

LOCALIZATION OF THE RING-INFECTED ERYTHROCYTE
SURFACE ANTIGEN (RESA) OF *PLASMODIUM FALCIPARUM*
IN MEROZOITES AND RING-INFECTED ERYTHROCYTES

BY G. V. BROWN, J. G. CULVENOR, P. E. CREWETHER, A. E. BIANCO,
R. L. COPPEL, R. B. SAINT, H.-D. STAHL, D. J. KEMP, AND R. F. ANDERS

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

Recently (1), we described a cloned polypeptide of *Plasmodium falciparum* representing part of an antigen found at the surface of erythrocytes infected with ring-stage parasites. This antigen (RESA) contains two separate blocks of tandem repeat sequences that encode antigenic determinants recognized by antibodies in the sera of individuals exposed to malaria (2). In this study we have used immunoelectron microscopy and immunoblotting to investigate the distribution of this antigen in mature parasites and at the surface of recently invaded erythrocytes.

Materials and Methods

Parasite cultures of *P. falciparum* isolate FCQ27/PNG were cultured in vitro in group O human erythrocytes (3) and were synchronized twice to within a 6-h spread of maturation using sorbitol (4). Infected cells from a single bulk culture were harvested at 4, 26, and 38 h after the second sorbitol treatment to yield ring-stage (>99%), trophozoite (>97%), and schizont (98% schizont, 2% rings) preparations, respectively. The parasitized erythrocytes were washed three times by centrifugation (500 g, 10 min) and resuspended in serum-free culture medium, and the pellets were stored at -70°C .

To prepare naturally released merozoites, supernatants of synchronous cultures were collected over a 2 h period of merozoite release, centrifuged to remove the majority of erythrocytes (550 g, 5 min), and sequentially passed through nylon sieves of 3.0 and 1.2 μm pore size (Versapor; Gelman Sciences, Inc., Ann Arbor, MI) (Mrema et al. [5]). Merozoites in the filtrate were concentrated by centrifugation (3,000 g, 10 min) and washed in three changes of serum-free culture medium containing 2% Trasylol (Bayer AG, Federal Republic of Germany).

Immunoelectron Microscopy. Samples of parasite culture were fixed with 0.25% glutaraldehyde for 10 min at room temperature, then diluted with 50 mM NH_4Cl in 0.1 M phosphate buffer, pH 7.4, and left in fresh 50 mM NH_4Cl in phosphate buffer for 30 min. Cells were then washed twice in phosphate buffer, dehydrated in 70% ethanol, and embedded in L. R. White resin, hard grade (London Resin Co. Ltd., Basingstoke, England). Sections were first incubated in 1% bovine serum albumin or ovalbumin in 0.05 M phosphate, pH 7.4, containing 0.25% Tween 20 (phosphate-Tween) for 5 min. They were then transferred to a drop of rabbit anti-RESA antiserum (diluted 1:100) or affinity-

This work was supported by the National Health and Medical Research Council of Australia, The Rockefeller Foundation, the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, the Australian National Biotechnology Programme Research Grants Scheme, and the John D. and Catherine T. MacArthur Foundation.

purified human anti-RESA antibodies (diluted as indicated) in phosphate-Tween with or without 1% ovalbumin added, for 30–60 min at room temperature. The sections were washed in phosphate-Tween and transferred to protein A gold (E-Y Laboratories, Inc.) diluted 1:10 in phosphate-Tween for 30–60 min. After further washing, the sections were stained with aqueous uranyl acetate. Isolated merozoites were fixed at 4°C in 0.25% glutaraldehyde for 10 min and then processed in the same way as cells.

Affinity Purification of Human Serum. A cDNA clone expressing RESA was used for affinity purification of specific antibodies (as described previously [(6)] from a pool of sera collected from healthy adults in Papua New Guinea.

Immunoblots. Merozoites and infected erythrocytes were incubated in phosphate-buffered saline containing 0.5% Triton X-100, 5 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone (all from Sigma Chemical Co., St. Louis, MO), 2.5 mM EDTA, and 2 mM iodoacetamide for 30 min at room temperature, and then centrifuged at 12,000 *g* for 10 min. Supernatants or pellets were then electrophoresed and blotted as described (6).

