

LOCALIZATION OF PROTECTIVE MALARIAL ANTIGENS BY IMMUNO-ELECTRON MICROSCOPY

MASAMICHI AIKAWA

The introduction of ferritin immuno-labelling in electron microscopy by Singer in 1959 has opened the new field of immuno-electron microscopy. The important factors to be considered when performing immuno-electron microscopy are: a) visualization of antigen-antibody reaction sites by labelling antibodies with electron opaque materials, b) suitable fixation without loss of antigenicity and structural details and c) accessibility of antibodies to intracellular antigens.

In recent years hybridoma technology has been used to produce many monoclonals against malarial parasites and various protective antigens have been identified (Coopel et al., 1984; Miller et al., 1984; Perrin et al., 1984). Immuno-electron microscopy may be able to characterize these monoclonals in relation to their reaction sites. In addition, by using this method, we will be able to understand the mechanism by which these proteins are transported within the parasites to the surface of *Plasmodium* and host erythrocytes. Furthermore, determining the location of protective antigens may assist in identifying which organelles may be used for the induction of immunity against malaria infection.

In this chapter, the localization of these antigens by using various monoclonals together with protein A-gold and cryoultramicrotomy or LR White embedding resin will be described. In addition, the technical aspects of immuno-electron microscopy will be discussed.

IMMUNO-ELECTRON MICROSCOPY

a) **Tissue fixation** – Tissue fixation has two main functions, namely to retain antigens in cells or tissues and to preserve tissue structure. Fixatives introduce intra- and inter-molecular crosslinks which decrease antigenicity. So when one chooses a fixative for immuno-electron microscopy, a compromise normally has to be made between preservation of the ultrastructure and retention of antigenicity. In general, a strong fixation is better for the preservation of the structure, but a weak one is better for the retention of antigenicity and the accessibility of antigens. Glutaraldehyde is more suitable for structural preservation than formaldehyde but the opposite is true for antigenicity retention (Tokuyasu, 1984). Regarding the selection of fixatives, most workers have used a mixture of glutaraldehyde and paraformaldehyde. We have been using a mixture of 1.0% formaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and have obtained relatively good results (Oka et al., 1984).

b) **Visualization of antigen-antibody reaction sites** – Since Singer (1959) introduced ferritin for immuno-labelling, many electron dense markers have been used for the visualization of antigen-antibody reaction sites. In 1966, Nakane & Pierce used horseradish peroxidase to localize antigens by conjugating antibody to horseradish peroxidase, thus establishing the enzyme-labelled antibody techniques for immuno-electron microscopy.

Recently, protein A-gold complexes have become popular for localizing antigens in cells (Roth, Bendayan & Orci, 1978). The wall of *Staphylococcus aureus* contains protein A which has the ability to interact with the Fc portion of IgG molecules. Protein A is an elongated protein with four distinct Fc-binding regions per molecule (Romano & Romano, 1977). The reaction of protein A with IgG does not involve the antigen-binding region of the immunoglobulin molecules, therefore, does not affect the antigen-antibody reaction. Colloidal gold is a negatively charged hydrophobic sol, the stability of which is maintained by electrostatic repulsion. Protein A stabilizes colloidal gold by shielding it from the effects of the electrolytes. By adding protein A to the colloidal gold suspension, the positively charged groups of the protein A interact with the negatively charged gold particles, forming a noncovalent complex. The advantages of the protein A-gold method include that: 1) the tissue can be aldehyde-fixed without interfering with the sensitivity or specificity of the immunological probe, 2) protein A reacts with the Fc fragment of IgG molecules from several species and 3) the protein A-gold technique requires only a small amount of specific antibodies.

c) **Accessibility of antibodies to intracellular antigens** – Immuno-staining of intracellular antigens with antibodies requires that antibodies must first pass through the plasma membrane

and then organelle membranes, to locate the target antigens. Therefore, the membranes must be damaged to make them permeable to antibodies. Also, cells generally contain a high concentration of protein which becomes rigidly cross-linked during fixation, thus inhibiting antibody penetration into the cell interior.

