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Immunoelectron Microscopy of Schüffner's Dots in Plasmodium vivax-*Infected Human Erythrocytes*

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Plasmodium vivax **induces morphologic alterations in infected host erythrocytes that are visible by light microscopy in Romanovsky-stained blood smears as multiple brick-red dots. These morphologic changes, referred to as Schüffner's dots, are important in the identification of this species of malarial parasite and have been associated by electron microscopy with caveola-vesicle complexes along the erythrocyte plasmalemma. We have produced a monoclonal antibody (MAb A 20) that identifies an antigen in *Plasmodium vivax*-infected erythrocytes that is associated with the caveola–vesicle complexes of the parasitized host cell. This monoclonal antibody reacts with air-dried *P vivax*-infected erythrocytes to produce a pattern by the indirect immunofluorescence test (IFT) that is evocative of Schüffner's dots. Immunoelectron microscopy of *P vivax*-infected human erythrocytes using MAb A 20 confirmed the location of this antigen within vesicles of caveola–vesicle complexes. On Western blots MAb A 20 recognized four polypeptides of 54, 64, 72, and 86 kd. MAb A 20 reacted by IFT with 90% of Sri Lankan isolates and with a Brazilian *P vivax* isolate, which indicates that the epitope identified by this monoclonal is conserved. (Am J Pathol 1988, 131: 48–52)**

MALARIAL PARASITES induce a variety of structural and antigenic changes in the membrane and cytoplasm of their host erythrocytes, including the development of caveolae, caveola–vesicle complexes, clefts, and knobs.[1–8] One of the first such structural modifications to be recognized by light microscopy was a stippling composed of fine, brick-red dots in the cytoplasm of *Plasmodium vivax*-infected erythrocytes.[9] This stippling was evident in Romanovsky stained blood smears and was termed Schüffner's dots. These dots can be demonstrated in properly stained smears of erythrocytes that contain maturing asexual stages of the parasite as well as gametocytes and are diagnostic of *P vivax* or *Plasmodium ovale* infection when they are present.[10]

Studies by electron microscopy have demonstrated caveola–vesicle (C-V) complexes in the membrane of erythrocytes infected with *P vivax* that are believed to correspond to Schüffner's dots. These structures are composed of alveolar-like clusters of small vesicles that are connected to the base of invaginations or caveolae in the erythrocyte membrane.[4] While little is known of the origin or the functional significance of these C-V complexes, horseradish peroxidase-labeled antibody from *P vivax*-infected monkeys reacts with these structures, which indicates that they contain malarial antigens.[4]

In the present study, we have produced a monoclonal antibody (MAb) that identifies an antigen associated with Schüffner's dots in *P vivax*-infected human erythrocytes. This antigen was localized in vesicles of C-V complexes by immunoelectron microscopy.

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Materials and Methods

Plasmodium vivax cannot be maintained in continuous *in vitro* culture; hence, parasites were obtained from infected blood donors among acute vivax malaria patients admitted to the General Hospital, Colombo. After voluntary informed consent, up to 20 ml of blood was drawn intravenously into heparinized tubes. Erythrocytes infected with trophozoites, schizonts, and gametocytes were isolated by a single-step Percoll (Pharmacia Fine Chemicals, Sweden) gradient as described previously.[11] Mouse monoclonal antibodies (MAbs) were produced against the asexual erythrocytic stages of *P vivax* by means of an immunization schedule and fusion technique which have been published previously.[12] Briefly, mice were immunized with three doses of purified parasitized erythrocytes, each consisting of 10^7 parasites composed mainly of schizonts and a few late trophozoites. Three days after the last immunizing dose, spleen cells were fused with the P3-X63-Ag8.653 (P3U1) myloma cell line, according to the method of Galfre et al.[13] Hybridoma clones producing antibodies to *P vivax* were selected by screening culture supernatants by the indirect immunofluorescence test (IFT) on smears of air-dried parasitized erythrocytes containing asexual stage parasites. Selected hybrids were cloned twice by limiting-dilution on thymocyte feeder layers.

The IFT was performed on smears of air-dried infected erythrocytes by the method described by Voller and O'Neil.[14] The IFT was also performed with live unfixed infected erythrocytes[15] for detection of antigens located on the surface of infected erythrocytes.