Results

RESA was detected by immunoelectron microscopy at the membrane of erythrocytes infected with ring-stage parasites but not in association with immature parasites within the erythrocyte (Fig. 1, *A* and *B*). In contrast, the membranes of erythrocytes containing mature parasites were not labeled, but gold particles were associated with electron-dense organelles presumed to be micronemes within the parasite cytoplasm (Fig. 2). Control antibodies to S antigens did not react with merozoites or the erythrocyte membrane.

The labeling of merozoites was clearly internal, with no indication of specific labeling of the merozoite surface. Labeling occurred in clusters away from the nucleus (Fig. 1, *C* and *E*) and occasionally over a rhoptry (Fig. 1*D*). In other merozoites, gold particles were more dispersed but were located near the rhoptries, which were particle-free. Similar distributions of gold were observed with both affinity-purified human antibodies and rabbit antibodies raised against the cloned antigen, although higher background labeling was evident with the affinity-purified human antibodies (Fig. 1, *B* and *G*). The specificity of the observed patterns of labeling was demonstrated by the different patterns, or by the lack of labeling when the same procedures were used with affinity-purified human antibodies or rabbit antisera to other cloned *P. falciparum* antigens (e.g., S antigen) (data not shown).

Antibodies to RESA produced several bands when reacted in immunoblots with asynchronously grown parasites lysed in electrophoresis sample buffer and fractionated on 7.5% sodium dodecyl sulfate (SDS)/polyacrylamide gels (Fig. 3*A*). The most prominent band was at M_r 155,000 and, in some experiments, resolved to a closely migrating doublet. A higher molecular weight polypeptide reacting with the anti-RESA antibodies varied in size in different isolates (Fig. 3*A*); it was at M_r 210,000 in isolate FC27. In addition, a smaller molecular weight polypeptide (M_r 80,000) was detected in some antigen preparations (Fig. 3*A*). The abundance of the M_r 210,000 polypeptide was greatest in schizonts (Fig. 3*B*). In contrast, the M_r 155,000 antigen was abundant in the merozoites, rings, and trophozoites with small amounts in schizonts (Fig. 3, *B* and *C*).

The solubility of RESA in detergents was determined to examine the nature of the interaction between RESA and the erythrocyte membrane. The M_r 210,000 polypeptide was soluble in solutions of the nonionic detergent Triton

