

Plasmodium falciparum Ring-Infected Erythrocyte Surface Antigen Is Released from Merozoite Dense Granules after Erythrocyte Invasion

JANETTA G. CULVENOR,* KAREN P. DAY, AND ROBIN F. ANDERS

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

Received 16 July 1990/Accepted 13 December 1990

Electron microscopy was used to study the fate of *Plasmodium falciparum* ring-infected erythrocyte surface antigen after merozoite invasion by using postembedding immunolabeling. The antigen was localized to small dense granules located centrally or laterally in free merozoites. In newly invaded erythrocytes, labeling was found in pockets of the parasitophorous vacuole space or in aggregates closely associated with the parasitophorous vacuole. These patterns indicate that ring-infected erythrocyte surface antigen is contained in merozoite dense granules that are released after merozoite invasion and not via apical rhoptry ducts at the time of merozoite attachment.

Ring-infected erythrocyte surface antigen (RESA) is a nonpolymorphic malaria antigen that has been cloned and completely sequenced for two *Plasmodium falciparum* isolates (8, 9, 13). Since antibodies were inhibitory to merozoite invasion *in vitro* (24) and immunization with RESA fusion polypeptides afforded partial protection to challenge infection in *Aotus* monkeys (7), this antigen is an important potential vaccine candidate. RESA was first localized by using immunofluorescence to the erythrocyte surface of *P. falciparum* ring trophozoite-infected cells (8, 20). Immunoelectron microscopy confirmed this association and also showed localization within merozoite dense granules that we initially termed micronemes (6). Recent data have shown that RESA is associated with the erythrocyte cytoskeleton of ring-stage parasites (14).

Structural studies with *Plasmodium knowlesi* by Bannister et al. (4) and Bannister and Mitchell (5) distinguished three types of membrane-bound organelles within merozoites: rhoptries (double, large, tear-shaped organelles terminating at the apex), micronemes (small tube-shaped structures associated with the rhoptries), and microspheres or dense granules (approximately 130 nm in diameter, intermediate in size, spherical, and more dense than micronemes and rhoptries). These authors also showed structural evidence that the dense granules were released during or after merozoite entry. Immunolabeling studies of *P. knowlesi* (22) and of the related protozoan *Sarcocystis muris* (12) followed the fate of labeled merozoite dense granules and confirmed release of their contents after merozoite junction formation. Analogous dense granules and micronemes have proved more difficult to distinguish ultrastructurally in *P. falciparum*, where merozoites are somewhat smaller. Mitchell and Bannister (19) have previously suggested that RESA may be located in dense granules or microspheres rather than in apically associated micronemes.

We have examined RESA localization by postembedding immunolabeling with isolates that produced the best ultrastructure after minimal glutaraldehyde fixation, monoclonal antibodies (MAbs), polyclonal antibodies, and 5-nm gold particles to give improved resolution over our earlier re-

ports. The studies described here show that *P. falciparum* RESA is localized to dense round granules (of up to 100 nm in diameter) in merozoites and to similar-sized aggregates of amorphous material in the parasitophorous vacuole of newly invaded erythrocytes similar to the labeled structures described by Torii et al. (22) for *P. knowlesi* merozoite granule release.

