (Malaysia, Hackeri, and Philippine) over 22 months and treated by chemotherapy. The gt11-infected Y1090 was grown to stationary phase on agar plates in L Broth medium with 50 μg of ampicillin per ml and induced at 42°C for 4 h. Then nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) were placed on top of the nearly confluent bacteriophage plaques, and the plates were incubated overnight at 37°C (12). The filters were removed and washed three times in 0.05% Tween 20 in phosphate-buffered saline (TPBS), blocked for 1 h in 1.0% gelatin in TPBS, washed three times in TPBS, incubated for 2 h in 1:100 anti-*P. knowlesi* hyperimmune serum diluted in TPBS,

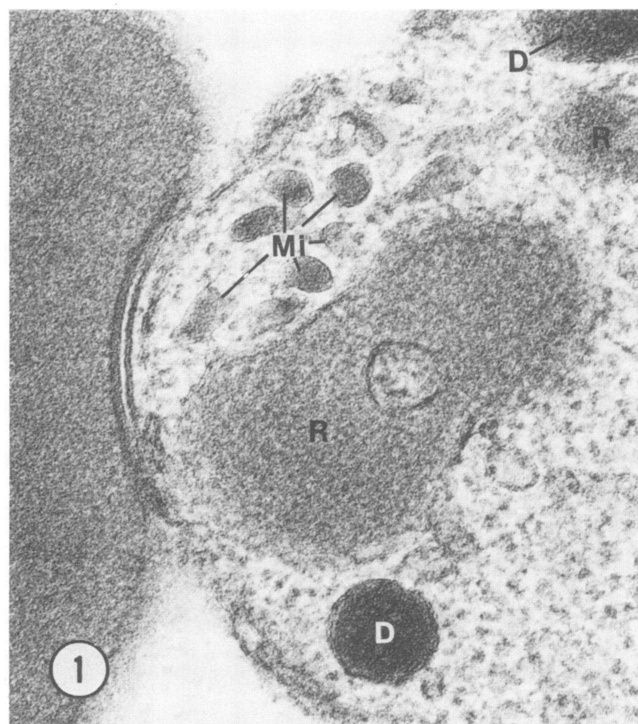

FIG. 1. Epon-embedded section of dense granules and micronemes of a *P. knowlesi* merozoite showing round dense granules (D), elongated micronemes (Mi), and rhoptries (R) in the apical-end region. Magnification, ×83,000.

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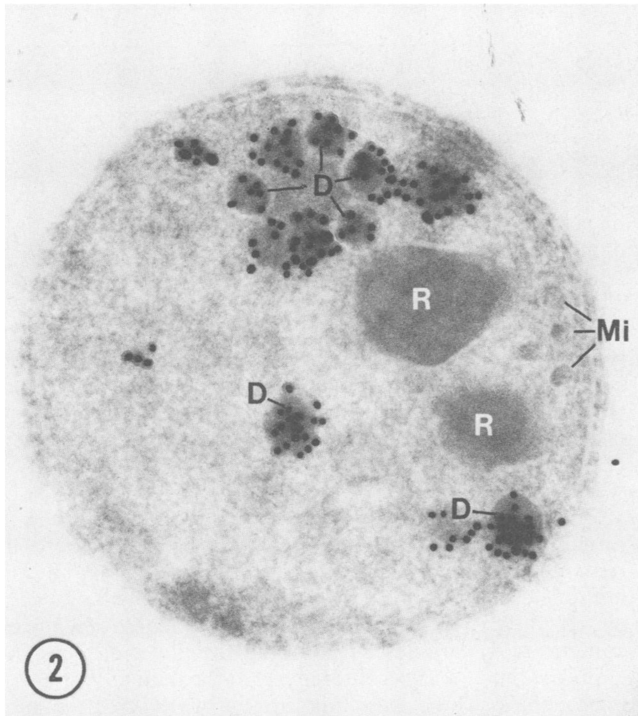

