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Electron Microscopy of Intracellular Protozoa

Annual/Final Report

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We performed immunoelectron microscopy to investigate the distribituon of malarial antigens in the various stages of malaria parasites. Monoclonal antibodies, which recognize different epitopes of the P. yoelii CS protein, reacted with the surface of oocyst and salivary gland sporozoites. However, a small percentage of developing oocysts did not express CS antigen on the surface of sporoblasts or sporozoites. These sporozoites were still capable of completing development and invading salivary glands. Their presence may explain in part the failure of CS vaccines to completely protect immunized animals against challenge. The non-CS antigen did not become

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abundant until late in sporogony. The Non-CS protein was most abundant within the cytoplasm and micronemes, although non-CS protein was also seen on the sporozoite surface.

After studying CS protein in oocysts and sporozoites, we studied the presence of CS protein in the EE stages of P. berghei. CS antigen could be localized on the parasitophorous vacuole membrane and pellicular complex of sporozoites which had recently invaded hepatocytes, and on electron-dense masses of sloughed CS antigen in the host cell cytoplasm. CS antigen persisted throughout the complete EE cycle of P. berghei on the surface of EE schizonts and incorporated into the plasma membrane of budding EE merozoites. These observations may indicate that malaria vaccine using CS protein may also affect the development of the EE stages. In addition, we studied the fate of dense granules during invasion of erythrocytes by P. knowlesi merozoites. Once merozoites entered host cells, dense granules moved to the pellicle and released their contents into the parasitophorous vacuole space, creating finger-like channels from the vacuole membrane. This is the first report showing the content of dense granules of malarial parasites is different from rhoptries and micronemes and involved in the formation of channels for the parasitophorous vacuole.

Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Localization of CS- and Non-CS antigens in the sporogonic stages of *Plasmodium yoelii*

Abstract: Monoclonal antibodies (MAbs) and colloidal gold probes were used to localize circumsporozoite (CS) protein and two unrelated polypeptides in developing oocysts and salivary gland sporozoites of the 17X (NL) strain of Plasmodium yoelii. MAbs NYS1, NYS2, and NYS3 recognized different epitopes of the P. yoelii CS protein and produced similar patterns of immunolabeling on developing oocysts and sporozoites. A small percentage of developing oocysts did not express CS antigen on the surface of sporoblasts or sporozoites, although internal labeling was associated with endoplasmic reticulum (ER). These sporozoites were still capable of completing development and invading salivary glands where they could be found adjacent to sporozoites with densely labeled surface coats. If these sporozoites are infective, their presence may explain in part the failure of CS vaccines to completely protect immunized animals against challenge. The non-CS antigen recognized by MAbs NYS4 did not become abundant until late in sporogony. This antigen was not associated with the surface of budding sporozoites, but was most abundant within the cytoplasm and micronemes. A second non-CS antigen identified by NYS5 first appeared in 7-day-old oocysts, although labeling was sparse. Small quantities of antigen appeared on the sporoblast membrane, cytoplasmic clefts and ER of oocysts and was associated with micronemes and the surface of budding and mature sporozoites. Since we do not yet know the role played by non-CS antigens in the biology of the parasite, further characterization of their function is needed before their potential as vaccine candidates can be determined.

Introduction

Charoenvit and associates\1/ produced monoclonal antibodies (MAbs) against sporozoites of P. yoelii. MAbs NYS1, NYS2 and NYS3 recognized the CS antigen, although each reacts with a different epitope. The remaining 2 MAbs, NYS4 and NYS5, recognized antigens which are not CS protein. The antigen recognized by NYS4 is a 140 kDa protein with a common determinant with the CS protein. The antigen recognized by NYS5 could not be found by Western blotting. Immunofluorescent microscopy (IFA) demonstrated that 3 MAbs (NYS1, NYS2 and NYS3) reacted with the sporozoite surface, although there were slight differences in patterns of reactivity. On the other hand, NYS4 produced a large patchy pattern that appeared to be both internal and on the surface of the sporozoite. MAb NYS5 produced a pattern that appeared to be on the surface but was restricted to polar regions. The precise location of antigens detected by these MAbs were difficult to determine because of the limited resolution of IFA. Therefore, we performed immunoelectron microscopy with particular attention to non-CS proteins, since we do not yet know what role these antigens play in the biology of the parasite.

Materials and Methods

Since the detailed description of the production of MAbs, ascitic fluids, IFA test, CSP reaction, SDS-PAGE and immunodetection and sporozoite neutralization have already been published by Charoenvit et\1/ these methods will not be described here.

Immunoelectron microscopy was performed on 4-, 7-, 10- and 15-day-old oocysts and salivary gland sporozoites of the 17X(NL) strain of P. yoelii. Infected midguts and salivary glands were fixed for 20 minutes at 4°C in 1% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 and were embedded at low temperatures in LR gold resin (London Resin Company). Sections were cut with a diamond knife, mounted on nickel grids and labeled with MAb's NYS1, NYS2, NYS3, NYS4 and NYS5 and 15 nm immunoglobulin-gold as described elsewhere.\2/

Results

Localization of CS protein by NYS1, NYS2 and NYS3

NYS1, NYS2 and NYS3 produced similar results when tested on 4-, 7- and 10-day-old oocysts and salivary gland sporozoites of P. yoelii.

Four-day-old oocysts did not show subcapsular vacuolization, cleft formation, or budding sporozoites. Little label was associated with the cytoplasm. Seven-day-old oocysts started to show synthesis of CS antigen. Gold label was seen in association

with the nuclear membranes, endoplasmic reticulum and plasma membrane (Fig. 1). The subcapsular space of developing oocysts also was labeled with gold particles. In some oocysts, the sporoblast cytoplasm had started to contract, showing deep clefts in its peripheral cytoplasm. Gold particles were seen on the surface of clefts and on fine granular electron-dense material in the subcapsular space. There were many sporozoites in 10-day-old oocysts. Gold particles were present mainly on the surface of sporozoites but some particles are also seen within the sporozoite cytoplasm (Fig. 2). The residual sporoblast cytoplasm was also labeled with gold particles. Membrane-bound electron opaque granules were also labeled with gold particles.

Mature sporozoites in salivary glands of infected mosquitoes were coated evenly with CS protein (Fig. 3). Label was also associated with micronemes (Fig. 3). Electron-opaque secretory material which surrounded the sporozoite was also labeled with gold particles.

A small percentage (<5%) of oocyst and salivary gland sporozoites did not express CS antigen on their surface (Figs. 4 and 5). A few gold particles were only limited to internal micronemes and perinuclear membranes of budding sporozoites and to the endoplasmic reticulum in the sporoblast cytoplasm. All sporozoites produced from oocysts without CS protein lacked CS protein on their surface. Oocysts without CS protein were often seen next to oocysts with CS protein in the same section.

Localization of non-CS protein (NYS4-non-CS protein) by NYS4.

Only a few random gold particles were associated with 6- and 7- day-old-oocysts. No labeling was found in the endoplasmic reticulum indicating that synthesis of this antigen had not started. Synthesis of NYS4-non-CS protein began late in sporogony, when budding of sporozoites started (Fig. 6). Dense labeling of gold particles was associated with the endoplasmic reticulum and with small membrane-bound, electron opaque granules in the sporoblast cytoplasm of 15-day-old oocysts (Fig. 6). These small membrane-bound electron opaque granules appeared to migrate into budding sporozoites. When salivary gland sporozoites were examined, micronemes, but not rhoptries, were densely labeled by MAb NYS4 (Fig. 7). No label was seen on the plasma membrane of sporozoites.

Localization of non-CS protein by NYS5 (NYS5-non-CS protein).

Similar to NYS4-non-CS protein, only a few gold particles were present in the sporoblast cytoplasm of the 7 day old oocysts. Antigen recognized by NYS5 appeared on the surface and cytoplasm of sporoblasts in 10-day-old oocysts (Fig. 8). Gold particles were seen in the cytoplasmic matrix as well as in the endoplasmic reticulum. Gold particles was associated with the micronemes and cytoplasm of salivary gland sporozoites as well as on the surface (Fig. 9).