P. falciparum isolate FCQ27/PNG (FC27) and the derived clone D10 were cultured and prepared for electron micros-

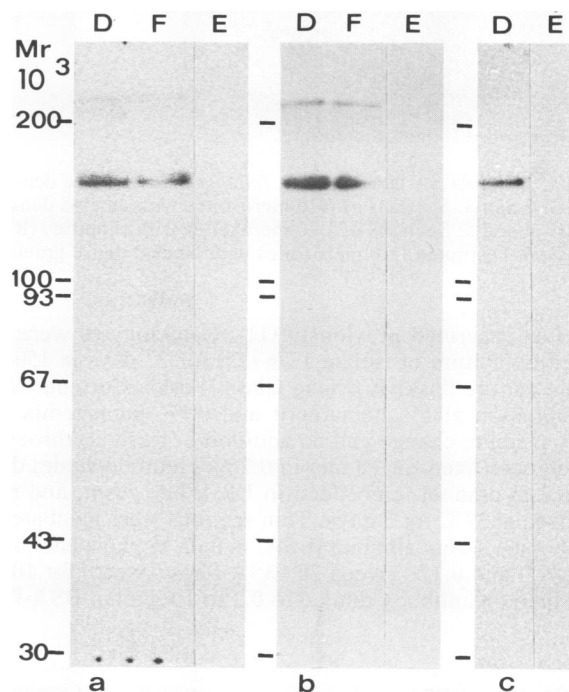


FIG. 1. Immunoblot of asexual stages from *P. falciparum* isolate D10 (D) or FC27 (F) or uninfected erythrocytes (E) fractionated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with RESA antibodies as follows: (a) human anti-4-mer, preparation H145; (b) human anti-4-mer, preparation H165; and (c) MAb 23/9 (anti-3' repeat segment).

* Corresponding author.

