Parasitol Res (1993) 79:579–586

Parasitology Research



# Accessibility and distribution of intraerythrocytic antigens of *Plasmodium*-infected erythrocytes following mild glutaraldehyde fixation and detergent extraction

**Mark F. Wiser [1,2], Lynn V.V. Faur [1], H. Norbert Lanners [2,3], Mary Kelly [2], Russell B. Wilson [2,4]**

[1] Department of Tropical Medicine, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA 70112, USA
[2] Interdisciplinary Program in Molecular and Cell Biology, Tulane University, New Orleans, LA 70112, USA
[3] Tulane Regional Primate Research Center, Covington, LA 70433, USA
[4] Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA 70112, USA

Received: 16 March 1993 / Accepted: 2 June 1993

**Abstract.** Malarial antigens on the surface of infected erythrocytes have been described by many investigators. However, few of these antigens have been unambiguously demonstrated to be exposed on the surface of erythrocytes. This study demonstrates that mild glutaraldehyde fixation results in the cytoplasmic face of the host membrane becoming accessible to antibody under conditions that normally do not expose the cytoplasmic face of uninfected erythrocytes. These results indicate that caution should be used in interpreting data on the membrane disposition of malarial antigens. Detergent extraction of the glutaraldehyde-fixed erythrocytes results in an increased permeabilization such that malarial antigens on the parasite surface and within the cytoplasm of the infected erythrocyte are accessible to antibody. The accessibility of these antigens was demonstrated by both immunofluorescence and two-color flow cytometry. The antigens within the host cytoplasm were not diffuse but associated with patchy aggregates. Analysis of the antigens associated with the cytoplasmic aggregates by immunoelectron microscopy indicated that they were not associated with membrane-bound compartments. The fixation and permeabilization protocol described herein will have useful applications for the characterization and analysis of malarial antigens.

The malarial parasite alters the host erythrocyte. These alterations include changes in the overall metabolism of the erythrocyte along with structural changes in the host erythrocyte membrane and cytoplasm. For example, the erythrocyte membrane of infected cells exhibits an increase in permeability to small-molecular-weight metabolites (Ginsberg 1990), reflecting the needs of an actively growing and replicating parasite. In addition, structural alterations such as knobs, caveolae, vesicles, and clefts are associated with the erythrocyte membrane and cytoplasm during *Plasmodium* infections (Atkinson and Aikawa 1990).

There is an intense interest in identifying parasite antigens expressed on the erythrocyte surface as potential vaccine candidates (Miller et al. 1986). However, the demonstration of malarial antigens on the surface of the erythrocyte has been problematic. One of the few definitive demonstrations of a *Plasmodium* protein expressed on the erythrocyte surface is a strain-specific high-molecular-weight protein, referred to as PfEMP1, that is believed to be involved in cytoadherence (Leech et al. 1984; Howard et al. 1988; Biggs et al. 1992). Most other claims of surface-exposed antigens either are not well documented or are controversial. For example, Pf155/RESA, initially believed to be exposed on the erythrocyte surface (Coppel et al. 1984), is now generally accepted to be localized to the cytoplasmic face of the erythrocyte membrane (Berzins 1991).

The experiments reported herein further emphasize the complications in interpreting data concerned with the localization of *Plasmodium* antigens. A mild glutaraldehyde fixation results in the exposure of the submembrane cytoskeleton in infected erythrocytes, whereas uninfected erythrocytes are for the most part unaffected. Extraction of the fixed erythrocytes with nonionic detergen exposes additional intraerythrocytic antigens of the parasitized cell. The fixation and permeabilization procedures described herein will be useful in the application of techniques such as immunofluorescence and flow cytometry to the study of how the malarial parasite affects and alters the host erythrocyte.

The other aspect of this study concerns a further characterization of malarial antigens associated with cytoplasmic inclusions within the infected erythrocyte (Wiser 1989). Analysis by immunofluorescence clearly demonstrates these antigens within the host cell cytoplasm. However, they are not associated with membrane-bound

*Abbreviations:* Ag, antigen; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; mAb, monoclonal antibody; MSP1, merozoite surface protein 1; PBS, phosphate-buffered saline; PfHRPII, *Plasmodium falciparum* histidine-rich protein II; RESA, ring-stage erythrocyte surface antigen

*Correspondence to:* M. Wiser

clefts or vesicles as indicated by immunoelectron microscopy.

## Materials and methods

### *Parasites*

The general procedures for maintenance of parasites and the isolation of infected erythrocytes have been described previously (Wiser and Schweiger 1985; Wiser et al. 1988). Briefly, mice (CD-1, Charles Rivers) were infected with either *Plasmodium berghei* (Bayer strain) or *P. chabaudi*. Both strains produce a rapidly fulminating infection and neither demonstrates a preference for reticulocytes. Anesthetized mice were exsanguinated following axillary incision and the infected blood was collected with a heparinized Pasteur pipet into a heparinized tube. Blood was passed over a CF-11 powdered cellulose (Whatman) column equilibrated with Hanks' balanced salt solution (HBSS) and then washed four times in HBSS.