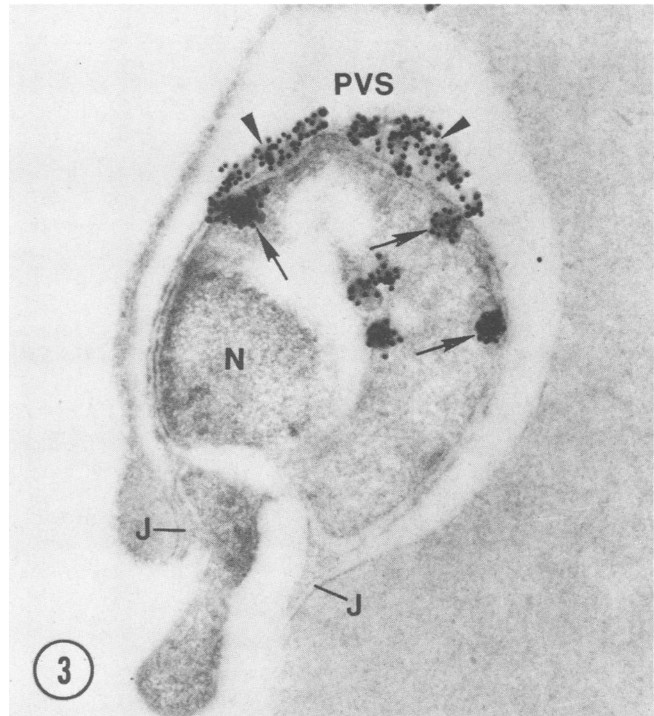

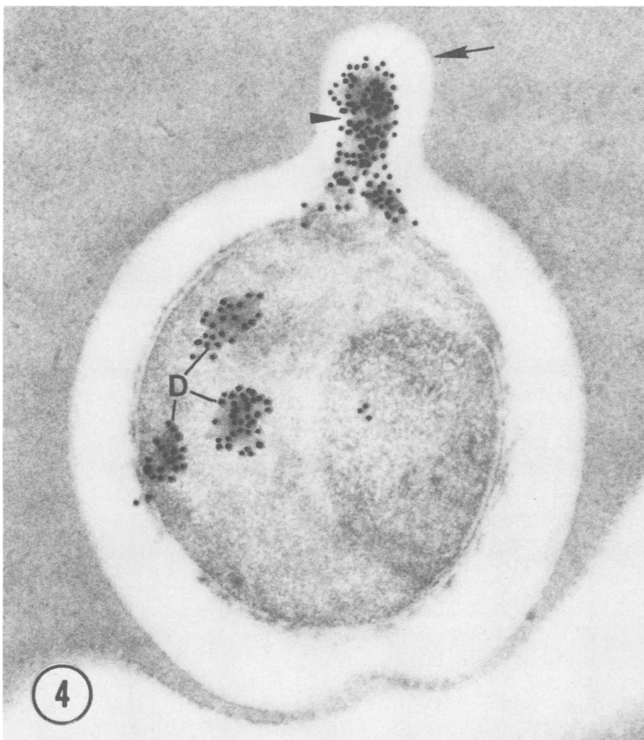

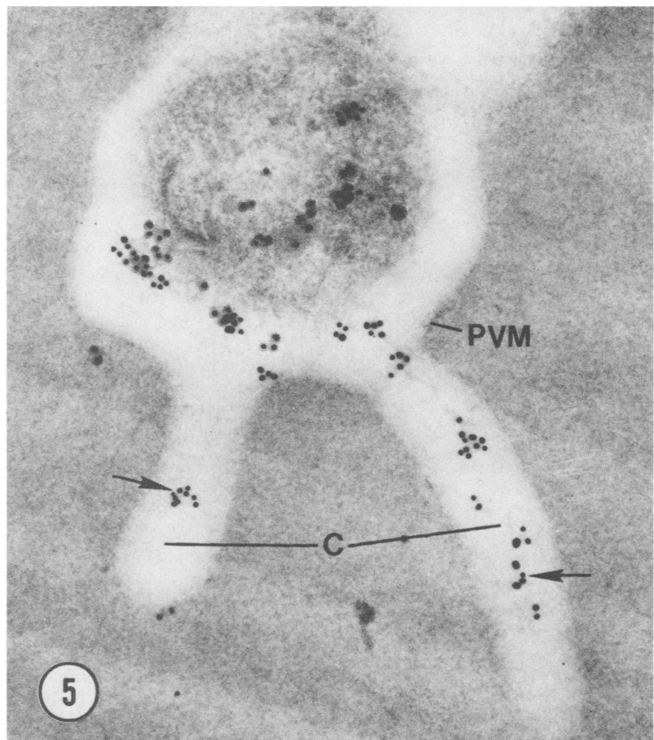

FIG. 2. LR white section of an extracellular merozoite. Dense granules (D) located in the anterior half of the merozoite are densely labeled with gold particles. Rhoptries (R) and micronemes (Mi) situated in the apical portion of the merozoite are not labeled. Magnification, ×55,000.

FIG. 3. Merozoite invading a rhesus monkey erythrocyte. Some dense granules (⟶) are seen at the surface of the merozoite. The presence of gold particles (▶) indicates that the contents are discharged into the parasitophorous vacuole space (PVS) through the pellicle. The junctions (J) are not labeled with gold. N, Nucleus. Magnification, ×34,000.

FIG. 4. Electron micrograph showing the invagination of the parasitophorous vacuole membrane (⟶) adjacent to the discharged dense-granule material (▶). D, Dense granules. Magnification, ×40,000.

FIG. 5. Long narrow channels (C) which extend from the parasitophorous vacuole membrane (PVM) are associated with gold particles (⟶). Magnification, ×40,000.