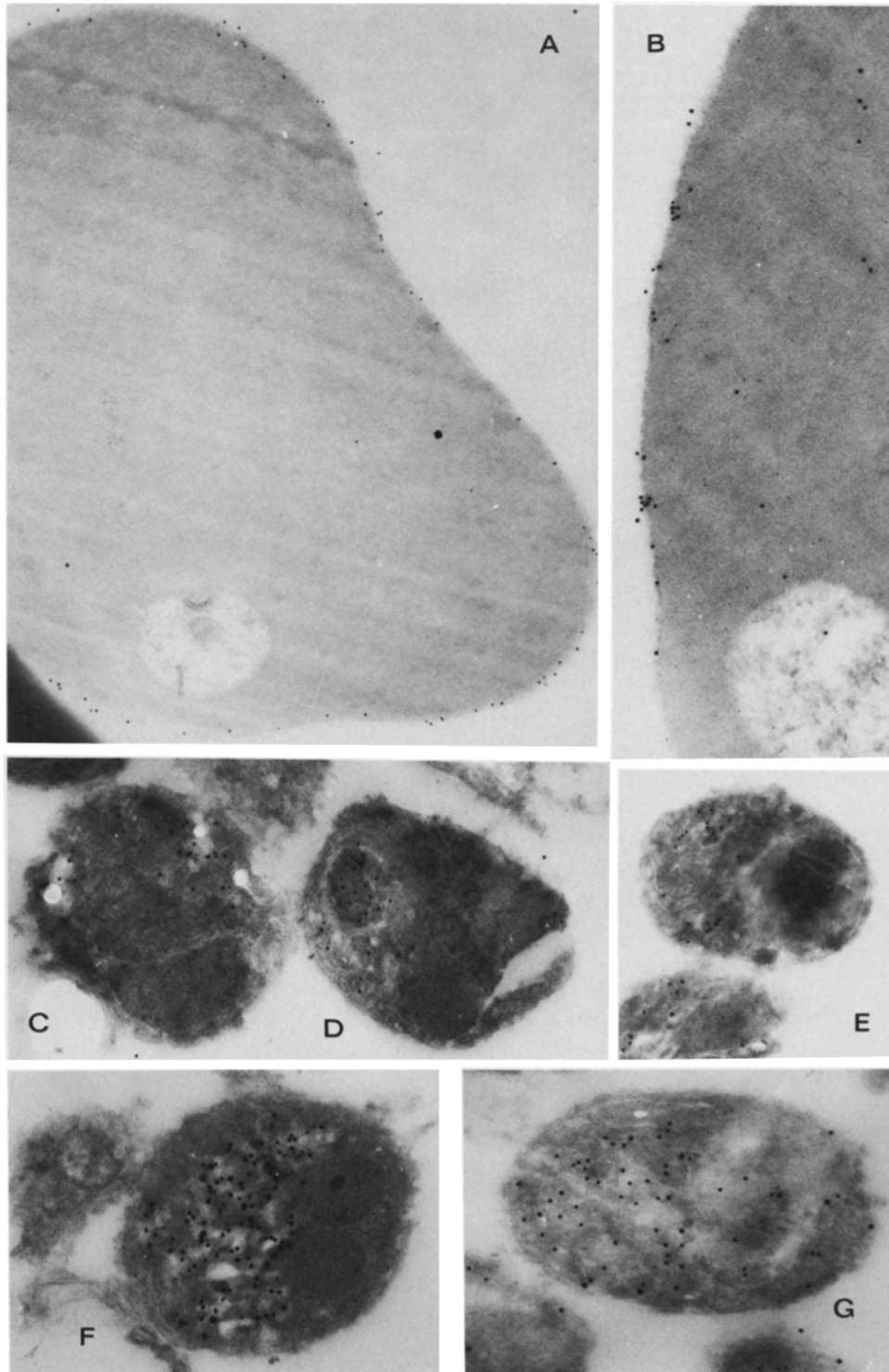


FIGURE 1. Immunoelectron micrographs showing sections of ring-infected erythrocytes reacted first with rabbit anti-RESA (A) or $\sim 2 \mu\text{g/ml}$ of affinity-purified human anti-RESA antibodies (B), then with protein A gold; other sections of merozoites were reacted with rabbit anti-RESA (C-F) or $0.5 \mu\text{g/ml}$ human anti-RESA antibodies (G), and then protein A gold. (A) $\times 35,600$; (B) $\times 56,500$; (C, D) $\times 49,700$; (E) $\times 42,000$; (F) $\times 59,200$; (G) $\times 57,500$.

