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Localization of Circumsporozoite Protein of *Plasmodium ovale* in Midgut Oocysts†

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Circumsporozoite (CS) proteins are the major proteins found on the surface of salivary gland sporozoites and are the protective antigens of several species of malaria parasites. Little is known about the distribution of CS proteins in developing oocysts, however. Immunoelectron microscopy with protein A-gold and a monoclonal antibody specific for the CS protein of *Plasmodium ovale* was performed to investigate the distribution of CS protein within developing *P. ovale* oocysts. There was an almost complete absence of label in immature oocysts prior to the development of sporoblasts. In contrast, sporoblasts and budding and free sporozoites in mature oocysts were labeled uniformly on the outer surfaces of their plasma membranes, indicating a uniform distribution of CS protein on these membranes. Gold particles were frequently associated with the cytoplasm of sporoblasts and sporozoites, as well as with the inner surface of the oocyst capsule. This is the first evidence that CS protein is present in oocyst sporozoites and sporoblasts of *P. ovale*.

Observations of circumsporozoite (CS) proteins in vitro and in vivo suggest that they are involved in the initial interaction of malarial sporozoites with target hepatocytes and are functionally important to the survival of sporozoites in the mammalian host (5, 6). These findings suggest, therefore, that CS proteins are not only the targets of the host immune response but are also prime candidates for vaccine development. Despite the importance of these proteins, studies of their origin and expression on the sporozoite surface are limited.

Only one study, reported by Fine et al. (4), has examined the distribution of CS proteins and their precursors on malarial sporozoites by immunoelectron microscopy. These researchers studied the distribution of CS proteins and their precursors on salivary gland sporozoites of *Plasmodium knowlesi* with cryoultramicrotomy, monoclonal antibody (MAb), and protein A-gold. These authors found that CS proteins were mainly localized on the sporozoite pellicle as well as in rhoptries and micronemes.

Procell et al. (P. M. Procell, W. E. Collins, F. H. Collins, and G. H. Campbell, manuscript in preparation) recently used MAb 110-54.3 to characterize the CS protein of *Plasmodium ovale*. When this MAb was used in Western blot analysis with *P. ovale* sporozoites, three distinct species-specific polypeptides were recognized. A single-antibody, two-site enzyme-linked immunosorbent assay was used to demonstrate the presence of a repeating epitope. In this study, we investigated the distribution of the CS protein in midgut *P. ovale* oocysts by using immunoelectron microscopy.

MATERIALS AND METHODS

MAb. An anti-*P. ovale* sporozoite MAb was produced and characterized previously (Procell et al., in preparation). Briefly, the MAb identified a repeating epitope on the surface of *P. ovale* sporozoites. This was confirmed with glutaraldehyde-fixed sporozoites in an indirect fluorescent-antibody assay and the circumsporozoite precipitin reaction. In addition, the MAb detected polypeptides with M_rs of 51,000, 57,000, and 67,000 in Western blot analysis. The MAb-containing ascitic fluid used in these experiments was produced by injection of the hybridoma cell line 110-54.3 into BALB/c × C57BL/6J F_1 mice.

Parasites. Individual *Anopheles stephensi* mosquitoes were allowed to feed through artificial membranes on chimpanzee blood infected with the Nigerian I/CDC strain of *P. ovale*. Mosquitoes were killed 10 to 12 days after feeding, and the midguts were dissected into 1.25% glutaraldehyde in 0.1 M phosphate buffer containing 4% sucrose (pH 7.3) and fixed for 10 min at room temperature. Specimens were then washed three times in 0.1 M phosphate buffer. They were dehydrated in a graded series of ethanol concentrations at progressively lower temperatures between 0 and −20°C and infiltrated with LR Gold resin containing 0.5% (wt/vol) benzoin methyl ether as an initiator (Polysciences, Inc.). Midguts were transferred to gelatin capsules and polymerized for 30 h at −20°C with UV light. Thin sections were cut with a diamond knife, mounted on nickel grids (no. 400), and processed for immunolabeling.