TABLE 1. Characterization of the merozoite apical organelles possibly involved in host cell invasion

Organelle	Shape	Size (nm)	Density	No. (range)	Point of release
Dense granule	Round	140 by 120	Medium to high	6.9 (4–10)[a]/section	Pellicle, adjacent to apex
Microneme	Ellipsoidal	100 by 40	Medium	7.2 (5–12)[a]/section	Unknown
Rhoptry	Teardrop	570 by 330	Medium	2	Ducts at apex

[a] Sections through 20 different merozoites were examined.

and washed five times with TPBS in 1 h. Antibodies were eluted by the modified method of Smythe et al. (10). The filters were incubated for 10 min in 100 mM boric acid–150 mM NaCl (pH 9.0), washed two times in PBS, incubated for 20 min in 100 mM glycine–150 mM NaCl (pH 2.8) to elute any bound antibody, and then washed three times and stored in TPBS (10). The solution of eluted antibody in glycine saline was neutralized with 2 M Tris, pH 8.0, and dialyzed overnight against PBS with 0.05% NaN_3, and the volume was reduced 60-fold in Centricon 30 microconcentrators (Amicon Corp., Lexington, Mass.). The antibody selected from the immune rhesus serum by this method immunoprecipitated several Triton X-100-soluble molecules from [^{35}S]methionine metabolically labeled schizonts; therefore, the protein of the dense granules recognized by this serum cannot be defined at this time.

For immunoelectron microscopy, extracellular merozoites and infected erythrocytes were fixed with 1% paraformaldehyde–0.1% glutaraldehyde in 0.1 M PBS at pH 7.3 and embedded in LR white resin (Polysciences, Inc., Warrington, Pa.) (1). Sections were etched with a saturated aqueous solution of sodium metaperiodate and incubated for 30 min in 0.1 M PBS containing 5% nonfat dry milk and 0.01% Tween 20 (PBS-milk-Tween). Grids were transferred to PBS-milk-Tween containing polyclonal rhesus monkey serum or nonimmune rhesus monkey serum and incubated overnight at 4°C. Grids were rinsed in 0.1 M PBS containing 1% bovine serum albumin (BSA) and 0.01% Tween 20 (PBS-BSA-Tween) and incubated for 1 h with rabbit anti-rhesus monkey immunoglobulin G diluted 1:100 in PBS-milk-Tween. After being washed in PBS-BSA-Tween, grids were incubated for 1 h with goat anti-rabbit immunoglobulin G conjugated to gold particles (15 nm in diameter; Janssen Pharmaceutica, Piscataway, N.J.) diluted 1:20 in PBS-milk-Tween. After final rinses in PBS-BSA-Tween and then distilled water, grids were stained with 2% uranyl acetate in 50% methanol and examined by a JEOL 100CX electron microscope. For transmission electron microscopy, specimens were fixed and embedded as previously described (2).

Electron microscopy showed that round dense granules were located near the rhoptries. They appeared to be intermediate in size between the rhoptries and micronemes and measured ~140 nm in diameter (Fig. 1). The matrix was more electron dense than the micronemes and rhoptries were. Table 1 summarizes apical organelles on the basis of results from our current study. By immunoelectron microscopy, dense granules were clearly distinguished from rhoptries and micronemes. In extracellular merozoites, gold particles were associated only with round dense granules, whereas no labeling of rhoptries or micronemes was observed (Fig. 2). During initial attachment of the apical end of the merozoite to the rhesus erythrocyte, discharge of the dense granule material did not occur. However, during the invasion process after junction formation, dense granules moved to the surface of the merozoites and their contents were discharged into the parasitophorous vacuole through the pellicle, mainly from the anterior lateral side of the merozoites. At this stage, an aggregate of the dense-granule contents labeled with gold particles was localized around the anterior surface of the merozoite (Fig. 3).

The parasitophorous vacuole membrane adjacent to the released contents of dense granules started to invaginate and form an elongated channel (Fig. 4). Gold particles were often seen extending into the channel from the merozoite pellicle (Fig. 5). However, gold particles were not located on the parasitophorous vacuole membranes. The surface membrane of the infected erythrocyte and the moving junctions were also not labeled with gold particles (Figs. 3–5).

Our study indicates that dense granules play a role in the formation of channels which extend from the parasitophorous vacuole membrane after erythrocyte entry by the merozoite. This suggestion is strengthened by the fact that all channels which extend from the vacuole membrane contain gold particles. Therefore, the function of dense granules appears to be different from those of rhoptries and micronemes, which are thought to be involved in the initial invasion process. The significance of the fingerlike channels that contained the contents of the dense granules is not clear. Although discharge of dense-granule content has not been described, these organelles also appear to be present in the merozoites of other species of *Plasmodium*. Since micronemes and dense granules are difficult to distinguish morphologically, antigens which were once thought to be present in micronemes or rhoptries may actually be present in dense granules. Reevaluation of published data, especially immunolocalization studies by fluorescence microscopy, may be required for further clarification of malaria biology, as these organelles may have similar functions in all members of the phylum Apicomplexa.

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