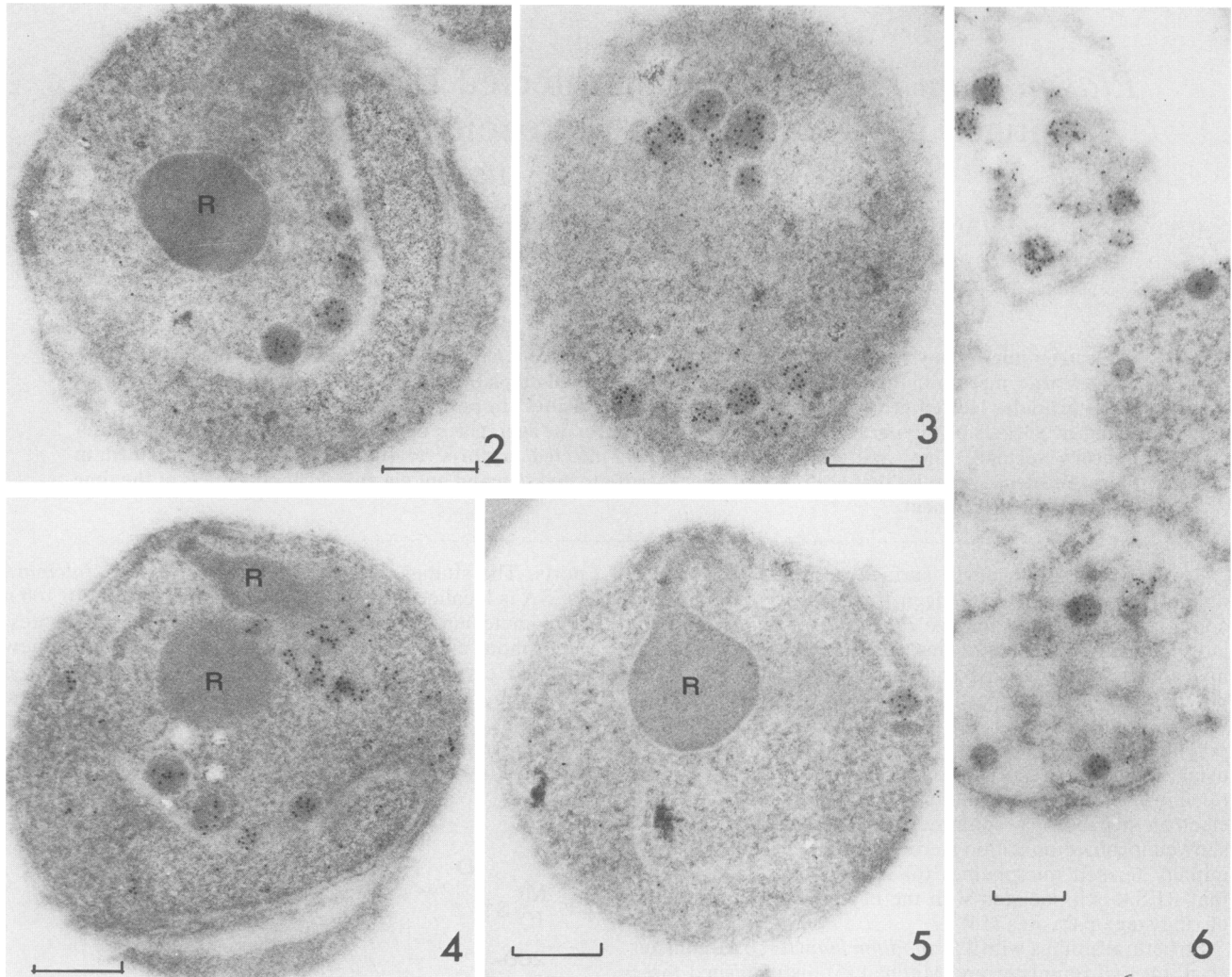


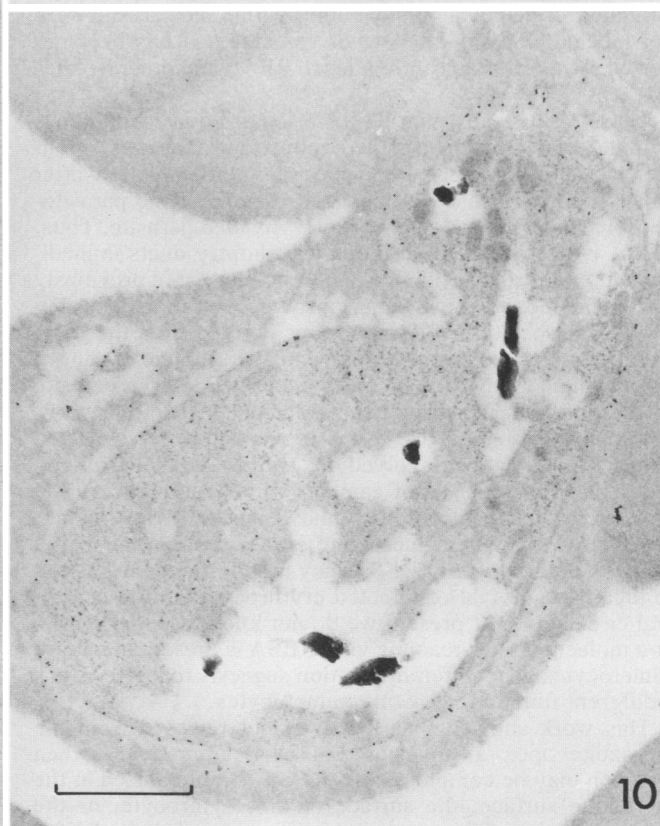
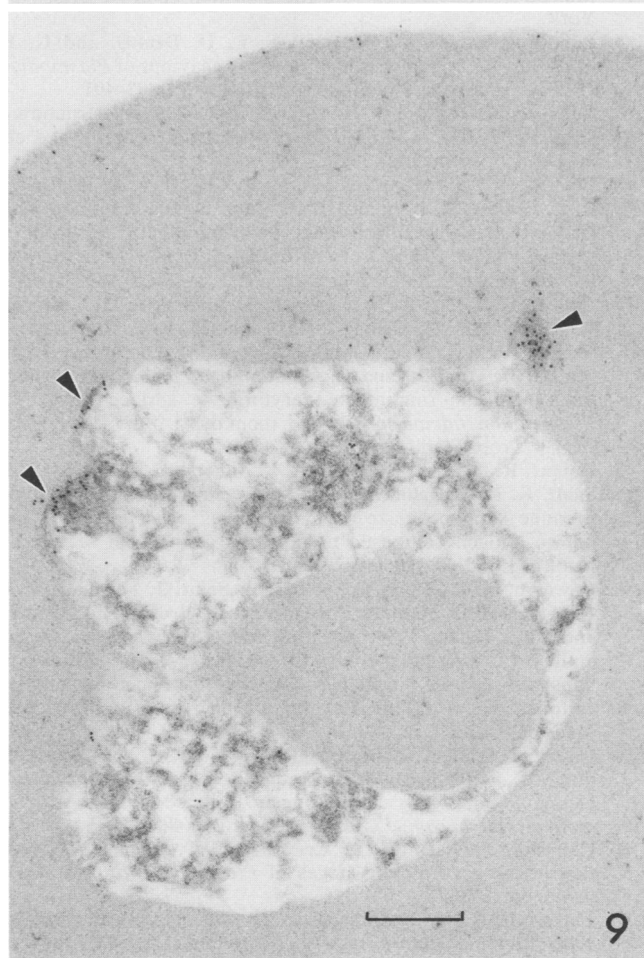
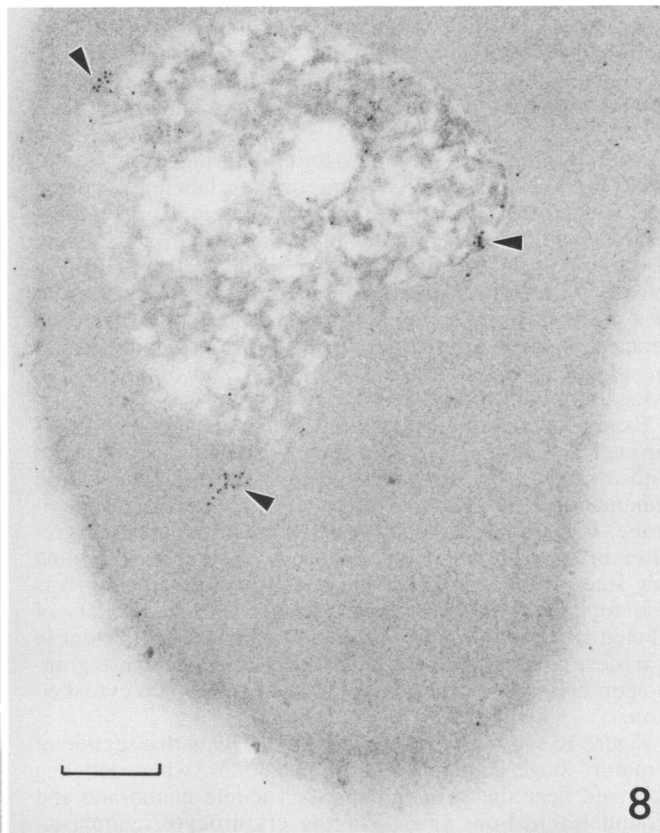
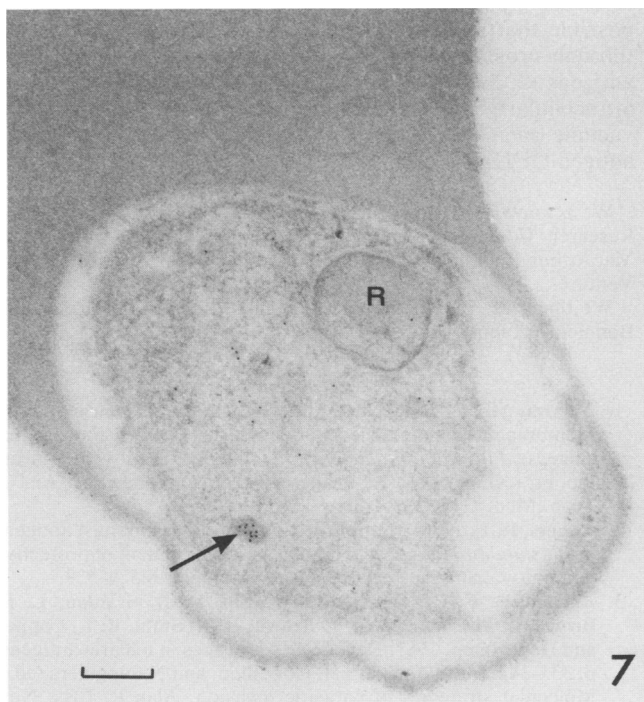
FIG. 2-6. RESA labeling of *P. falciparum* merozoite dense granules (protein A-5-nm gold; bars, 0.2 μ m).
 FIG. 2 and 3. Sections of D10 merozoites with labeled dense granules after incubation with MAb23/9.
 FIG. 4 and 5. Sections of D10 merozoites with rhoptries (R) in view after incubation with human anti-RESA, preparation H145.
 FIG. 6. Disrupted D10 merozoites with labeled dense granules (MAb23/9).