In order to overcome these problems, a number of new embedding resins have appeared in recent years. The development of Lowicryl (Carlemain, Garairto & Villiger, 1982) has made possible the embedding of tissue at temperatures of -30°C or below, where the denaturation and extraction of protein during dehydration is minimised. The recently formulated acrylic resin LR White also has a number of advantageous characteristics (Newman, Jasani & Williams, 1982). It is used at low temperatures like Lowicryl K4M, and is hydrophilic, so that ultrathin sections permit full penetration of aqueous solutions. Tissue thus prepared shows an improved antigenicity over fully dehydrated tissue. These embedding resins have been applied to various kinds of tissue and are well suited for immuno-electron microscopy. However, cryoultramicrotomy (Tokuyasu, 1984) appears to give better results for the preservation of antigenicity. In this chapter, both cryoultramicrotomy and LR White embedding method will be discussed.

d) Cryoultramicrotomy – In cryoultramicrotomy, ice provides the rigidity that is required for cutting tissues into ultrathin sections. Upon returning to room temperature, the sections regain their original hydrated state. Actually, to improve the freezing characteristics and the plasticity of frozen pieces, fixed specimens may be infused with a solution containing hydrophilic substances before freezing (Tokuyasu, 1973, 1983). Low molecular weight substances such as sugars or polyethylene glycol are effective modifiers of the physical characteristics of the cell interior. When sections are thawed and exposed to water, these hydrophilic substances dissipate. If proteins are included in the infusion solution, they can be cross-linked with an aldehyde fixative to form an insoluble network, which aids the preservation of intracellular relationships.

Since harsh treatments such as dehydration in chemical solvents or embedding in hydrophobic materials are not required, cryoultramicrotomy offers advantages that open many possibilities in both the observations of cellular structure and the localization of specific macromolecules in tissues and cells by immunocytochemistry.

The following protocol is used for cryoultramicrotomy:

- 1) Fix erythrocytes in an appropriate fixative
- 2) Wash in PB (pH 7.3) three times
- 3) Cross-link in 30% bovine serum albumin for 10 min with 0.5% glutaraldehyde
- 4) Wash in PB
- 5) Infuse with 1.3M sucrose in PB for three hours
- 6) A small piece of the sample is mounted onto a metal stud and then frozen in liquid nitrogen
- 7) Cryoultramicrotomy at -91°C
- 8) Pick up sections with a wire loop containing 2.3M sucrose in PB and place them on grids
- 9) Immunostaining
- 10) Protein A-gold labelling
- 11) The grids with sections are fixed again in 1.25% glutaraldehyde in PB for 10 min.
- 12) Stain with neutral 2% uranyl acetate for 30 min.

e) LR White resin embedding method – Although the acrylic embedding medium Lowicryl (Chemische Werke Lowi, Waldkraiburg, West Germany) was produced for immunocytochemistry (Carlemain, Garairto & Villiger, 1982), this was unsatisfactory for *Plasmodium* because of poor polymerization and poor penetration of medium onto the tissue and poor preservation of antigenicity. Recently, another acrylic resin, LR White (London Resin Co., Basingstoke, V.K.) has become available (Newman, Jasani & Williams, 1982). This has several advantages such as simple embedding procedure, the use of partially dehydrated tissue, low lipid solvency and improved preservation of antigenicity.

LR White resin is designed for use at room temperature, but can be used at temperatures as low as -20°C with little loss of fluidity. Use of the resin at low temperature provides maximum protection from the denaturing effects of ethanol, but does introduce artifacts into the tissue. Following post-staining with lead citrate, a "salt and pepper" background may be present in the

sections. The artifact probably results from the different rates of penetration of the various components of the resin into the tissue at low temperature.

The following protocol is used for embedding blood cells infected with *Plasmodium*:

- 1) Wash blood cells three times with 0.1 M buffer (pH 7.3) after fixation
- 2) 30% ethanol for 10 min at 4°C
- 3) 50% ethanol for 10 min at -20°C
- 4) 70% ethanol for 10 min at -20°C
- 5) One part 95% ethanol and two parts of LR White resin for 10 min at -20°C
- 6) Three changes of LR White resin (60 min each) at -20°C
- 7) Fresh LR White resin overnight at 4°C
- 8) Transfer sample to capsules and polymerize at 37-40°C for five days
- 9) Thin sectioning
- 10) Immunolabelling using 1% BSA in 0.1 M PBS with 0.001% tween -20 (Tween -20 provides minimal background staining)
- 11) Protein A-gold labeling
- 12) Stain grids for 10 min in aqueous uranyl acetate and for 2 min in lead citrate.