Discussion

The distribution of circumsporozoite protein in oocysts and salivary gland sporozoites has been studied by immunoelectron microscopy in P. malariae, P. ovale, P. knowlesi, P. berghei and P. falciparum.\3-7/ Based on these data, synthesis of CS protein appears early in sporogony before the differentiation of sporozoites. CS protein becomes abundant in the oocyst cytoplasm before sporozcites begin to bud. The presence of CS antigen in the subcapsular space might indicate sloughing of CS antigen from the surface of the oocysts.

The presence of CS antigen in the micronemes has been described in P. knowlesi, P. malariae, and P. berghei.\3,5,6/ In addition, rhoptries of P. knowlesi were also shown to possess CS protein\5/. However, cross-reactivity of MAb NYS2 with NYS4-non-CS protein on Western blots might indicate that labeling of micronemes in P. yoelii sporozoites could be a cross-reaction.\1/

It is interesting to note that a small percentage of oocysts and salivary gland sporozoites did not show CS protein on their surface. We do not know if these sporozoites are capable of being transmitted or whether they are infective. If they are infective, their presence may explain in part why immunization with sporozoite vaccines is not completely protective.\8-10/ It is unclear what molecules are on the surface of these sporozoites. Presence of internal reactivity with MAb's to the CS protein suggests that the antigen is not processed or exported to the surface of the

developing sporozoites. Alternatively, only a small fragment of the CS antigen may be exported to the surface or a new antigenic variant that is not recognized by any of the 3 MAbs may have arisen. Since the CS protein is believed to play such an important role in sporozoite motility and host cell recognition and invasion, it is clear that this phenomenon needs to be investigated further.

NYS4-non-CS protein appeared in the oocyst much later than CS protein detected by NYS1, NYS2 and NYS3. The NYS4 non-CS protein was first evident in oocysts with budding sporozoites and was always associated with small membrane-bound electron opaque granules in the oocyst cytoplasm. These granules appear to migrate to the budding sporozoites, carrying NYS4-non-CS protein into the sporozoites. These granules might be precursors of micronemes, since the size and shape are similar to micronemes and micronemes of the sporozoite also contain NYS4-non-CS protein.

The distribution of NYS5-non-CS protein is similar to that of NYS4 non-CS protein. Both antigens are mainly located in micronemes and sporozoite cytoplasm although NYS5-non-CS protein is on the surface of the sporozoite. Also, they appear late in the oocyst. However, NYS5 non-CS protein was not associated with small membrane-bound electron opaque granules in the oocyst. This may indicate that synthesis of NYS5 non-CS protein is different from that of NYS4 non-CS protein.

Our immunoelectron microscopy demonstrated that the distribution of NYS4 non-CS protein of P. yoelii is different from that of CS protein. Since the role on non-CS protein in the

biology of the parasite is still not clear, further investigation of their function may be required before their potential as vaccine candidates can be determined.

Acknowledgements

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Figure legends

Fig. 1 Section of a 7-day-old P. yoelii oocyst incubated with MAb NYS1 against CS protein and immunoglobulin-gold. Gold particles are associated with the endoplasmic reticulum (ER), cytoplasmic matrix and subcapsular areas (arrow). X 20,000

Fig. 2 Section of a 10-day-old oocyst incubated with MAb NYS2 against CS protein and immunoglobulin-gold. Gold label (arrow) is associated with the surface of clefts and of budding sporozoites (S). The oocyst capsule space (CS) is also labeled with gold particles. X 36,000

Fig. 3 Section of a salivary gland sporozoite (S) incubated with MAb NYS3 against CS protein and immunoglobulin-gold. Gold label is associated with the surface (arrow), and micronemes (Mi). X 44,000

Fig. 4 Section of a 10-day-old oocyst incubated with MAb NYS1 against CS protein and immunoglobulin-gold. Gold particles are only associated with perinuclear membranes (arrow) and endoplasmic reticulum (ER) Label is not on the surface (S) of the sporoblast or budding sporozoites. X 21,000

Fig. 5 Section of two salivary gland sporozoites incubated with MAb NYS2 against CS protein and immunoglobulin-gold. Sporozoite at the right (S1) is labeled on the surface (arrow) and micronemes (Mi). The sporozoite at the left (S2) shows gold label particles only over micronemes (Mi). X 40,000

Fig. 6 Section of a 15-day-old oocyst incubated with MAb NYS4 against non-CS protein and immunoglobulin-gold. Dense labeling of gold particles is mainly associated with membrane-bounded electron dense granules (D). These granules are also in the cytoplasm of budding sporozoites (S). Label is not present on the surface of budding sporozoites. X 40,000

Fig. 7 Section of a salivary gland sporozoite incubated with MAb NYS4 against non-CS protein and immunoglobulin-gold. Abundant gold particles are associated with sporozoite cytoplasm and micronemes (Mi). Few gold particles are on the surface. X 45,000

Fig. 8 Section of a 10-day-old oocyst incubated with MAb NYS5 against non-CS protein and immunoglobulin-gold. Only a few gold particles (arrow) are associated with the cytoplasmic matrix, plasma membrane (P), and subcapsular spaces (S). X 37,000

Fig. 9 Section of a salivary gland sporozoite incubated with MAb NYS5 against non-CS protein and immunoglobulin-gold. Gold particles are associated with micronemes (Mi), cytoplasm, and the sporozoite surface (arrow) X 30,000