Immunolabeling. Sections were processed for immunocytochemical labeling by first floating the grids on drops of MAb in 0.1 M phosphate buffer (pH 7.3) with 0.9% NaCl, 1% bovine serum albumin, and 0.01% Tween 20 (PBS-BSA) for 2 h. Incubations were performed at room temperature. After being washed with PBS-BSA, grids were incubated on drops of rabbit antimouse immunoglobulin G (10 μg/ml in PBS-BSA) for 1 h. Grids were rinsed thoroughly in PBS-BSA and then floated on a drop of protein A-gold particles (Janssens Pharmaceutica, Beerse, Belgium) diluted at a 1:10 ratio in PBS-BSA and incubated for 1 h. Sections were washed in PBS-BSA and distilled water and stained with 2% uranyl acetate in 50% methanol before examination in a 100 CX electron microscope (JEOL, Ltd., Tokyo, Japan).

To evaluate the specificity of labeling, the following controls were performed: (i) normal-mouse ascitic fluid was

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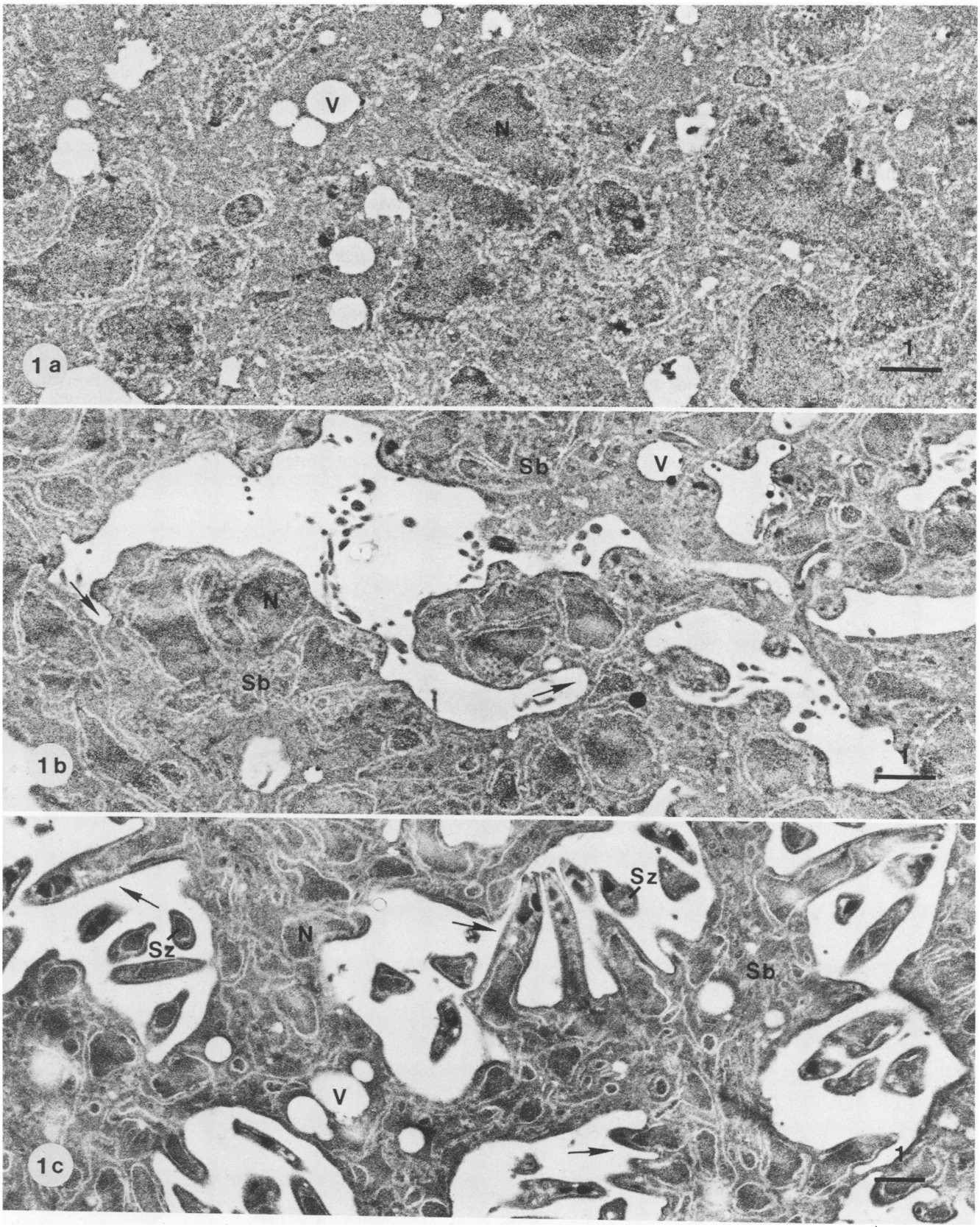