LOCALIZATION OF PROTECTIVE MALARIAL ANTIGENS USING VARIOUS MONOCLONALS

a) **Localization of protective antigens of *P. knowlesi* by cryoultramicrotomy** – Miller et al. (1984) described a protein composed of two subunits 143 and 140kD on the merozoites' surface of a primate parasite, *P. knowlesi*. Immune sera from mice immunized with the 143/140kD proteins were demonstrated to partially block primate erythrocyte invasion by the merozoites *in vitro*. Thus, the equivalent of the 143/140kD protein in human malarial parasites may be important as a possible candidate against which a malaria vaccine can be developed.

In collaboration with Miller et al., we performed immuno-electron microscopy utilizing cryoultramicrotomy, antibody to 143/140kD protein and protein A-gold in order to determine the precise localization of this protein in *P. knowlesi* (Aikawa et al., 1986). Gold particles were not seen associated with young trophozoites but appeared in the parasite cytoplasm as the parasites grew to multinucleate schizonts (Fig. 1). In presegmenter-schizonts, gold particles were associated with the well-developed endoplasmic reticulum, the parasite plasma membrane, and the parasitophorous vacuole membrane. The surface of merozoites was also covered with gold particles (Fig. 2). Maurer's clefts, which appeared in *Plasmodium* infected erythrocytes, were associated with gold particles.

These observations suggest that 143/140kD protective malarial proteins may be synthesized in the endoplasmic reticulum of *P. knowlesi* schizonts before being transported to the surface of the schizonts and merozoites. Shedding of the merozoite surface coat may be responsible for the presence of the 143/140kD proteins in the parasitophorous vacuole and Maurer's clefts (Fig. 5).

b) **Localization of the 150/130kD antigens in sexual and asexual blood stages of *P. falciparum*-infected human erythrocytes** – Masuda et al. (1986) described the properties of an antigen present in all asexual and sexual blood stages of *P. falciparum* using a monoclonal, (MAb) 9B11 obtained by immunizing mice with viable gametocytes. MAb recognizes a 150 and 130kD polypeptides which share common properties with pf 155kD and the ring-infected erythrocyte surface antigen (RESA). By indirect immunofluorescence microscopy, MAb 9B11 produced an intense diffuse fluorescence in gametocyte infected erythrocytes and also in erythrocytes infected with asexual parasites. In order to determine the precise subcellular localization of the antigens recognized by MAb 9B11 in the various developmental stages in *P. falciparum*-infected erythrocytes, we utilized immuno-electron microscopy (Uni et al., 1986) using both cryoultramicrotomy and LR White resin, in collaboration with Nussenzweig et al.

This antigen is found to be associated with the membrane of newly-infected human erythrocytes and the cytoplasm of ring stage parasites (Fig. 3). During differentiation of the parasite to the trophozoite stage, the antigens are no longer detectable on the erythrocyte membrane, while gold particles become more numerous within the parasite and in the erythrocyte cytoplasm adjacent to the parasite. As the parasites develop into schizonts, more antigen appears within the parasites, and some of it appears to diffuse into the erythrocyte cytoplasm (Fig. 4). At the segmented schizont stage, many intraparasitic gold particles are associated with rhoptries and micro-

nemes of developing merozoites. Likewise, gold particles are associated with the rhoptry-microsome complex in free merozoites. These antigens are also found in erythrocytes infected with gametocytes. These subcellular localization patterns are similar to those described for the ring-infected erythrocyte surface antigen (RESA) (Brown et al., 1985) (Fig. 6).