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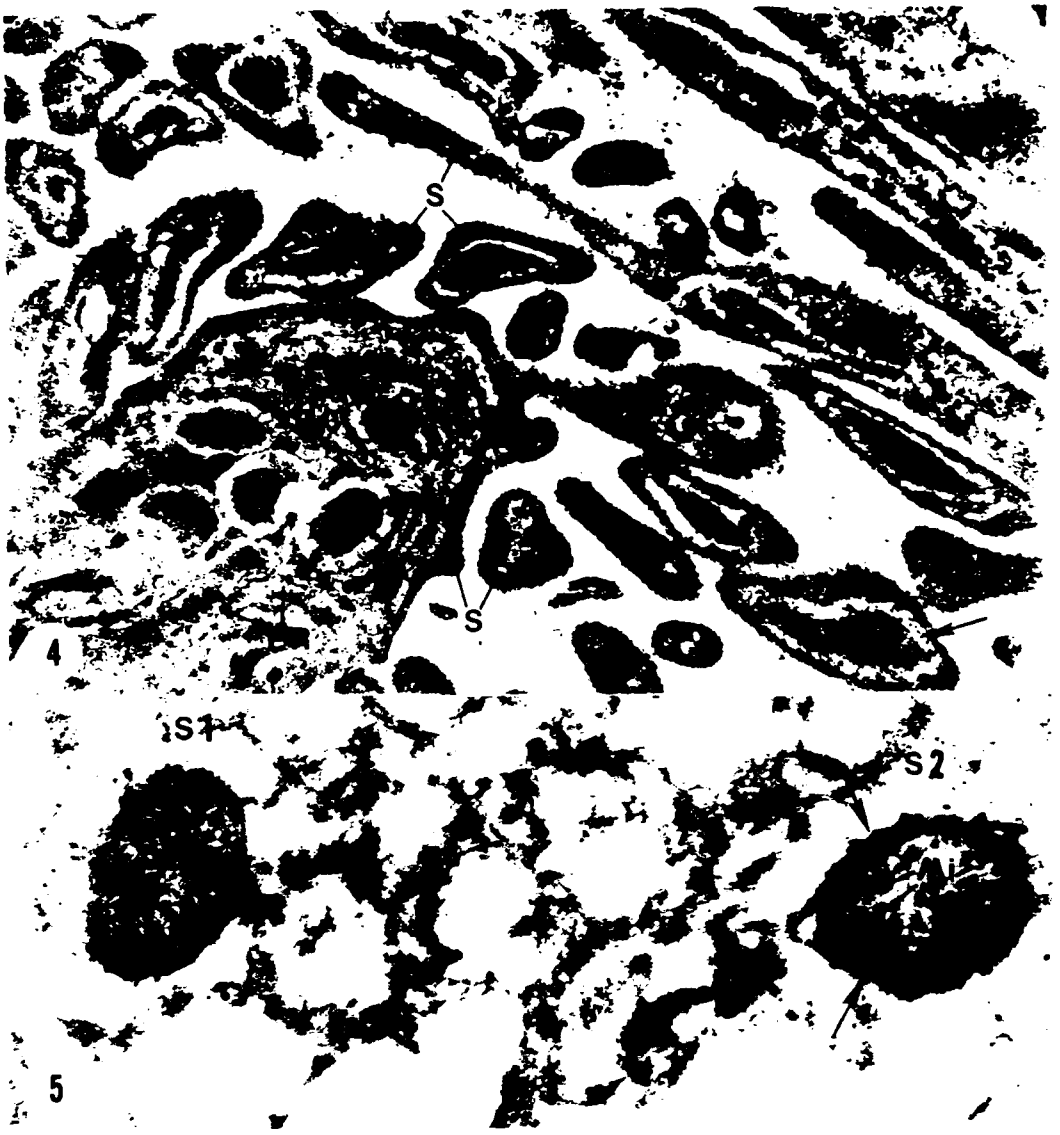
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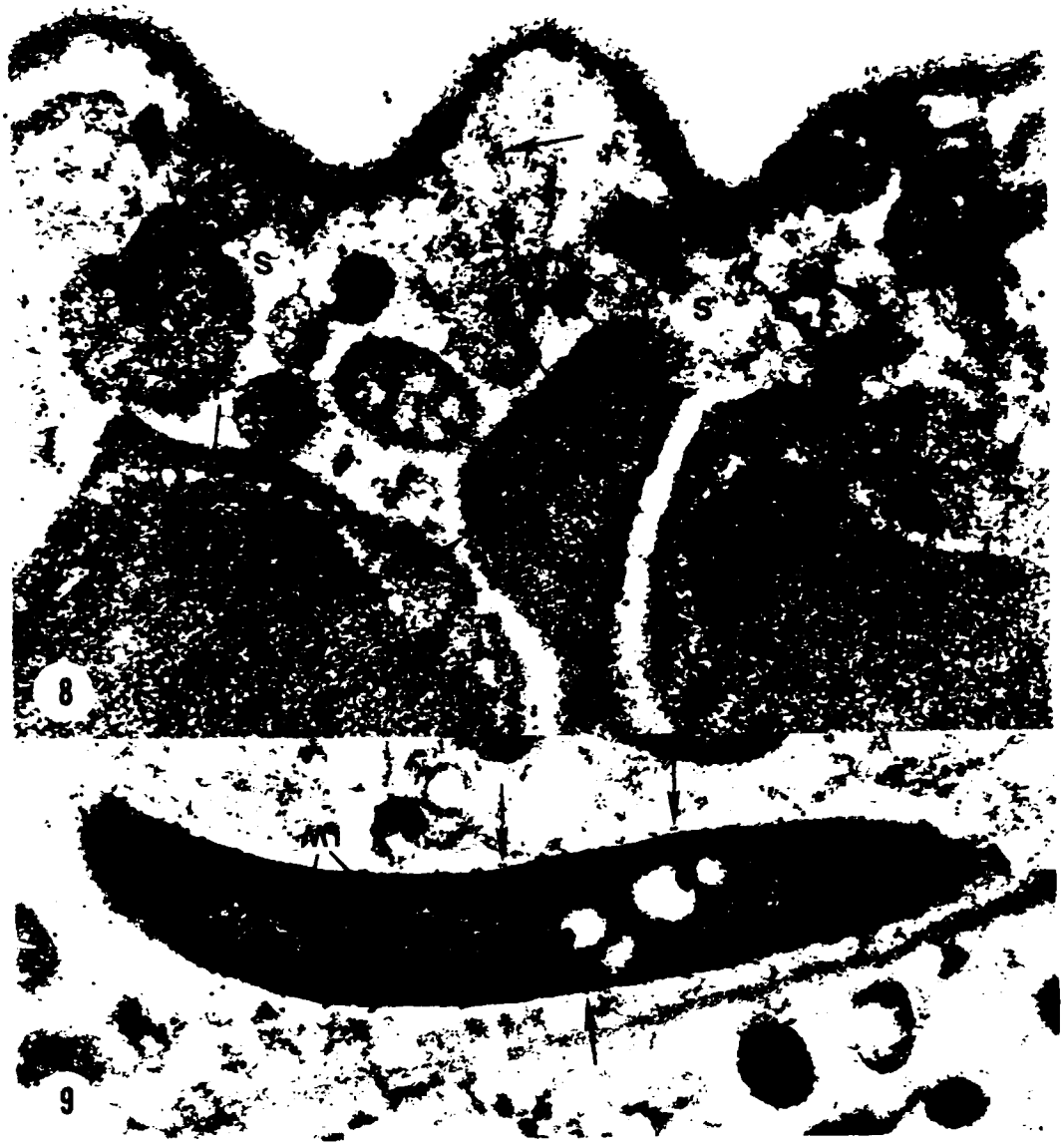
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Expression of Plasmodium berghei Circumsporozoite Antigen on the Surface of Exoerythrocytic Schizonts and Merozoites

ABSTRACT: The intracellular distribution of circumsporozoite (CS) antigen was traced by immunoelectron microscopy within in vitro cultures of P. berghei exoerythrocytic (EE) schizonts with monoclonal antibody (MAb) 3D11 to the immunodominant repeat region of the P. berghei CS protein. CS antigen could be localized on the parasitophorous vacuole (PV) membrane and pellicular complex of recently invaded sporozoites and on electron-dense masses of sloughed CS antigen in the host cell cytoplasm. CS antigen persisted throughout the complete EE cycle of P. berghei on the surface of EE schizonts and was incorporated into the plasma membrane of budding EE merozoites. Erythrocytic merozoites were not labeled by MAb 3D11, indicating that these two populations of merozoites differ in antigenic composition. Significant internal labeling occurred in 50-hour EE schizonts in association with the limiting membranes of peripheral vesicles and with short, tube-like structures attached to their outer surfaces. These vesicles contained an electron-dense flocculent material which was also present in the PV space. Association of CS antigen with the limiting membranes of these vesicles suggests that they either develop as endocytotic invaginations of the schizont plasma membrane or transport newly synthesized CS antigen from the endoplasmic reticulum and Golgi of developing EE schizonts to the parasite surface.

INTRODUCTION

The association of circumsporozoite (CS) proteins with the plasma membrane and parasitophorous vacuole (PV) membrane that surround exoerythrocytic (EE) parasites was first suggested by immunocytochemical studies of Plasmodium berghei EE parasites that were grown in cultured hepatoma (HepG2-A16) cells. \1/ These results demonstrated that CS antigen could be detected throughout the EE cycle and on EE merozoites. Similar studies of P. berghei \2, 3/, P. falciparum \4-6/, P. vivax \7, 8/, and P. cynomolgi \9/ have also identified CS antigen within EE parasites, but have not followed the fate of CS antigen through a complete EE cycle by immunoelectron microscopy. Since CS protein is the primary candidate for a sporozoite vaccine and may elicit cytotoxic T-lymphocytes that recognize CS antigen within EE parasites \10, 11/, precise knowledge about its fate and distribution in infected hepatocytes is important.