copy as described previously (11). Gametocytes were prepared by culture of isolate 1776 (15) for 22 days in 150-cm² tissue culture flasks (Corning Glass Works, Corning, N.Y.) by initiation at 5% hematocrit and 0.5% parasitemia with daily medium change and no addition of fresh erythrocytes. Tissue was fixed for 10 min in 0.25% glutaraldehyde, dehydrated in ethanol, embedded in LR White resin, and polymerized at 37°C for 5 days. Thin sections were incubated on 1% bovine serum albumin (BSA) in 0.05 M phosphate buffer (pH 7.4) and 0.1% Tween 20 (BSA-Phos-Tween) for 10 min and then on antibody diluted to 0.1 to 1 μ g/ml in BSA-Phos-

Tween for 1 h at room temperature, washed in Phos-Tween, and then incubated on protein A-5 or 10-nm gold particles (Janssen) diluted 1:10 in BSA-Phos-Tween. After further washing, sections were stained with uranyl acetate.

MAb 23/9 (mouse immunoglobulin G2a) was isolated on protein A-Sepharose. This MAb was prepared from mice injected with a β -galactosidase fusion protein containing the C-terminal 3' repeat region of RESA (8) and reacted primarily with the 3' repeat region of RESA when tested by ELISA (3). Human antibodies to the RESA 3' repeat (preparation H145) were affinity purified on the dodecameric synthetic

FIG. 7-10. RESA labeling of early trophozoites and a mature gametocyte.
 FIG. 7. View of a D10 merozoite after erythrocyte invasion, with rhoptry (R) present, a labeled dense granule (arrow), and no label over the erythrocyte plasma membrane (MAb 23/9; protein A-5-nm gold; bar, 0.2 μ m).
 FIG. 8 and 9. Views of early FC27 ring-trophozoite stage parasites with pockets of labeled aggregates (arrowheads) in or near the parasitophorous vacuole (human anti-RESA, preparation H145; protein A-5-nm gold; bars, 0.2 μ m).
 FIG. 10. Section of a mature strain 1776 gametocyte with labeling mainly over the parasitophorous vacuole membrane and the erythrocyte cytoplasm (MAb 23/9, protein A-10-nm gold; bar, 1.0 μ m).



peptide (EENV)₃ coupled to BSA and then Sepharose (10). The specificity of these reagents was tested by immunoblotting (Fig. 1) by the method of Crewther et al. (10). The antibodies reacted with D10 and FC27 asexual stages to give a major band of M_r 155,000; a minor band (M_r 215,000) of unknown identity was also detected by the human antibodies.

Antibodies reacted with merozoites and ring-stage trophozoites found in the sections. In merozoites, labeling occurred over round dense granules of up to 100 nm in diameter (Fig. 2 through 5). Similar granules in damaged merozoites were also labeled (Fig. 6), suggesting that this is the way RESA is released into culture supernatants (14). Labeled dense granules were frequently found in the central area near the concave region of the merozoite nucleus (Fig. 2 and 4) and at the lateral sides of merozoites (Fig. 3 and 5) but not in association with the neck of the rhoptry.

Examples of early trophozoite labeling are shown in Fig. 7 through 9. Figure 7 shows a recently invaded erythrocyte with a rhoptry organelle still present and a labeled dense granule but without label over the erythrocyte plasma membrane. Figures 8 and 9 show labeled medium-density aggregates in or near the parasitophorous vacuole surrounding ring-stage trophozoites; in these cells, some labeling was also found over the erythrocyte surface. The pockets of labeled material, which were similar in size to the dense granules, presumably represent newly released dense granule contents, which then travel to the erythrocyte cytoskeleton.

Figure 10 shows the reaction of MAb 23/9 with a section of a mature-stage gametocyte of isolate 1776, where labeling occurred near the parasitophorous vacuole membrane and around leached-out spaces of the erythrocyte cytoplasm. Granules termed osmiophilic bodies within the parasite were not labeled. Similar labeling of gametocytes has been reported by others using antibodies to RESA and other isolates (21, 23).

These results show that RESA is localized in small, dense granules of *P. falciparum* merozoites, and that these granules move to the lateral regions of the merozoite at or after entry and are released in localized regions of the parasitophorous vacuole space of the newly invaded parasite. Thus, RESA is not released via the apical rhoptry ducts immediately after apical junction formation, as suggested previously (6, 7). The movement to the erythrocyte membrane possibly involves association with membrane derived from the parasitophorous vacuole membrane, similar to the movement of knob protein material, which is synthesized later in parasite maturation and also becomes associated with the erythrocyte cytoskeleton (1, 16). It has previously been suggested that RESA may be involved in erythrocyte invasion by merozoites and release of gametes from erythrocytes (21). However, our localization studies suggest RESA is not involved in the initial merozoite invasion steps but has a function after invasion; RESA may modify the cytoskeleton to facilitate survival of infected erythrocytes in the peripheral circulation. At present we do not know whether RESA or a molecule cross-reacting with RESA is present in mature gametocytes. The different location suggests that RESA has a different function in mature gametocytes.