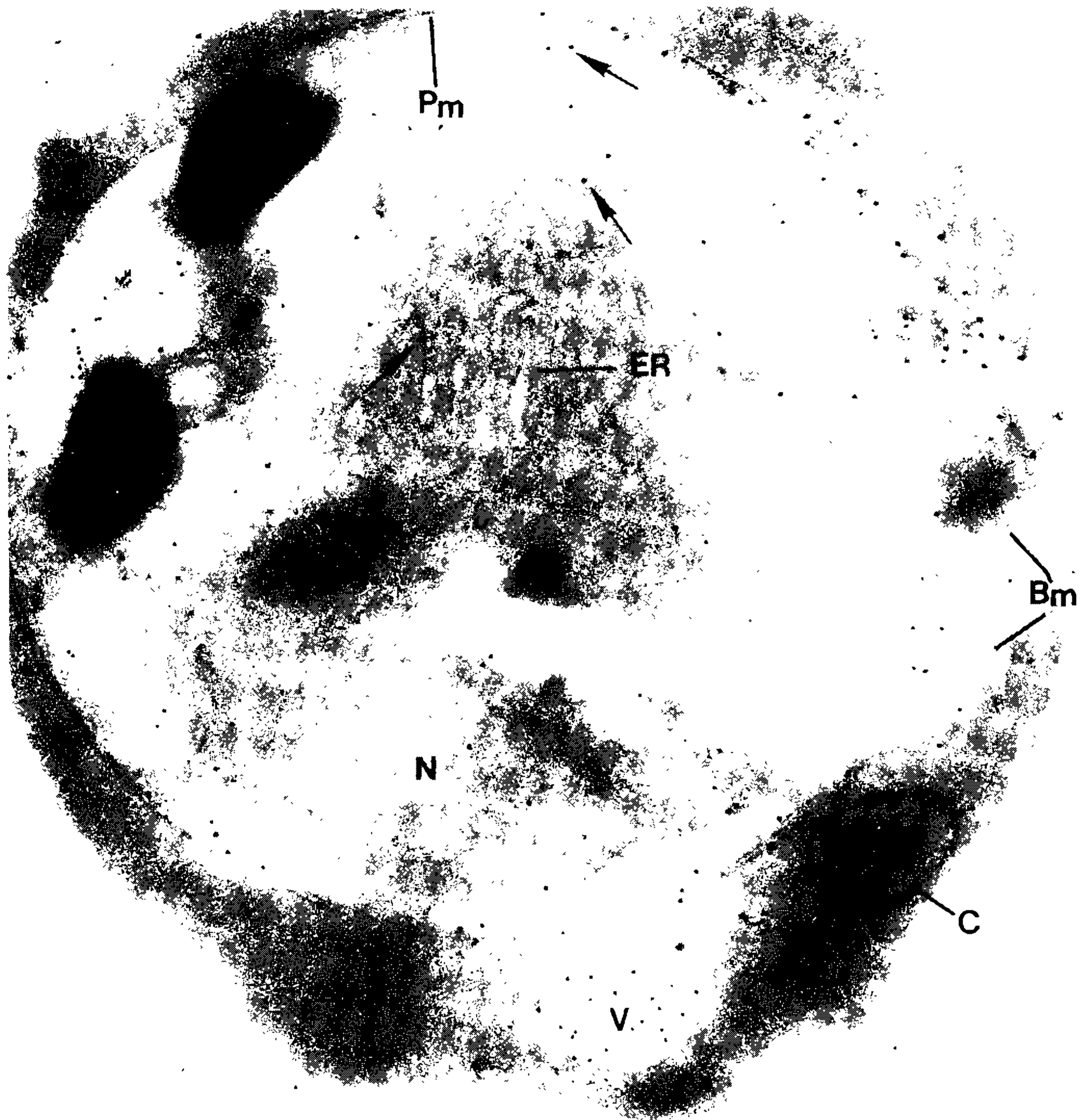


Fig. 1: Electron micrograph of a cryosectioned pre-segmenter-schizont budding merozoites (Bm). Gold particles which indicate the presence of 143/140kD proteins, are seen over an array of endoplasmic reticulum (ER). Gold particles (arrow) emanating from the endoplasmic reticulum appear to approach the plasma membrane of the parasite (Pm). The erythrocyte cytoplasm contains membrane-bound Maurer's clefts (C) and vacuoles (V). Gold particles are associated with both structures. X34,000 (Aikawa et al., *Europe J. Cell Biol.*, 1986, in press).



Fig. 2: *P. knowlesi* merozoites (M) within a parasitophorous vacuole (Pv). Gold particles identifying the presence of 143/140kD proteins are seen along the merozoite surface membrane (Mm), the parasitophorous vacuole membrane (Pvm) and within the vacuolar space. X33,000. (Aikawa, M. et al., *European J. Cell Biol.*, 1986, in press).



Fig. 3: Electron micrograph of a cryosectioned ring-infected erythrocyte demonstrating gold particles (arrow) on the erythrocyte surface as well as in the cytoplasm of ring forms (R). Note the absence of gold particles on the surface of the uninfected erythrocyte (arrowhead). X20,000. Inset: high magnification of a ring-infected erythrocyte with gold particles on the membrane. X50,000. (Uni, S. et al., *Am. J. Trop. Med. Hyg.*, 1986, in press).

Fig. 4: Electron micrograph of a cryosectioned trophozoite (T)-infected erythrocyte. Gold particles (arrow) are not only found within the parasite, but also in the erythrocyte cytoplasm adjacent to the parasite. X38,000. (Uni, S. et al., *Am. J. Trop. Med. Hyg.*, 1986, in press).