Purified infected erythrocytes containing asexual blood stage parasites of *P vivax* were extracted under reducing conditions in 5% 2-mercaptoethanol, electrophoretically separated on 5–15% sodium dodecyl sulfate (SDS gradient gels and electroeluted onto nitrocellulose paper. The reactivity of MAbs with antigens thus isolated was determined by standard techniques of Western blotting as described previously.[12]

Samples for immunoelectron microscopy were fixed for 10 minutes in 1% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and washed with 0.1 M phosphate buffer. The lightly fixed cells were dehydrated with an ethanol series at progressively lower temperatures between 4 C and −20 C and embedded in LR White Resin as described by Atkinson et al.[16] Ultrathin sections were cut with a diamond knife and collected on nickel grids. Grids were etched for 1 hour on drops of saturated aqueous sodium metaperiodate, washed with distilled water, and blocked for 30 minutes on drops of 5% nonfat dry milk in 0.1 M phosphate buffer, pH 7.3, with 0.9% NaCl and 0.01% Tween 20 (PBS-Tween). Grids were incubated for 2 hour at room temperature on drops of primary antibody diluted with 1% bovine serum albumin in PBS-Tween (PBS-BSA-Tween), washed with PBS-BSA-Tween, and incubated for 1 hour on drops of a rabbit anti-mouse IgG secondary antibody (ICN Biomedicals, Costa Mesa, Calif) diluted with PBS-BSA-Tween. Grids were washed again with PBS-BSA-Tween and incubated for 1 hour on drops of protein A–gold (Janssen Life Sciences, Piscataway, NJ) diluted 1/20 with PBS-BSA-Tween. Finally, the grids were washed with PBS-BSA-Tween, fixed for 15 minutes with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, washed with distilled water, and stained for 25 min in 2% uranyl acetate in 50% methanol. The grids were carbon-coated and examined with a JEOL 100CX electron microscope.

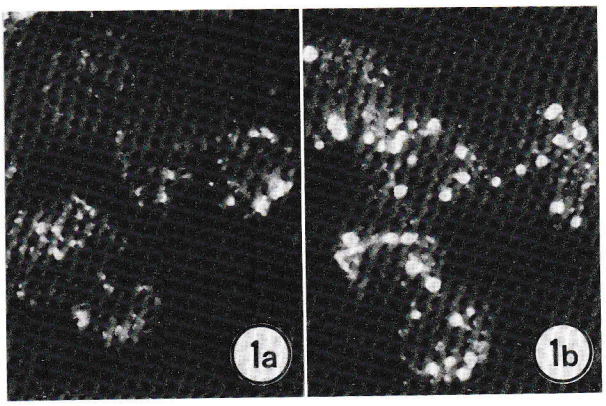

Figure 1—Immunofluorescence staining patterns produced by MAb A 20 on air-dried *P vivax*-infected erythrocytes that contain asexual stages of the parasite. (×2500) **a**—Staining pattern consists of a series of fine dots that are distributed over the surface of infected erythrocytes. **b**—Staining pattern consists of coarse dots that are superimposed on a finely speckled background.

Control grids were incubated with a mouse monoclonal IgG1 antibody that specifically labeled the parasitophorous vacuole of *P vivax*-infected cells, followed by secondary antibody and protein A–gold, or were incubated with secondary antibody and protein A–gold or protein A–gold alone.

Results and Discussion

Monoclonal antibody A 20 of gamma 1 isotype reacted with air-dried *P vivax*-infected erythrocytes by IFT to produce an immunofluorescent staining pattern that was reminiscent of Schüffner's dots as seen in Romanovsky-stained blood films. Two patterns were observed. One was characterized by a series of fine dots that were distributed over the entire infected erythrocyte (Figure 1a), and the second was composed