FIG. 1. (a) Young oocyst of *P. ovale* in the midgut epithelium of *A. stephensi*. The oocyst contains several nuclei (N) and vacuoles (V). (b) More-mature oocyst of *P. ovale*. The vacuoles enlarged, coalesced, and formed large clefts (arrows) that subdivided the oocyst cytoplasm into sporoblasts (Sb). (c) Mature *P. ovale* oocyst containing many mature sporozoites (Sz). Sporozoites were formed by budding from sporoblasts (arrows). Bars indicate 1 μm.

used in place of MAb, and (ii) preparations were incubated with protein A-gold alone.

RESULTS

Midgut oocysts from mosquitoes killed between 10 and 12 days after the infective-blood meal ranged in maturity from young, immature forms to fully mature oocysts containing well-developed sporozoites. Oocyst development was similar to that of other *Plasmodium* species (1). Briefly, oocysts became increasingly vacuolated by the retraction of the oocyst plasma membrane from the oocyst wall and by the formation of numerous cytoplasmic clefts. The clefts eventually coalesced to form a number of interconnected lobes from which sporozoites budded (Fig. 1).

The binding of MAb 110-54.3 differed in immature and mature oocysts. Gold particles were not found in immature, nonvacuolated oocysts. Specific label was first observed in the cytoplasm of more-mature, slightly vacuolated oocysts. In highly vacuolated oocysts in which sporozoite budding had not begun, gold particles were randomly distributed in the cytoplasm and distributed irregularly and in patches on the surface of the sporoblast and the oocyst capsule (Fig. 2). In contrast, MAb 110-54.3 bound primarily to the plasma membrane of sporoblasts that contained budding sporozoites (Fig. 3). These membranes were covered entirely in a dense, uniform fashion with gold particles. A similar distribution was observed on the inner surface of the oocyst capsule. Intracellular gold particles were consistently associated with the cytoplasm. Some label appeared on the endoplasmic reticulum and around the nucleus. Very few gold particles were found within vacuoles in the oocyst cytoplasm. No gold binding occurred in the oocysts, sporoblasts, and sporozoites when they were incubated with normal-mouse ascitic fluid. Additionally, nonspecific binding of protein A-gold was very low after incubation of sections with protein A-gold alone.