This work showed that RESA is not released from the merozoite apex at invasion but at a later stage, when invasion may be complete; thus RESA is not exposed at the merozoite surface, the surface of the erythrocyte, or the apical junction. This raises the question of how RESA antibodies, which are reported to be growth inhibitory, may

be acting. Since many antibodies to RESA do show various levels of cross-reactions with several other antigens, it is possible that inhibitory antibodies may be acting primarily through cross-reaction with other parasite or erythrocyte antigens (2, 3, 18). Alternatively, antibodies may be acting intracellularly, as has been reported for a parasitophorous vacuole membrane antigen, the circumsporozoite-related antigen QF116 (17).

We acknowledge support from the National Health and Medical Research Council of Australia, the John D. and Catherine T. MacArthur Foundation, and the Australian Malaria Vaccine Joint Venture.

We thank M. B. Moloney and H. Dillon for cell culture and E. Bonnici for typing.

REFERENCES

1. Aikawa, M., Y. Uni, A. T. Andrutis, and R. J. Howard. 1986. Membrane-associated electron-dense material of the asexual stages of *Plasmodium falciparum*: evidence for movement from the intracellular parasite to the erythrocyte membrane. *Am. J. Trop. Med. Hyg.* 35:30-36.
2. Anders, R. F. 1986. Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective immunity against malaria. *Parasite Immunol.* 8:529-539.
3. Anders, R. F., N. Barzaga, P.-T. Shi, D. B. Scanlon, L. E. Brown, L. M. Thomas, G. V. Brown, H. D. Stahl, R. L. Coppel, and D. J. Kemp. 1987. Repetitive sequences in malaria antigens, p. 333-342. *In* N. Agabian, H. Goodman, and N. Noguiera (ed.), *Molecular strategies of parasitic invasion*. Alan R. Liss, New York.
4. Bannister, L. H., G. A. Butcher, E. D. Dennis, and G. H. Mitchell. 1975. Structure and invasive behaviour of *Plasmodium knowlesi* merozoites *in vitro*. *Parasitology* 71:483-491.
5. Bannister, L. H., and G. H. Mitchell. 1989. The fine structure of secretion by *Plasmodium knowlesi* merozoites during red cell invasion. *J. Protozool.* 36:362-367.
6. Brown, G. V., J. G. Culvenor, P. E. Crewther, A. E. Bianco, R. L. Coppel, R. B. Saint, H.-D. Stahl, D. J. Kemp, and R. F. Anders. 1985. Localization of the ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* in merozoites and ring-infected erythrocytes. *J. Exp. Med.* 162:774-779.
7. Collins, W. E., M. Pappaioanou, R. F. Anders, G. H. Campbell, G. V. Brown, D. J. Kemp, J. R. Broderick, R. L. Coppel, J. C. Skinner, P. M. Procell, J. M. Favaloro, L. M. Corcoran, N. S.-F. Ma, G. F. Mitchell, and C. C. Campbell. 1988. Immunization trials with the ring-infected erythrocyte surface antigen of *Plasmodium falciparum* in owl monkeys (*Aotus vociferans*). *Am. J. Trop. Med. Hyg.* 38:268-282.
8. Coppel, R. L., A. F. Cowman, R. F. Anders, A. E. Bianco, R. B. Saint, K. R. Lingelbach, D. J. Kemp, and G. V. Brown. 1984. Immune sera recognize on erythrocytes a *Plasmodium falciparum* antigen composed of repeated amino acid sequences. *Nature (London)* 310:789-792.
9. Cowman, A. F., R. L. Coppel, R. B. Saint, J. Favaloro, P. E. Crewther, H. D. Stahl, A. E. Bianco, G. V. Brown, R. F. Anders, and D. J. Kemp. 1984. The ring-infected erythrocyte surface antigen (RESA) polypeptide of *Plasmodium falciparum* contains two separate blocks of tandem repeats encoding antigenic epitopes that are naturally immunogenic in man. *Mol. Biol. Med.* 2:207-221.
10. Crewther, P. E., A. E. Bianco, G. V. Brown, R. L. Coppel, H.-D. Stahl, D. J. Kemp, and R. F. Anders. 1986. Affinity purification of human antibodies directed against cloned antigens of *Plasmodium falciparum*. *J. Immunol. Methods* 86:257-264.
11. Culvenor, J. G., and P. E. Crewther. 1989. S-antigen localization in the erythrocytic stages of *Plasmodium falciparum*. *J. Protozool.* 37:59-65.
12. Entzeroth, R., J. F. Dubremetz, D. Hodick, and E. Ferreira. 1986. Immunoelectron microscopic demonstration of the exocytosis of dense granule contents into the secondary parasitophorous vacuole of *Sarcocystis muris* (Protozoa, Apicomplexa).