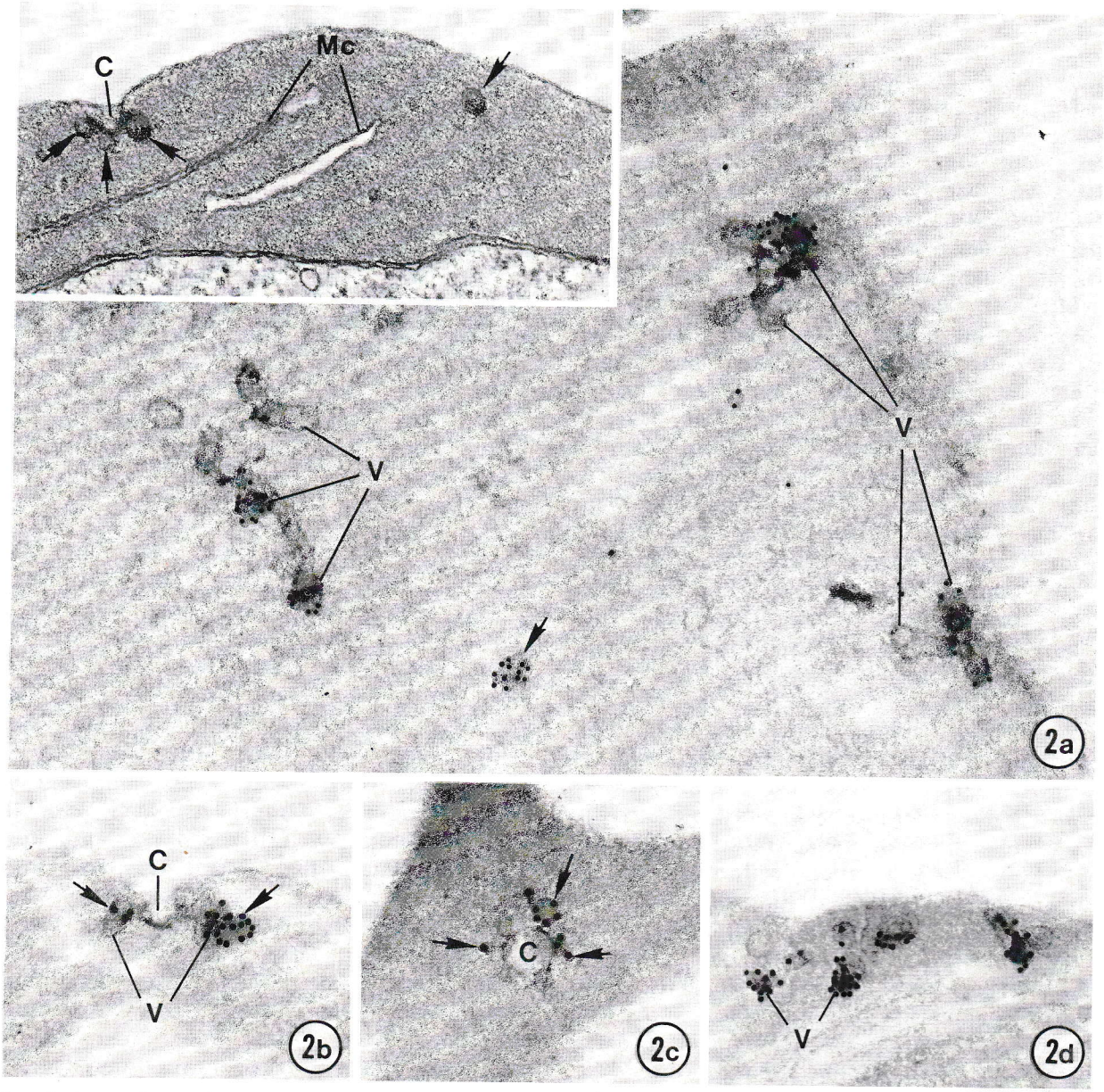

Figure 2—Immunoelectron micrographs of sections of *P vivax*-infected erythrocytes that were incubated with MAb A 20, secondary antibody, and protein–A gold. **a**—Oblique section near the surface of an infected erythrocyte. Electron-dense gold particles are associated with alveolar clusters of vesicles (*V*) that surround the bases of caveolae (not visible in this section) as well as with an isolated vesicle that is deeper in the erythrocyte cytoplasm (*arrow*). (×64,000) **Inset**—Standard transmission electron micrograph of a glutaraldehyde-osmium fixed *P vivax*-infected cell. Note caveola (*C*) with alveolar cluster of vesicles (*arrows*), as well as an isolated vesicle (*arrow*) that is located deeper in the erythrocyte cytoplasm. Several membranous clefts (*Mc*) are present in the erythrocyte cytoplasm. (×54,000) **b**—Gold particles (*arrows*) are associated with vesicles (*V*) that surround a caveola (*C*). Note the absence of gold particles in the cavity formed by the caveola. (×56,000) **c**—Oblique section through the base of a caveola (*C*). Gold particles (*arrows*) are associated with vesicles that surround the caveola (*C*). (×56,000) **d**—Gold particles are associated with vesicles (*V*) that are located deeper in the erythrocyte cytoplasm. (×56,000)

of coarse dots that were superimposed on a finely speckled background (Figure 1b). These staining patterns were obtained with early and late trophozoites, schizonts, and mature gametocytes. IFT reactivity of this MAb with ring-infected erythrocytes was not investigated, because of difficulties in purifying infected cells with this stage of parasite development. Immunofluorescent staining was not obtained when the IFT was performed with MAb A 20 on live unfixed parasitized erythrocytes. In addition, MAb A 20 did not cross-react by IFT with air-dried smears of parasitized erythrocytes containing asexual stages of *Plasmodium falciparum* or *Plasmodium cynomolgi,* a vivax-type simian malarial parasite that characteristically produces Schüffner's dots in the infected erythrocyte.