In this study, we examined the distribution of CS antigen from early stages of host cell invasion to the release of mature EE merozoites in P. berghei-infected HepG2-A16 cells. These results extend previous light and electron microscopic observations of CS antigen on the surface of EE parasites and merozoites. \1, 8, 9/

MATERIALS AND METHODS

Cell Culture

Human hepatoma cloned cell line HepG2-A16 was cultured on coverslips in MEM (Earl's) supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin in 5% CO_2 in air at 37°C as described previously. \12/ Plasmodium berghei sporozoites were isolated aseptically from the salivary glands of Anopheles stephensi mosquitoes which had fed 14 days earlier on mice infected with the ANKA strain of Plasmodium berghei. \12/

Immunoelectron microscopy

Monolayers of HepG2-A16 hepatoma cells were incubated with 500,000 P. berghei sporozoites for 3 hr at 37°C, carefully scraped from the coverslips with a rubber policeman and fixed for 1 hr at 4°C in 1% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Other coverslips were incubated for 24, 50 and 72 hours before cells were removed and fixed. Some 50-hour cultures were fixed for 30 min at room temperature in 1.25% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, with 4% sucrose. The suspensions of fixed cells were washed by centrifugation in three changes of 0.1 M phosphate buffer, pH 7.4 and dehydrated in 10-min changes of 30%, 50%, 70%, and 95% ethanol at progressively lower temperatures between 4°C and -20°C and infiltrated and polymerized in LR Gold Resin (London Resin Company, Basingstoke, England) at -20°C as described previously. \13/ Thin sections were cut with a diamond knife and collected on unsupported 300 mesh nickel grids.

Sections were etched in drops of a saturated aqueous solution of sodium metaperiodate, rinsed with distilled water and blocked with a solution containing 5% nonfat dry milk, 0.9% NaCl and 0.01% Tween 20 in 0.1 M phosphate buffer, pH 7.3 (PBS-Milk-Tween). The sections were labeled in drops of mouse monoclonal 3D11 diluted to a final concentration of 5 μg/ml with PBS-Milk-Tween. The mouse monoclonal recognized the repeat region of the P. berghei CS protein and was produced and characterized as described by Yoshida et al.. \14/ After incubation with the primary antibody, grids were washed with a solution containing 1% bovine serum albumin (Fraction V) (ICN Immunobiologicals), 0.9% NaCl and 0.01% Tween 20 in 0.1 M phosphate buffer, pH 7.3 (PBS-BSA-Tween). Grids were incubated in drops of a rabbit anti-mouse IgG antibody (ICN Immunobiologicals) diluted to 25 μg/ml with PBS-BSA-Tween. After incubation in the secondary antibody, the grids were washed with PBS-BSA-Tween and incubated in drops of 5 nm protein A-gold (Jannsen Life Sciences Products) or drops of 15 nm goat anti-rabbit IgG immunoglobulin-gold (Jannsen Life Sciences Products) diluted to 1/20 with PBS-BSA-Tween. After incubation with colloidal gold probes, grids were washed with buffer, fixed for 15 min with 2.5% glutaraldehyde in 0.1 M phosphate buffer to stabilize the gold, rinsed with distilled water, dried, stained with uranyl acetate and Reynold's lead citrate, carbon coated and examined with a JEOL 100CX electron microscope.

Method specificity was confirmed by incubating control sections with a nonrelevant mouse MAb in place of the primary antibody, with secondary antibodies and colloidal gold probes, or with colloidal gold probes alone.

RESULTS

3 Hour Cultures

Cultured HepG2-A16 cells which were fixed at 3 hours after sporozoite invasion contained numerous intracellular sporozoites and differentiating trophozoites. A few sporozoites were still present in 24 hour cultures. Dense label was associated with all three membranes of the pellicular complex of intracellular sporozoites and with internal micronemes (Fig. 1, inset). Label was present on the PV membrane and was also associated with small masses of amorphous electron-dense material in the host cell cytoplasm (Fig. 1).

24 Hour Cultures

After 24 hours of culture, most developing EE parasites were spherical and contained some remnants of the sporozoite pellicle (Fig. 2). MAb 3D11 densely labeled the surface and closely-apposed PV membrane of 24 hour EE schizonts (Fig. 2, inset). Some scattered labeling was associated with the parasite cytoplasm. Cytostomes and internal vesicles were not observed.

50 Hour Cultures

Dense label was associated with the surface of multinucleated 50 hour EE schizonts (Figs. 3, 4). Some label was associated with the PV membrane, but the density of labeling was significantly less than at 24 hours. This difference was particularly

evident around bump-like expansions of the PV that contained flocculent material (Fig. 5). The PV membrane that bounded these expansions was usually devoid of CS antigen.

Specific labeling of structures within the schizont cytoplasm was first observed in 50 hour EE schizonts and corresponded to the appearance of membrane-bounded vesicles. These vesicles varied in size and were located in the peripheral cytoplasm (Figs. 3-6). Small peripheral vesicles measured 200-500 nm in diameter and contained densely-packed flocculent material. These vesicles formed grape-like clusters immediately beneath the surface of the schizont (Figs. 3, 4). MAb 3D11 densely labeled the limiting membranes of these internal vesicles as well as short 50-150 nm tubule-like structures that were connected to the outer surface of the vesicles (Figs. 5-6).

Pocket-like invaginations of the schizont membrane and larger peripheral vesicles measuring 600-800 nm in diameter were also present, but not as common as the small vesicles (Figs. 5, 6). The PV membrane often formed bump-like expansions around the flocculent material that filled these pockets (Fig. 5). Labeling was associated with the schizont plasma membrane and with short tubule-like structures that extended into the schizont cytoplasm around the periphery of the invaginations, but only rarely with the PV membrane (Fig. 5). Large vesicles contained loosely or densely packed flocculent material and usually occurred alone or in small clusters of 3-4 vesicles. MAb 3D11 labeled the limiting membranes of larger vesicles as well as short tubule-like structures connected to their outer surface (Fig. 6).

70 Hour EE Schizonts

Maturing EE schizonts were divided into numerous large cytomeres by development of deep clefts in the schizont cytoplasm (Fig. 7). MAb 3D11 densely labeled the surface of the developing clefts. Internal vesicles were not observed at this stage of development. As cytomeres developed, the parasite cytoplasm retracted from the PV membrane to form an expanded PV space (Fig. 7). CS antigen remained associated with the schizont plasma membrane and with some electron-dense material in the PV space. Some scattered labeling was associated with the PV membrane at this stage of development, but was limited to small patches of adherent electron-dense material.

At the final stages of differentiation, merozoites budded from the surface of cytomeres (Fig. 8). MAb 3D11 labeled the surface of budding merozoites, but no internal structures. Sections of erythrocytic merozoites that were fixed and embedded by similar procedures were not labeled by MAb 3D11 (Fig. 8, inset).

DISCUSSION

Previous immunocytochemical studies of P. berghei EE schizonts by light and electron microscopic techniques have localized CS antigen on the plasma membrane of EE schizonts. \1-3/ Limited ultrastructural studies of Lowicryl-embedded 24- and 50-hour-old schizonts also reported some labeling of the PV membrane around 24-hour-old parasites and sparse labeling on the surface of 50-hour-old EE merozoites. \2-3/ Results of the present study confirm these observations; however, we identified

significantly more labeling on the surface of schizonts and EE merozoites than reported previously. These differences in labeling intensity probably reflect the better preservation of antigenicity and immunoreactivity that is possible with LR Gold resin. \15/

Ultrastructural observations in our study demonstrate the association of CS antigen with the PV membrane around recently invaded sporozoites. We observed a clear decrease in labeling on the PV membrane during EE development, suggesting that no additional CS antigen becomes associated with the expanding membrane around growing EE schizonts. A similar decrease in immunoreactivity has also been reported for the PV membrane around P. cynomolgi EE schizonts. \9/ Association of CS antigen with the PV membrane during host cell invasion and early EE development has also been observed in cultures of P. cynomolgi \9/ and P. vivax (C. Atkinson, M. Aikawa, S. Aley and M. Hollingdale, unpublished data). All three of these species are capable of completing EE schizogony in vitro. Interestingly, CS antigen was not detected on the PV membrane around hepatoma cell cultures of recently invaded P. falciparum sporozoites. \13/ Failure of P. falciparum sporozoites to transform into early trophozoites and complete EE development in this cell type suggests that interaction of CS antigen with the PV membrane may be one factor that is important for early intracellular survival and development.