- Eur. J. Cell. Biol. **41**:182-188.
13. Favaloro, J. M., R. L. Coppel, L. M. Corcoran, S. J. Foote, G. V. Brown, R. F. Anders, and D. J. Kemp. 1986. Structure of the RESA gene of *Plasmodium falciparum*. Nucleic Acid Res. **14**:8265-8277.
 14. Foley, M., L. J. Murray, and R. F. Anders. 1990. The ring-infected erythrocyte surface antigen protein of *Plasmodium falciparum* is phosphorylated upon association with the host cell membrane. Mol. Biochem. Parasitol. **38**:69-76.
 15. Forsyth, K. P., G. Philip, T. Smith, E. Kum, B. Southwell, and G. V. Brown. 1989. Diversity of antigens expressed on the surface of erythrocytes infected with mature *Plasmodium falciparum* parasites in Papua New Guinea. Am. J. Trop. Med. Hyg. **41**:259-265.
 16. Howard, R. J. 1988. *Plasmodium falciparum* proteins at the host erythrocyte membrane: their biological and immunological significance and novel parasite organelles which deliver them to the cell surface, p. 111-145. In P. T. Englund and A. Sher (ed.), The biology of parasitism: a molecular and immunological approach, MBL lectures in biology. Alan R. Liss, New York.
 17. Kara, U. A. K., D. J. Stenzel, L. T. Ingram, G. R. Bushell, J. A. Lopez, and C. Kidson. 1988. Inhibitory monoclonal antibody against a (myristylated) small-molecular-weight antigen from *Plasmodium falciparum* associated with the parasitophorous vacuole membrane. Infect. Immun. **56**:903-909.
 18. Mattei, D., K. Berzins, M. Wahlgren, R. Udomsangpetch, P. Perlmann, H. W. Griesser, A. Scherf, B. Müller-Hill, S. Bonnefoy, M. Guillotte, G. Langsley, L. P. Da Silva, and O. Mercereau-Puijalon. 1989. Cross-reactive antigenic determinants present on different *Plasmodium falciparum* blood-stage antigens. Parasite Immunol. **11**:15-30.
 19. Mitchell, G. H., and L. H. Bannister. 1988. Malaria parasite invasion: interactions with the red cell membrane. CRC Crit. Rev. Oncol. Hematol. **8**:255-310.
 20. Perlmann, H., K. Berzins, M. Wahlgren, J. Carlsson, A. Björkman, M. E. Patarroyo, and P. Perlmann. 1984. Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum*. J. Exp. Med. **159**:1686-1704.
 21. Quakyi, I. A., Y. Matsumoto, R. Carter, R. Udomsangpetch, A. Sjolander, K. Berzins, P. Perlmann, M. Aikawa, and L. H. Miller. 1989. Movement of a falciparum malaria protein through the erythrocyte cytoplasm to the erythrocyte membrane is associated with lysis of the erythrocyte and release of gametes. Infect. Immun. **57**:833-839.
 22. Torii, M., J. H. Adams, L. H. Miller, and M. Aikawa. 1989. Release of merozoite dense granules during erythrocyte invasion by *Plasmodium knowlesi*. Infect. Immun. **57**:3230-3233.
 23. Uni, S., A. Masuda, M. J. Stewart, I. Igarashi, R. Nussenzweig, and M. Aikawa. 1987. Ultrastructural localization of the 150/130 Kd antigens in sexual and asexual blood stages of *Plasmodium falciparum*-infected human erythrocytes. Am. J. Trop. Med. Hyg. **36**:481-488.
 24. Wählin, B., M. Wahlgren, H. Perlmann, K. Berzins, A. Björkman, M. E. Patarroyo, and P. Perlmann. 1984. Human antibodies to a M_r 155,000 *Plasmodium falciparum* antigen efficiently inhibit merozoite invasion. Proc. Natl. Acad. Sci. USA **81**:7912-7916.