DISCUSSION

Sporozoites obtained from oocysts and salivary glands are morphologically very similar but are biologically quite different. Salivary gland sporozoites appear to be more infective and can be used to vaccinate mammalian hosts (8, 9). Animals immunized with salivary gland sporozoites can acquire protective immunity to sporozoite-induced infection. Antibodies from immunized animals react with a single immunodominant protein that covers the surface of the

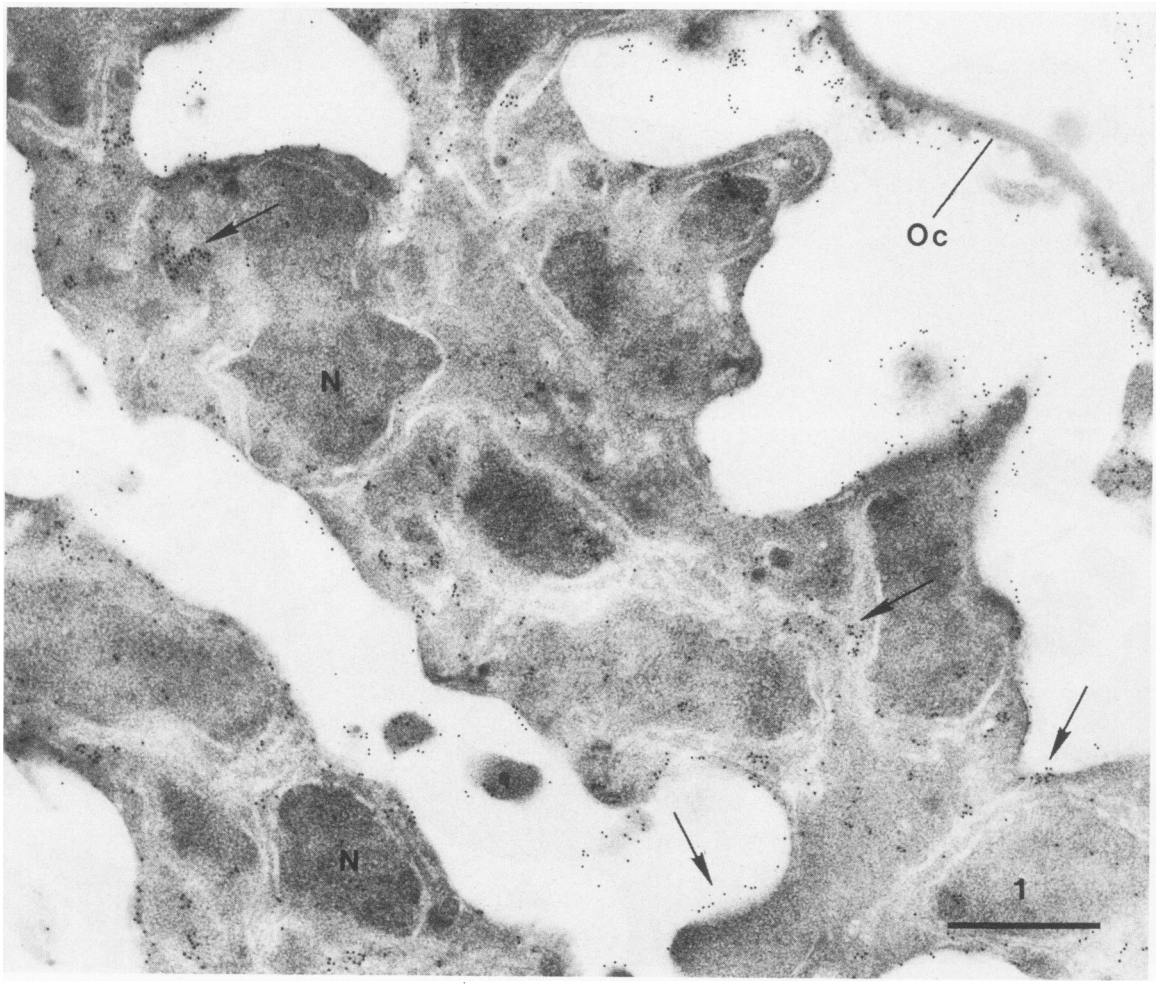

FIG. 2. Immature oocyst of *P. ovale* labeled with MAb 110-54.3 and protein A-gold. Gold particles (arrows) were distributed irregularly in the cytoplasm and on membranes of developing sporoblasts and oocyst capsules (Oc). Binding of MAb 110-54.3 was specific for both cytoplasm and membranes. The distribution was irregular and in patches on the surface of the sporoblast. Bar indicates 1 μm. N, Nucleus.