A number of workers have described the sloughing of CS antigen from sporozoites that have recently invaded HepG2-A16 cells and mosquito salivary glands. \1, 2, 16/ We found similar electron-dense masses of CS antigen in the cytoplasm of host cells in 3- and 24-hour-old cultures when undifferentiated sporozoites were still

present. Similar masses were not detected in 50- and 70-hour-old cultures, suggesting that sloughed CS antigen may eventually be degraded by the host cell.

Suhrbier et al. \2/ observed spotty or punctate labeling of 48 hour EE schizonts by immunofluorescence antibody techniques with MAb 3D11 and suggested that the spots might correspond to peripheral vesicles described by Meis et al. \17/ We found dense label on the limiting membranes of these internal vesicles and on short, tube-like structures on their outer surface. Based on morphological evidence, Meis et al. \17, 18/ postulated that these vesicles originate from the endoplasmic reticulum and Golgi of the parasite and transport flocculent material to the PV space where it may aid in external digestion of the host cell cytoplasm. Localization of CS antigen on the membranes of these vesicles indicates that it is either 1) synthesized during EE development and simultaneously exported with peripheral vesicles to the surface of the parasite, 2) internalized from the schizont plasma membrane by the formation of endocytotic vesicles (e.g. Fig. 5), or 3) cycled intracellularly by some combination of these two hypotheses. The short tubule-like structures that we observed on the outer surface of internal peripheral vesicles have not been previously reported in malarial EE schizonts and are similar in structure to endosomal or CURL (compartment for uncoupling of receptor and ligand) vesicles. \19/ If they have a similar function, the tube like structures may be important in recycling or transport of CS antigen to the surface of the schizont as occurs in eucaryotic cells during uncoupling and sorting of receptors and ligands after receptor-mediated endocytosis.

Persistence of CS antigen on the surface of P. berghei EE schizonts suggests that it plays an important role in mediating intracellular development, although its precise

function remains unknown. We found that MAb 3D11 bound to EE merozoites but not erythrocytic merozoites that were fixed and labeled by similar procedures, indicating that a true antigenic difference exists between these two populations of merozoites. Association of CS antigen with the surface of EE merozoites may be important in maintaining a state of premunition in infected hosts that are repeatedly exposed to sporozoites. \20/ Since sporozoites encounter a variety of cell types during passage through tissues of their mosquito and mammalian hosts, it is possible that CS antigen has a number of different but related functions that are important in motility, recognition and invasion of host cells, regulation of host-parasite interactions between EE schizonts and their host cells, and modulation of immune responses in infected hosts.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Electron micrographs of HepG2-A16 hepatoma cells that were fixed 3 hours (inset) and 24 hours after invasion of P. berghei sporozoites and labeled with MAb 3D11, secondary antibody, and protein A-gold. 1. Intracellular sporozoite fixed 24 hours after invasion. Gold label is associated with the surface of the sporozoite (S) and with masses of electron-dense material (EDM) in the host cell cytoplasm. X 36,400. Inset. Intracellular sporozoite fixed 3 hours after invasion. Gold label is associated with the sporozoite pellicle (arrow), intracellular micronemes (Mi), and with the surrounding PV membrane (PV) and space. X 56,000.

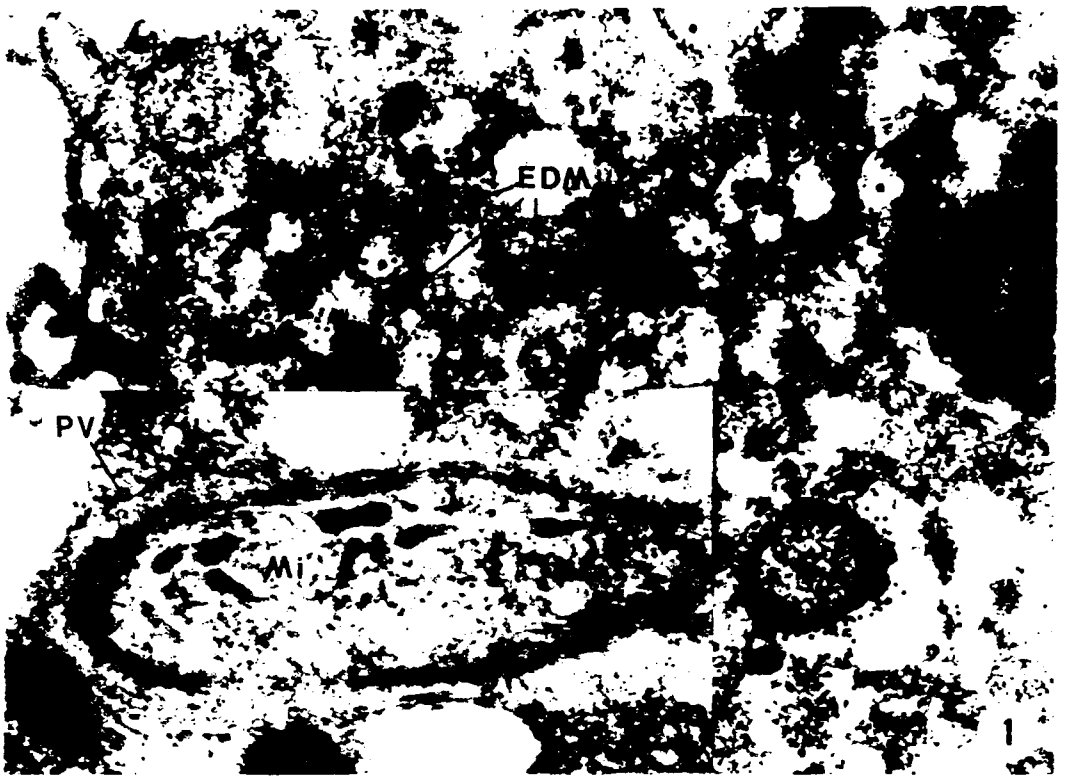
Fig. 2. Electron micrograph of a 24-hour-old P. berghei EE schizont that was incubated with MAb 3D11 and 15 nm goat, anti-mouse IgG colloidal gold. Dense label (arrows) is associated with the surface and closely apposed PV membrane. Internal structures include nuclei (N), mitochondria (mt), and vacuolated areas (V). X 21,000. Inset. Higher magnification of the schizont surface. Note remnants of the sporozoite pellicle (arrows). X 46,000.

Fig. 3. Low magnification electron micrograph of a 50-hour-old P. berghei EE schizont that was incubated with MAb 3D11 and 15 nm goat, anti-mouse IgG colloidal gold. Dense label (arrows) is associated with the surface of the EE schizont and with the limiting membranes of peripheral internal vesicles (Vs). Internal structures include nuclei (N) and vacuolated areas (V). X 12,000.

Figs. 4-6. Higher magnification electron micrographs of 50-hour-old P. berghei EE schizonts that were incubated with MAb 3D11 and 15 nm goat anti-mouse IgG colloidal gold. 4. Dense label is associated with the surface of the EE schizont and with limiting membranes of peripheral vesicles (Vs). The PV membrane (PV) and flocculent material (Fm) within peripheral vesicles and the PV space are not labeled by MAb 3D11. X 44,200. 5. Large pocket-like invagination in the schizont surface. Label is associated with tube-like extensions (arrows) which extend from the schizont plasma membrane into the schizont cytoplasm around the periphery of the invagination. Some scattered label is associated with flocculent material (Fm) in the PV space. Note absence of label on the PV membrane (PV). X 36,000. 6. Internal peripheral vesicles that contain flocculent material (Fm). Gold label is associated with the limiting membrane of the vesicles and with attached tube-like structures (arrows). X 44,200.