Merozoite Surface Antigens

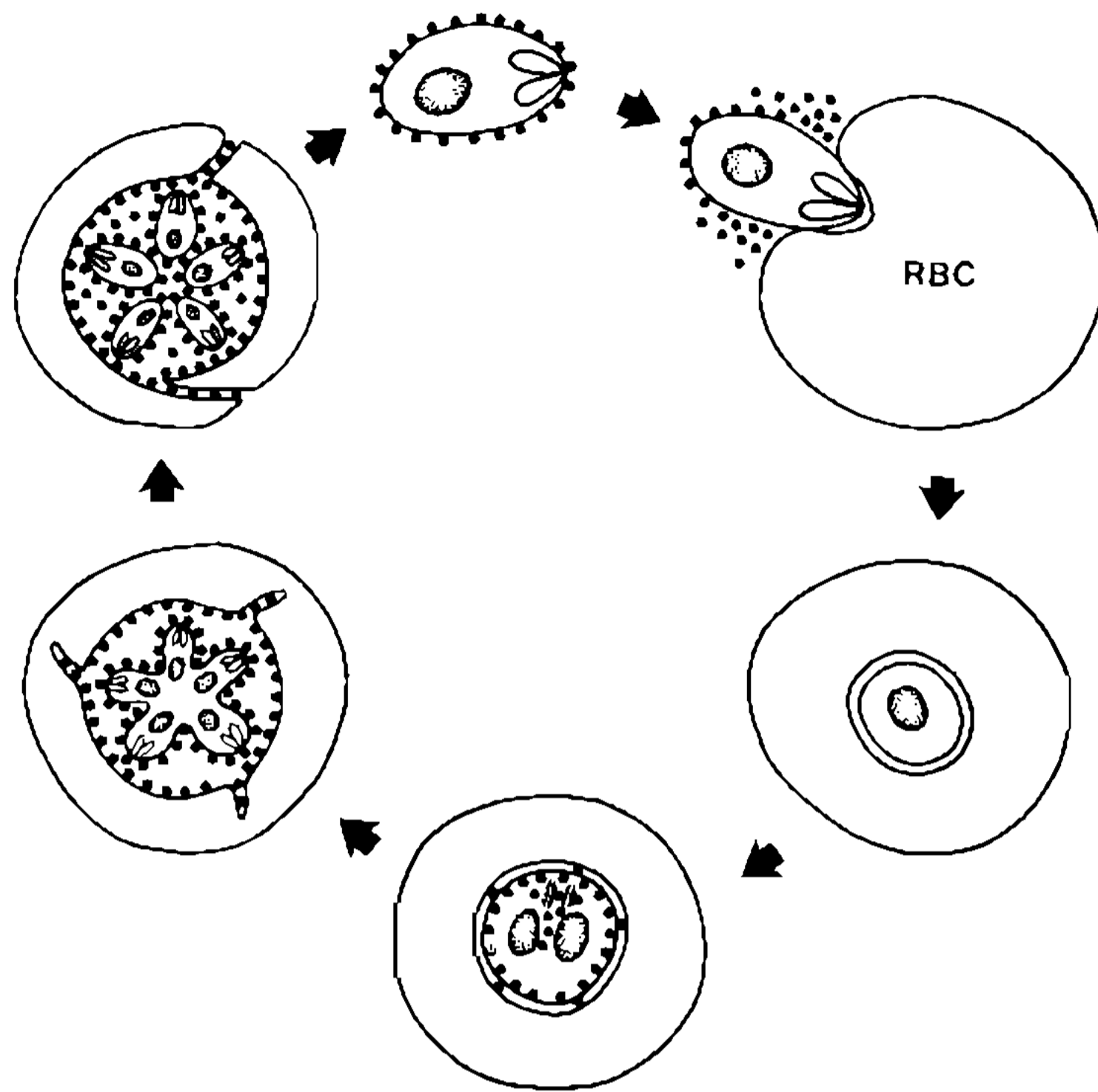


Fig. 5: A schematic diagram showing the distribution of merozoite surface antigens (●) during the various stages of the plasmodial life cycle.