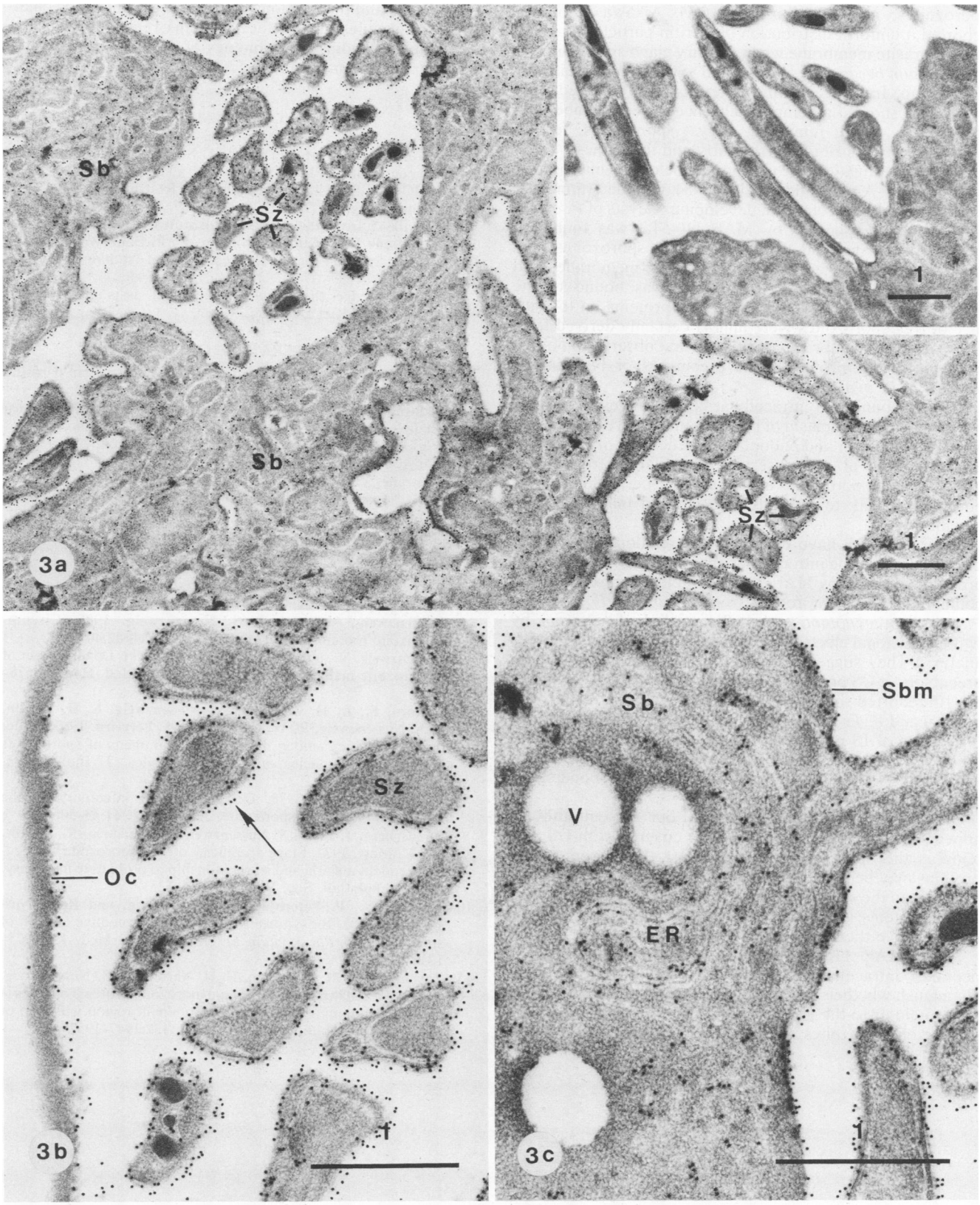

FIG. 3. (a) Mature oocyst of *P. ovale* labeled with MAb 110-54.3 and protein A-gold. The cytoplasm and surface of the sporoblast (Sb) and the cytoplasm and pellicle of sporozoites (Sz) were covered with gold particles. Inset: Mature oocyst incubated with normal-mouse ascitic fluid as control. Few gold particles were associated with the parasite. (b) Gold particles were uniformly bound over the sporozoite pellicle (arrow) and on the inner surface of the oocyst capsule (Oc). (c) Gold particles were bound to the plasma membrane of the sporoblast (Sbm). Some binding of MAb 110-54.3 occurred over the endoplasmic reticulum (ER), but no gold particles were found in vacuoles (V). Bars indicate 1 μm.

sporozoites, namely CS protein (11). Aikawa et al. (2) reported a uniform distribution of ferritin particles over the entire parasite membrane when salivary gland sporozoites of *Plasmodium berghei* were incubated with a MAb specific for CS protein. In contrast, 50% of sporozoites obtained from the oocyst showed a complete absence of ferritin label. On the surface of the remaining oocyst sporozoites, ferritin particles were distributed irregularly and in patches.

In this investigation, we used immunoelectron microscopy with protein A-gold and MAb 110-54.3 to determine the distribution of CS proteins in developing oocysts of *P. ovale*. The antigen recognized by MAb 110-54.3 was found on surface membranes and in the cytoplasm of sporozoites from developing oocysts and on the sporoblast membranes and oocyst capsule. Since MAb 110-54.3 was bound to the membrane of budding sporozoites, CS proteins on the surface of free sporozoites were almost certainly derived from the sporoblast membrane. This is the first observation of the presence of CS protein or its precursors or both in the oocysts of *P. ovale*.