Immunoelectron microscopy of *P vivax*-infected

erythrocytes using MAb A 20 and protein A–gold revealed a distribution of gold particles that was confined primarily to vesicles, but not caveolae, of C-V complexes (Figure 2a–2d). Gold particles were occasionally detected within vesicles that were located deeper in the cytoplasm of the infected erythrocyte (Figure 2d), but were not associated directly with the parasite. Control grids incubated with a different monoclonal specific for parasite proteins in the parasitophorous vacuole space labeled this compartment around mature asexual parasites, but not C-V complexes (not shown). Gold particles were not found on sections incubated with secondary antibody and protein A–gold or protein A–gold alone.

On Western blots MAb A 20 reacted with four polypeptides of approximate molecular weights 54, 64, 72, and 86 kd (Figure 3). These polypeptides could either be a repertoire of different cross reacting antigens such as have been previously described in malarial parasites.[17] Alternatively, they could be degradation products of a single antigen. The latter hypothesis is favored by the highly localized distribution of the antigen in the parasitized erythrocyte.

Previous studies by electron microscopy demonstrated that the plasmalemma of *P vivax*-infected erythrocytes exhibits many small invaginations or caveolae that measure approximately 90 nm in diameter (Figure 2A, inset). The flattened base of these caveolae are connected to smaller 50-nm vesicles that surround the caveola in an alveolar fashion. The portion of each vesicle in contact with a caveola appears to open into the caveolar space. The characteristic stippling or Schüffner's dots of *P vivax*-infected erythrocytes is believed to result from the deposition of stain in these C-V complexes.[4]

The origin of vesicles of the C-V complexes has been the subject of considerable speculation, and it is still unclear whether they arise from the erythrocyte surface membrane, ie, by endocytosis,[4] or from the parasite membrane and traverse the cytoplasm of the erythrocyte to fuse with the caveolae to form C-V complexes.[5] Because the antigen identified in this study is probably a parasite molecule, rather than an altered host component, the vesicles with which it is associated may arise from the parasite membrane and traverse the cytoplasm to reach the surface of the erythrocyte. Failure to observe gold label within the caveolae of C-V complexes may indicate that the antigen recognized by MAb A 20 is rapidly released through the caveolae as these structures fuse with vesicles in the erythrocyte cytoplasm. Such a function would be in agreement with studies of *P falciparum* that have shown the active secretion of malarial proteins from parasitized erythrocytes.[18] Alternatively,

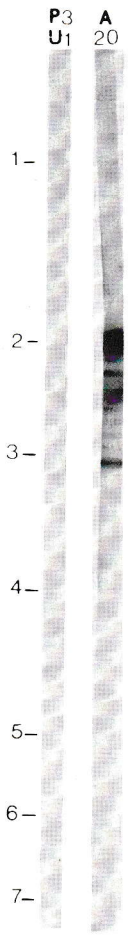

Figure 3—Western blot of *P vivax* parasites (asexual erythrocytic stages) extracted under reducing conditions and reacted with hybridoma culture supernatants of MAb A 20 and the control myeloma cell line (P3U1). Molecular weight markers: 1, 200 kd; 2, 97.4 kd; 3, 68 kd; 4, 43 kd; 5, 25.7 kd; 6, 18.4 kd; 7, 14.3 kd.

the absence of gold label within caveolae and the failure to observe immunofluorescence staining in live, unfixed parasitized erythrocytes might indicate that the antigen recognized by MAb A 20 remains associated with vesicles beneath the erythrocyte surface, where it may function in the uptake of host proteins, as occurs during internalization of the transferrin receptor in *P falciparum*-infected erythrocytes.[19]

We have previously demonstrated a high degree of polymorphism among antigens of the asexual erythrocytic stages of *P vivax*.[12] Only a few antigens are conserved among different parasite isolates prevalent in nature, and the rest exhibit a high degree of polymorphism.[12] MAb A 20 reacted by IFT with 90% of Sri Lankan isolates and with a Brazilian *P vivax* isolate, indicating that the epitope recognized by MAb A 20 is conserved. Thus, this monoclonal may have potential value as a diagnostic tool for *P vivax* infection.

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