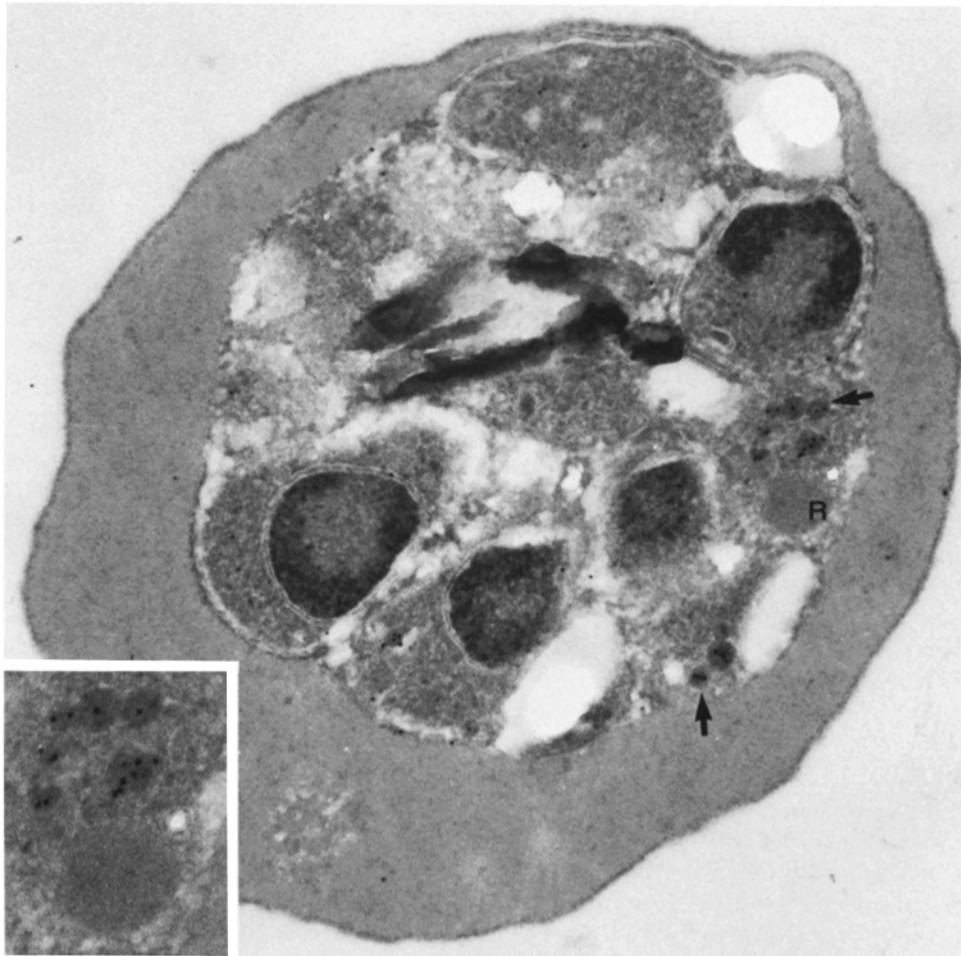


FIGURE 2. Immunoelectron micrograph showing the location of RESA (\rightarrow) in small dense vesicles, presumably micronemes, within the developing merozoites in a schizont, detected with rabbit anti-RESA and protein A gold. The rhoptries (*R*) are unlabeled. $\times 41,700$; inset, $\times 73,000$.

X-100, as was most of the M_r 155,000 polypeptide present in merozoites (Fig. 3*B*). In contrast, the bulk of the M_r 155,000 antigen in rings and other life-cycle stages was insoluble in Triton X-100 but could be solubilized in electrophoresis sample buffer containing SDS and 2-mercaptoethanol (Fig. 3, *B* and *C*).

Discussion

RESA is probably first synthesized in the maturing trophozoite as an M_r 210,000 polypeptide, although it is possibly a crossreacting molecule. RESA is particularly abundant in merozoites, but almost entirely as an M_r 155,000 polypeptide. We did not detect it on the merozoite surface and therefore conclude that RESA is not involved in the initial interactions between merozoite and erythrocyte and is distinct from the *P. falciparum* glycoprotein-binding proteins of about the same size (7). The location of gold particles in small clusters

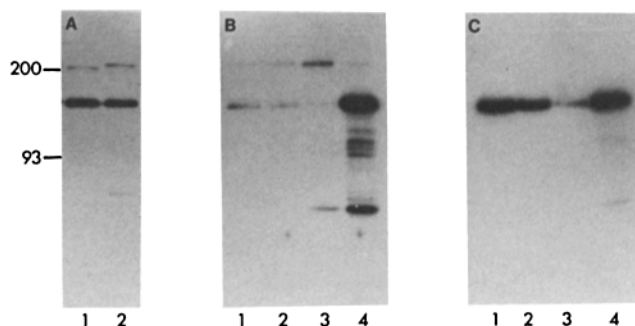


FIGURE 3. (A) Immunoblot of asynchronous cultures of two isolates of *P. falciparum* lysed in electrophoresis sample buffer and probed with anti-RESA antibodies. (B, C) Immunoblots of *P. falciparum* (lane 1) ring stages, (2) mature trophozoites, (3) schizonts, and (4) merozoites, using affinity-purified human antibodies to RESA. (B) Antigens extracted in Triton X-100. (C) Antigens insoluble in Triton X-100 but soluble in electrophoresis sample buffer. Radioactive molecular weight markers (Amersham International, Buckinghamshire, England) were myosin (200,000 mol wt) and phosphorylase b (93,000).