We also found that intracellular gold particles were associated with the cytoplasm of budding and mature sporozoites and the cytoplasm and endoplasmic reticulum of the sporoblast. Fine et al. (4) observed a similar distribution in salivary gland sporozoites of *P. knowlesi*, i.e., gold particles bound uniformly to the outer plasma and inner pellicular membranes.

Several studies have examined the relationship between CS proteins and the intracellular sporozoite polypeptides (IS proteins) detected by MAbs to CS proteins. Santoro et al. (7) compared CS and IS proteins from *P. berghei*, *P. knowlesi*, *Plasmodium cynomolgi*, and *Plasmodium falciparum* by two-dimensional electrophoretic analysis and tryptic peptide analysis. They suggested that IS proteins were intracellular precursors of CS proteins. Cochrane et al. (3) and Yoshida et al. (10) reported similar results with IS and CS proteins of *P. berghei* and *P. knowlesi*. Moreover, they suggested that the processing of IS proteins involves the cleavage of peptides relatively rich in basic residues, since the PIs of CS proteins were consistently lower than those of corresponding IS proteins.

On the basis of these findings and our present study, it appears that sporozoites are already covered with CS proteins as they bud from the sporoblast. Precursors to CS proteins may be stored in the cytoplasm of sporoblasts prior to export to the sporoblast membrane. Continued production of CS proteins after sporozoites reach the salivary glands may enhance their infectivity.

Since MAb 110-54.3 identified three different polypeptides, intracellular gold particles did not permit us to distinguish whether specific binding was to precursors of the CS protein or to the processed CS protein expressed on the surface of sporozoites. The biosynthesis and production of CS proteins and the relationships between higher-molecular-weight molecules and surface proteins remain obscure. A better understanding of the biology of CS proteins may be of considerable scientific and practical interest.

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