Figs. 7-8. Electron micrographs of 70-hour-old P. berghei EE schizonts that have been incubated with MAb 3D11 and 15 nm goat, anti-mouse IgG colloidal gold. 7. The schizont has been divided into cytomeres (Cy) by formation of clefts (arrows)

in the parasite cytoplasm. Most label is associated with the surface of cytomeres and material in the PV space. Internal vesicles with flocculent material are no longer evident. Little label is associated with the PV membrane (PV). Note numerous nuclei (N) in the cytoplasm of cytomeres. X 29,000. 8. EE merozoites (M) with prominent rhoptries (R) that are budding from the surface of a cytomere (Cy). Label (arrows) is associated with the surface of the merozoites, but not with internal structures. X 25,000. Inset. Section of an erythrocytic merozoite that was incubated with the same dilutions of MAb 3D11 and 15 nm goat, anti-mouse IgG colloidal gold. Gold label is not associated with the surface of the erythrocytic merozoite. Note rhoptries (R). X 19,500.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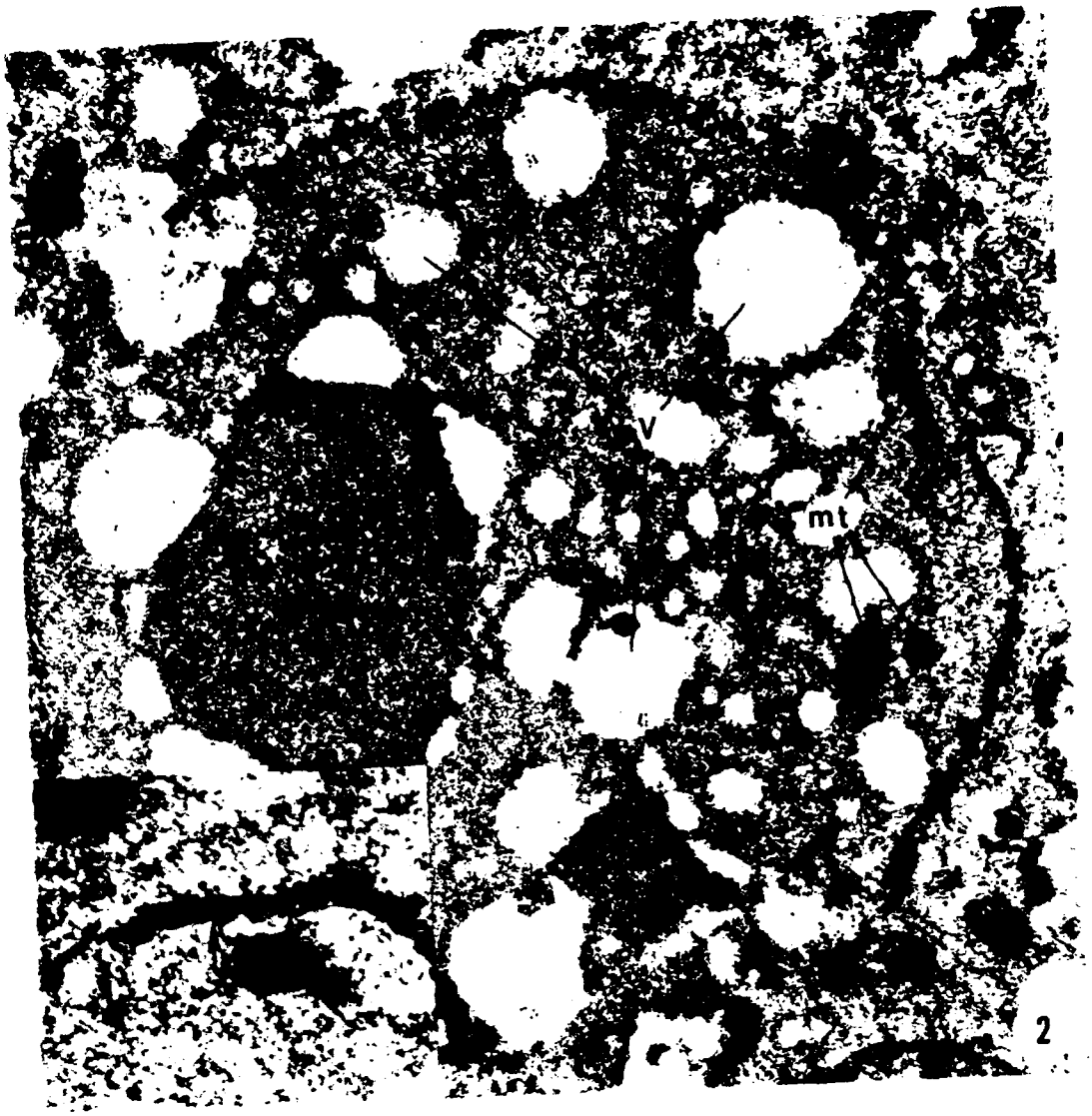
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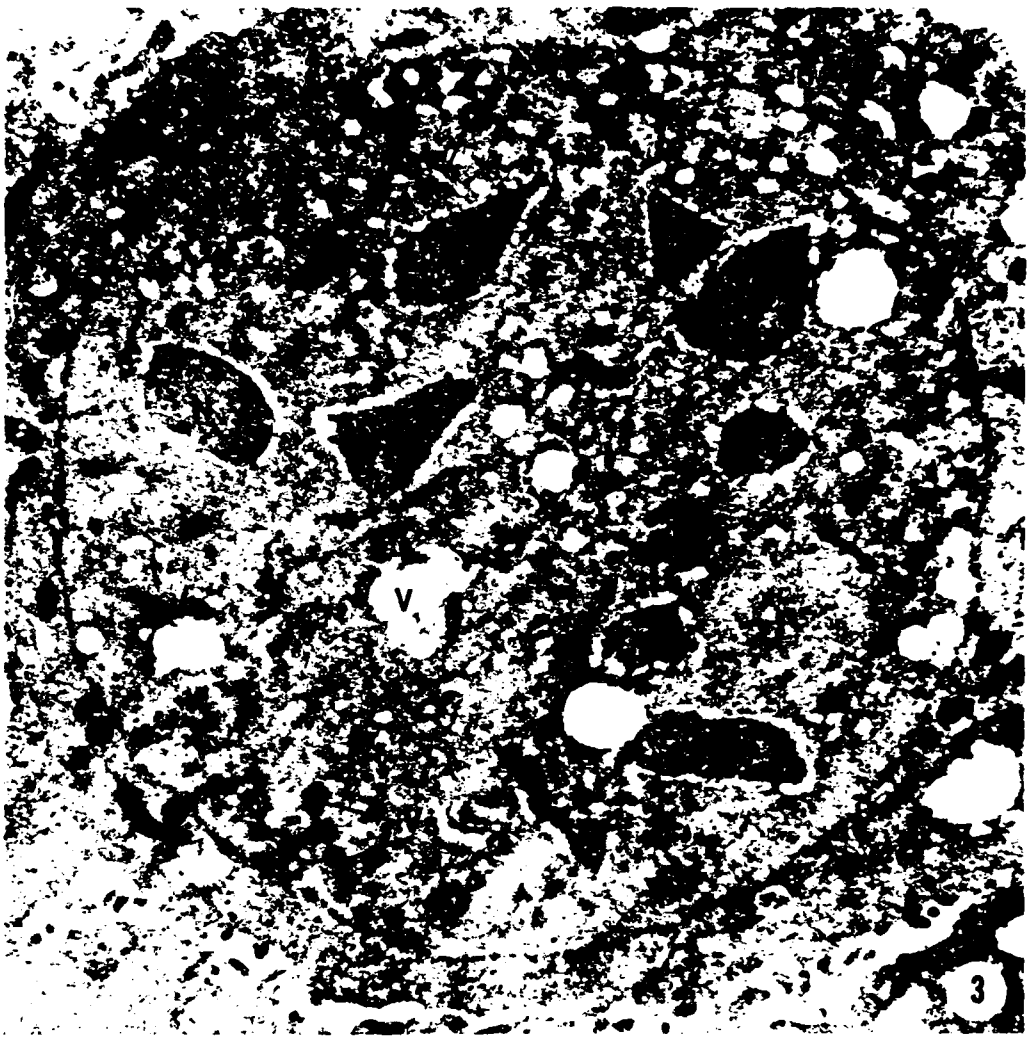
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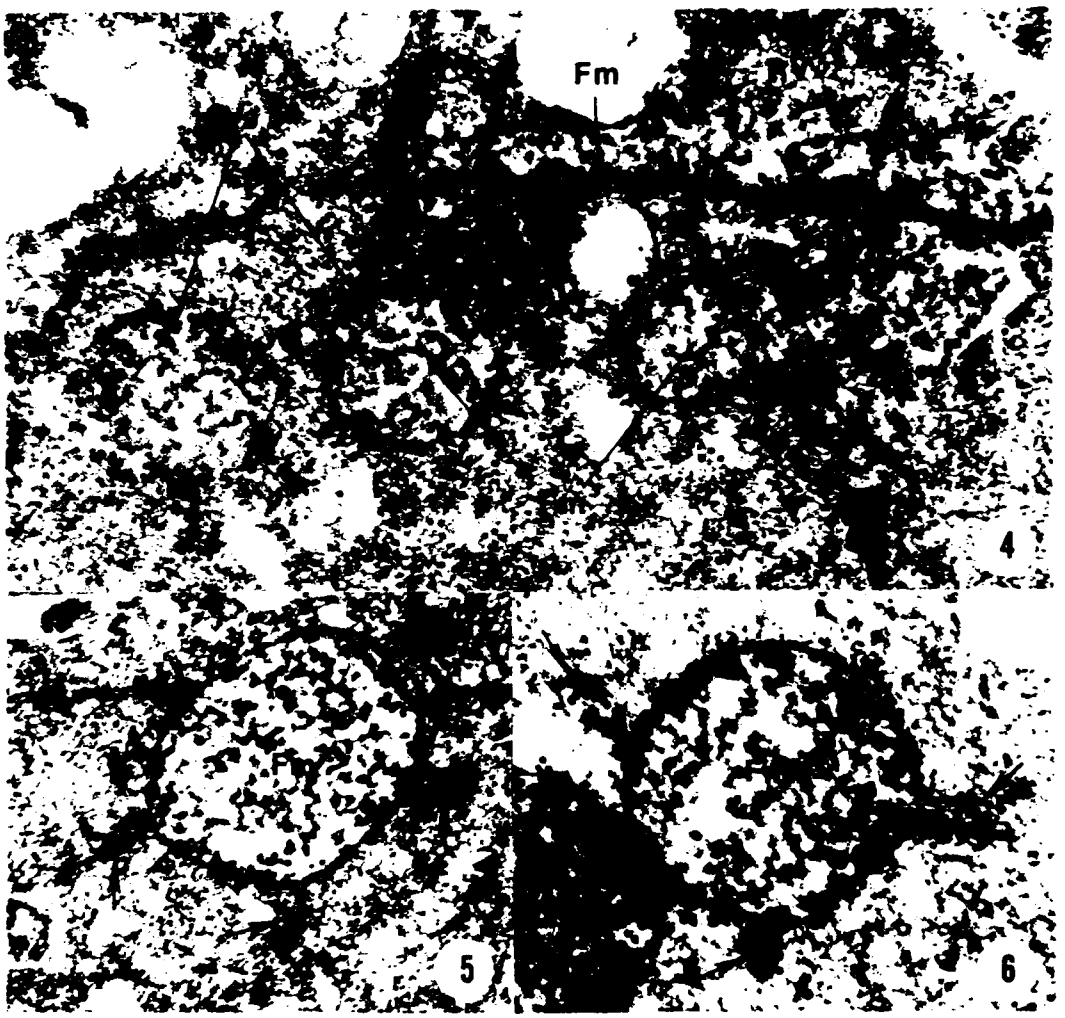
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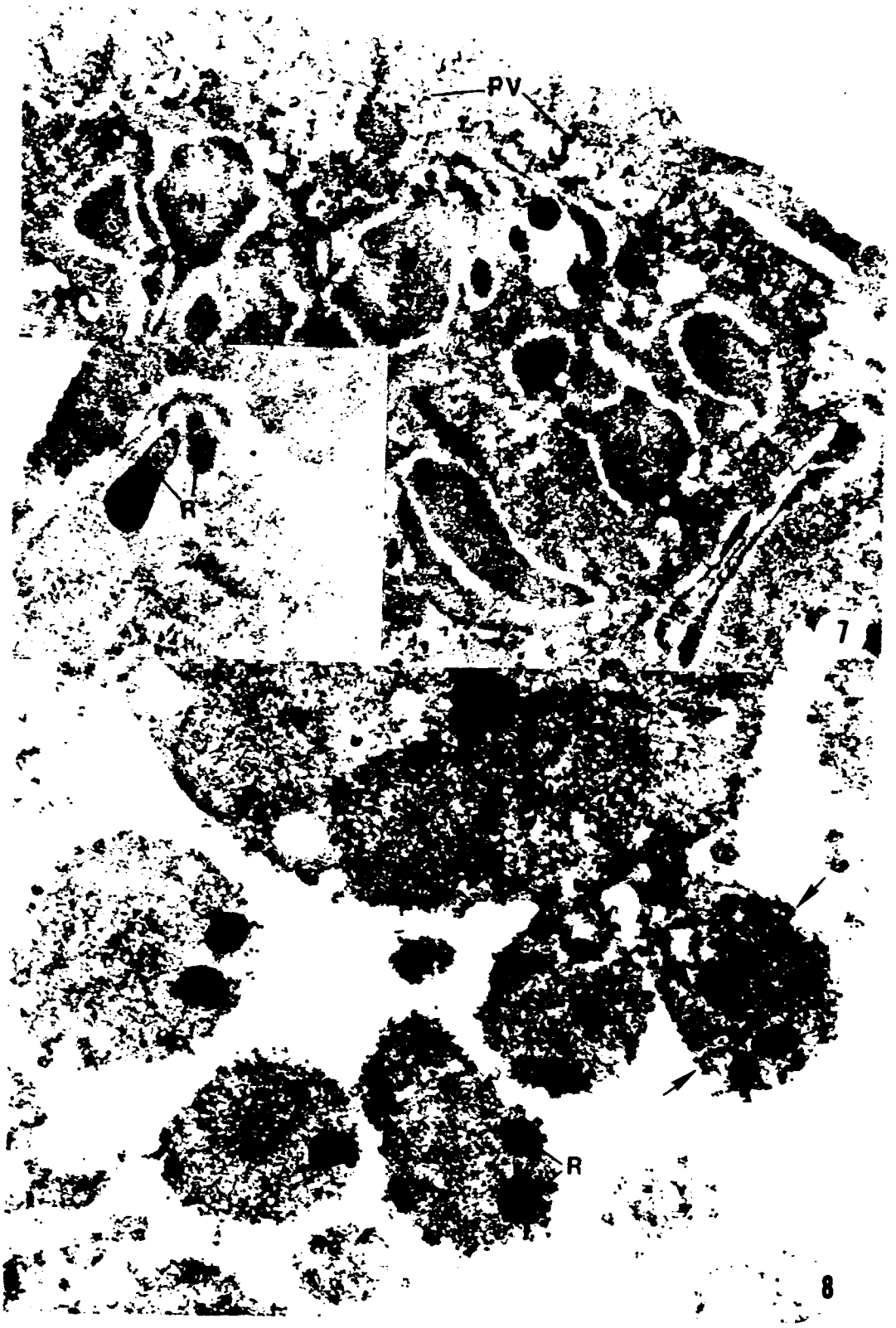
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Release of Merozoite Dense Granules During