adjacent to rhoptries indicates that RESA may be located within the micronemes, an association most clearly seen in maturing merozoites within the unruptured schizont. The finding of gold on rhoptries in some merozoites suggests that the initial step in the release process may involve transfer of RESA from the micronemes to the rhoptry before release from the apical pore, around the time of merozoite invasion. Presumably, RESA is then transferred to the erythrocyte membrane of the recently invaded cell. Its function is unknown, but the amphipathic nature of its most common constituent repeat sequence (Glu-Glu-Asn-Val) (1) provides a mechanism for dramatic effects on a membrane.

The exact location of RESA at the surface of the invaded erythrocyte is not clear. None of the affinity-purified human antibodies and only one of four antisera produced in rabbits against the cloned antigen reacted with untreated erythrocytes. However, all antisera gave strong fluorescence on ring-infected erythrocytes that had been lightly glutaraldehyde fixed and air dried, consistent with the results of Perlmann et al. (8), who have also described an M_r 155,000 antigen in the membrane of erythrocytes infected with immature parasites.

The persistence of RESA at the surface of the ring-infected cell for at least 24 h after merozoite invasion may indicate a function unrelated to the invasion process. Although RESA in merozoites is largely soluble in Triton X-100 it becomes Triton insoluble when transferred to the erythrocyte membrane. Such characteristics are similar to those of putative cytoadherence molecules (9). This suggests that at least part of RESA is internal to the lipid bilayer and anchored to the membrane cytoskeleton. However, no classical membrane-spanning segment has been found in the portions of the RESA gene that have been sequenced (2).

Summary

Immuno-electron microscopy with protein A gold has been used to determine the subcellular location of the ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum*. RESA was associated with dense vesicles presumed to be micronemes within merozoites. RESA was not detected on the surface of

merozoites but was located at the membrane of erythrocytes infected with ring-stage parasites. RESA within merozoites was largely soluble in the nonionic detergent Triton X-100, but was insoluble in this detergent when associated with the erythrocyte membrane.

We acknowledge the excellent technical assistance of Mary-Lou O'Halloran and Sheevaun Carey. We thank Andrew Abbot for help with electron microscopy and Graham Mitchell for continued support.

Received for publication 23 January 1985 and in revised form 21 May 1985.

References

1. 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 (Lond.)*. 310:789.
2. 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. The RESA polypeptide of *Plasmodium falciparum* contains two separate blocks of tandem repeats encoding antigenic epitopes that are naturally immunogenic in man. *Mol. Biol. Med* In press.
3. Trager, W. T., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science (Wash. DC)*. 193:673.
4. Lambros, C., and J. P. Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65:418.
5. Mrema, J. E. K., S. G. Langreth, R. C. Jost, K. H. Rieckmann, and H. G. Heidrich. 1982. *Plasmodium falciparum*: isolation and purification of spontaneously released merozoites by nylon membrane sieves. *Exp. Parasitol.* 54:285.
6. Stahl, H.-D., P. E. Crewther, R. F. Anders, G. V. Brown, R. L. Coppel, A. E. Bianco, G. F. Mitchell, and D. J. Kemp. 1985. Interspersed blocks of repetitive and charged amino acids in a dominant immunogen of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA*. 82:543.
7. Perkins, M. E. 1984. Surface proteins of *Plasmodium falciparum* merozoites binding to the erythrocyte receptor, glycophorin. *J. Exp. Med.* 160:788.
8. Perlmann, H., K. Berzins, M. Wahlgren, J. Carlsson, A. Bjorkmann, 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.
9. Leech, J. H., J. W. Barnwell, L. H. Miller, and R. J. Howard. 1984. Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. *J. Exp. Med.* 159:1567.