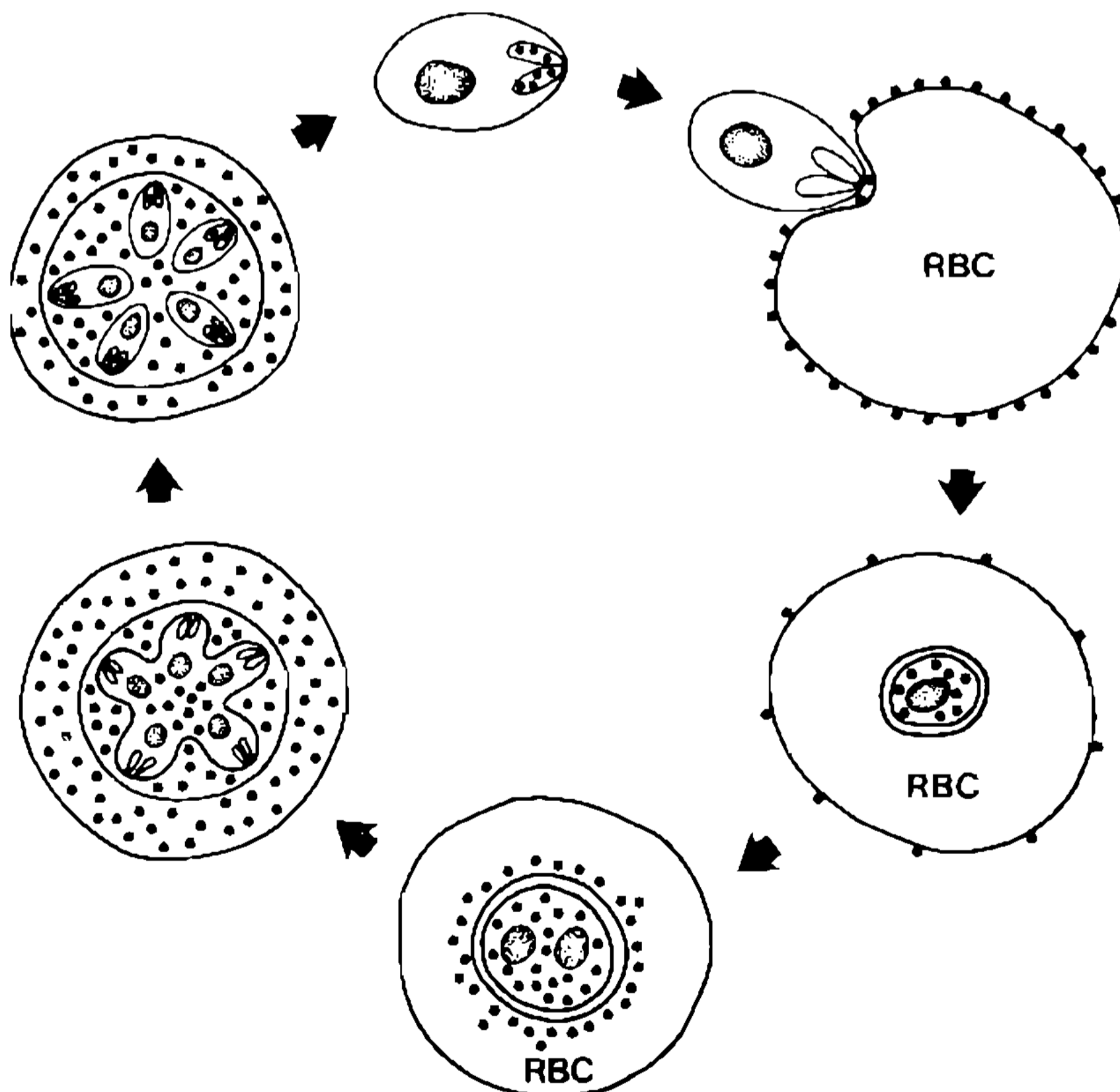


Fig. 6: A schematic diagram showing the distribution of the 150/130kD antigens (●) during the various stages of the plasmodial life cycle.

CONCLUSION

Immuno-electron microscopy has become one of the major techniques in the study of the molecular structure of cells and tissues in recent years. The improvement of this technique and the introduction of new embedding resins have advanced our knowledge in immunocytochemistry.

As we have demonstrated, immuno-electron microscopy is a very useful method for localizing protective malarial antigens in *Plasmodium* as well as in host cells. The localization of these protective antigens within specific organelles of *Plasmodium* may assist in identifying which particular subcellular organelles of *Plasmodium* can be used for the induction of immunity against malaria infection. In addition, immuno-electron microscopy helps to understand how these protective proteins are synthesized within the parasites. As more protective malarial proteins are identified, these proteins may be classified by immuno-electron microscopy according to their localization within *Plasmodium*.

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