Erythrocyte Invasion by *Plasmodium knowlesi*

ABSTRACT: We studied by immunoelectron microscopy the fate of dense granules during invasion of erythrocytes by *P. knowlesi* merozoites. Once merozoites entered host cells, dense granules moved to the pellicle and released their contents into the parasitophorous vacuole space, creating finger-like channels from the vacuole membrane. This is the first report showing that the content of dense granules of *Plasmodium* is different from rhoptries and micronemes and involved in the formation of channels from the parasitophorous vacuole.

Three types of membrane-bound organelles, namely rhoptries, micronemes and dense granules have been described in *Plasmodium knowlesi* merozoites (see Table 1). During invasion, the apical end of the merozoite attaches to the erythrocyte plasma membrane and junction formation occurs, the parasitophorous vacuole is formed and the parasite invades into the erythrocytes (2, 3, 7). It has been suggested that the contents of the rhoptries discharge into the erythrocyte membrane during invasion and assist the merozoite invasion process (2, 10, 12) and the micronemes connect with rhoptries, acting as a reservoir for rhoptries.

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 have been described in *Sarcocystis muris* (5, 6) and *Toxoplasma gondii* (4). The dense granules from these coccidia species were demonstrated 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 (5).

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

Merozoites of *Plasmodium knowlesi* (Malayan H strain) were prepared from schizont-infected Rhesus erythrocytes as described previously (9). Free merozoites were mixed and incubated 3 min with Rhesus erythrocytes in RPMI media with 2% fetal bovine serum before fixation for electron microscopy. With some samples merozoite invasion was arrested by the addition of 10 μg/ml of cytochalasin B in 0.1% DMSO.

Filter lifts of induced wild-type lambda gt11 in *Escherichia coli* (Y1090) were used to select antibody from anti-*P. knowlesi* hyperimmune monkey serum. The gt11-infected Y1090 was grown in stationary phase on agar plates in LB medium with 50 μg/ml ampicillin and induced at 42°C for 4 h, then nitrocellulose filters (BA85 Schleicher and Schuell) were placed on top of the near confluent phage plaques and the plates were incubated overnight at 37°C (13). The filters were removed and washed 3X in TPBS, blocked 1 h in 1.0% gelatin in TPBS, washed 3X in TPBS, incubated 2 h in 1:100 anti-*P. knowlesi* hyperimmune sera (8) diluted in TPBS, washed 5X with TPBS in 1 h, incubated 10 min in 100 nM boric acid 150 mM NaCl pH 9.0, washed 2X in PBS. incubated 20 min in 100 mM glycine 150 mM NaCl pH 2.8 to elute any bound antibody, and the filters were washed 3X and stored in TPBS (11). The solution of eluted antibody in glycine saline was neutralized with 2 M tris pH 8.0, dialyzed overnight against PBS 0.05% NaN_3. and concentrated 60X in Centricon 30 (Amicon) microconcentrators. The antibody selected from the immune Rhesus serum by this method is obviously binding crossreactive epitopes of the induced wild-type phage/bacteria and of the merozoite dense granules. To date, we have been unable to identify by immunochemical methods a specific antigen of *P. knowlesi* to which this antibody preparation reacts, even though it reacts very specifically with the dense

granules by immunoelectron microscopy.

For immunoelectron microscopy, extracellular merozoites and infected erythrocytes were fixed with 0.05% paraformaldehyde, 0.1% glutaraldehyde in 0.1M phosphate buffered saline (PBS) at pH 7.3, and embedded in LR white resin (Polysciences, Inc., Warrington, PA) (1). Sections were etched with saturated aqueous solution of sodium metaperiodate and incubated for 30 min in 0.1M 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 0.1M 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 IgG diluted 1:100 in PBS-Milk-Tween. After washing in PBS-BSA-Tween, grids were incubated for 1 h with goat anti-rabbit-IgG conjugated to gold particles (15 nm diameter) (Janssen Pharmaceutica, Piscataway, NJ) diluted 1:20 in PBS-Milk-Tween. Followed by final rinse 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 shaped dense granules were located near the rhoptries. They appeared to be intermediate in size between the rhoptry and microneme and measured ~140 nm in diameter (Fig. 1). The matrix was more electron dense than micronemes but appeared to be less dense than the rhoptries. By the immunoelectron microscopy, dense granules were clearly distinguished from rhoptries

and micronemes. In extracellular merozoites, gold particles were only associated with round dense granules, while no labeling was observed over the rhoptry or micronemes (Fig. 2). During initial attachment of the apical end of the merozoite with the rhesus erythrocyte, discharge of the dense granule material did not occur. However, during the late stage of the invasion process, 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 granules' contents labelled with gold particles were localized about the anterior surface of the merozoite (Fig. 3).

The parasitophorous vacuole membrane adjacent to the released contents of dense granules started to invaginate forming an elongated channel (Fig. 4). Often gold particles were 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, 4, 5).

The presence of dense granules in the merozoites and their discharge during the invasion are also observed on *Sarcocystis muris*. The contents of the granules appeared to be secreted by exocytosis into a secondary parasitophorous vacuole. Interestingly, the secretion of *S. muris* dense granules also occurred lateral to the conoid region and remained as an aggregate which formed invagination of the host cell membrane lining the parasitophorous vacuole. (5, 6). Their findings are very similar to what we describe here in *P. knowlesi* merozoites. Apparently the content of dense granules are discharged through different routes from that of the rhoptries and micronemes.

Our study indicates that dense granules appear to play a role for the formation of channels which extend from the parasitophorous vacuole membrane after erythrocyte entry by the merozoite. Therefore, the function of dense granules appears to be different from rhoptries and micronemes. The latter organelles are thought to be involved in the initial invasion process. The significance of the finger-like channels formed by the dense granules is not clear at this moment, but the channel may become a part of Maurer's clefts when the parasite grows within the host cell. Although discharge of dense granules' 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 thought to be present in micronemes or rhoptries in the past may actually be present in dense granules. Re-evaluation of the past published data, especially immunolocalization studies by fluorescent microscopy, may be required for further clarification of malaria biology.

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Figure Legends

Fig. 1 Epon embedded sections of dense granules and micronemes of a *P. knowlesi* merozoite. a) round dense granules (D) X 64,000
b) elongated micronemes (Mi) X 64,000

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. X 50,000

Fig. 3 A Merozoite invading a rhesus monkey erythrocyte. Some dense granules (arrows) are seen at the surface of the merozoite. The presence of gold particles (arrow heads) indicates that the contents are discharged into the parasitophorous vacuole space (PVS) through the pellicle. The junctions (J) are not labeled. Nucleus: (N) X 34,000

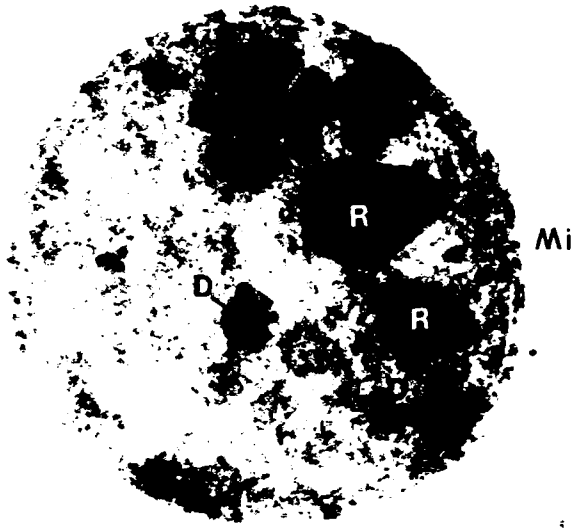
Fig. 4 Electron micrograph showing the invagination of the parasitophorous vacuole membrane (arrow) adjacent to the discharged dense granule material (arrow head). Dense granules: (D) X 40,000

Fig. 5 Long narrow channels (C) which extend from the parasitophorous vacuole membrane (PVM) are associated with gold particles (arrow). X 38,000

Table 1. Relative characterization of the merozoite organelles localized at the apical end and thought to be involved in host cell invasion

Organelles	Shape	Size (nm)	Density	Number (range)	Point of Release
		Relative to Other Structures			
Dense granule	Round	140 X 120	Medium	6.9 (4 -10)* per section	Through Pellicle Adjacent apex
Microneme	Ellipsoidal	100 X 40	Low	7.2(5 - 12)* per section	Unknown
Rhoptry	Tear-drop	570 X 330	High	2	Ducts at Apex

* Sections through 20 different merozoites were examined.



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