### *Antibody reagents*

The monoclonal antibodies (mAbs) used in this study have been described previously. MAb-13.5 recognizes a 93-kDa protein of *P. chabaudi* associated with the host erythrocyte membrane, and mAb-16.3 recognizes a similar 65-kDa protein of *P. berghei* (Wiser et al. 1988). MAb-16.8 recognizes a different epitope on the same 65-kDa *P. berghei* protein, and mAb-17.4 recognizes a 45-kDa *P. berghei* protein associated with the erythrocyte membrane (Wiser et al. 1988). MAb-T4.3, mAb-I2.6, and mAb-W3.5 recognize *P. berghei* antigens with molecular weights of 120, 31, and 13 kDa, respectively, that are associated with inclusion bodies within the host erythrocyte cytoplasm (Wiser 1986). MAb-F4.4 recognizes a 230-kDa antigen, commonly referred to as MSP1, found on the surface of schizonts and merozoites (Wiser 1986). A rabbit polyclonal antisera against spectrin was obtained from Sigma (S1515), and rabbit antisera against ankyrin and band 4.1 were generously supplied by Dr. J. Morrow (Yale University). Culture supernatant medium from unfused myeloma cells was used as a negative control. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rabbit IgG served as the secondary antibodies. Table 1 summarizes the characteristics of the antibodies used in this study and identifies the figures demonstrating the immunofluorescence results.

### *Immunofluorescence*

Infected erythrocytes, isolated and washed as described above, were suspended at 5% hematocrit in HBSS, and glutaraldehyde was added at a final concentration of 0.025%. Following a 10-min incubation (all operations were carried out at room temperature unless noted otherwise), 2 vols. of 0.1 *M* glycine in phosphate-buffered saline (PBS; 20 m*M* phosphate, pH 7.2, containing 0.15 *M* NaCl) were added and followed by another 10-min incubation. The fixed erythrocytes were recovered by centrifugation at 500 *g* for 5 min, washed twice in HBSS containing 1% bovine serum albumin (HBSS-BSA), and resuspended at half the original volume (i.e., approximately 10% hematocrit). In some cases the fixed erythrocytes were extracted with 0.1% Triton X-100 for 20 min.

The fixed erythrocytes were incubated for 1 h with the primary antibodies described above, washed three times in HBSS-BSA, incubated for 1 h with either FITC-conjugated anti-mouse or anti-rabbit IgG, and washed three additional times. If desired, the parasites were counterstained with ethidium bromide (10 μg/ml). The samples were examined for epifluorescence under UV illumination.

### *Flow cytometry*

Infected erythrocytes were fixed, extracted with detergent, incubated with primary and FITC-conjugated secondary antibodies, and counterstained with ethidium bromide in the manner described above for the immunofluorescence samples. The samples were analyzed for FITC and ethidium bromide fluorescence using logarithmic amplifiers on a Coulter Elite flow cytometer equipped with an argon laser (Hialeah, Fla.).

### *Electron microscopy*

*P. berghei*-infected erythrocytes were collected and washed in HBSS as described above. Infected erythrocytes were fixed for 10 min at room temperature (RT) with 1% paraformaldehyde and 0.2% glutaraldehyde in 0.1 *M* sodium phosphate buffer (pH 7.4; Aikawa and Atkinson 1990). Without prior osmication or uranyl acetate treatment, the samples were embedded in London Resin (LR) White, sectioned, and mounted on either gold or nickel grids. To expose more antigenic sites, sections were $H_2O_2$-etched and then blocked for 30 min with PBS containing 5% nonfat dried milk and 0.01% Tween-20 followed by a washing with PBS containing 1% BSA (fraction V) and 0.01% Tween-20. The washed

**Table 1.** Antibodies used in the immunofluorescence studies

| Antibody | Antigen mol. wt. (kDa) | Species | Cellular location | Figure[a] | Reference[b] |
|---|---|---|---|---|---|
| mAb-13.5 | 93 | *Plasmodium chabaudi* | Erythrocyte membrane | 1c, d | Wiser et al. (1988) |
| mAb-16.3 | 65 | *P. berghei* | Erythrocyte membrane | 1a, b | Wiser et al. (1988) |
| mAb-16.8 | 65 | *P. berghei* | Erythrocyte membrane | –[c] | Wiser et al. (1988) |
| mAb-17.4 | 45 | *P. berghei* | Erythrocyte membrane | –[c] | Wiser et al. (1988) |
| mAb-I2.6 | 31 | *P. berghei* | Erythrocyte cytoplasm | 2a–d | Wiser (1986) |
| mAb-W3.5 | 13 | *P. berghei* | Erythrocyte cytoplasm | Not shown[d] | Wiser (1986) |
| mAb-T4.3 | 120 | *P. berghei* | Erythrocyte cytoplasm | –[c] | Wiser (1986) |
| mAb-F4.4 | 230 | *P. berghei* | Parasite membrane | 2e, f | Wiser (1986) |
| Anti-spectrin[e] | 220/240 | Mammalian | Erythrocyte membrane | 1e–h | Sigma |

[a] The figures in this paper demonstrating the immunofluorescence pattern
[b] The reference describing the initial characterization of the mAbs
[c] These mAbs were tested but resulted in no immunofluorescence
[d] The results obtained with mAb-W3.5 are not shown but are qualitatively the same as those obtained with mAb-I2.6
[e] Antibodies against ankyrin and band 4.1 also exhibited the same results as the anti-spectrin antibodies

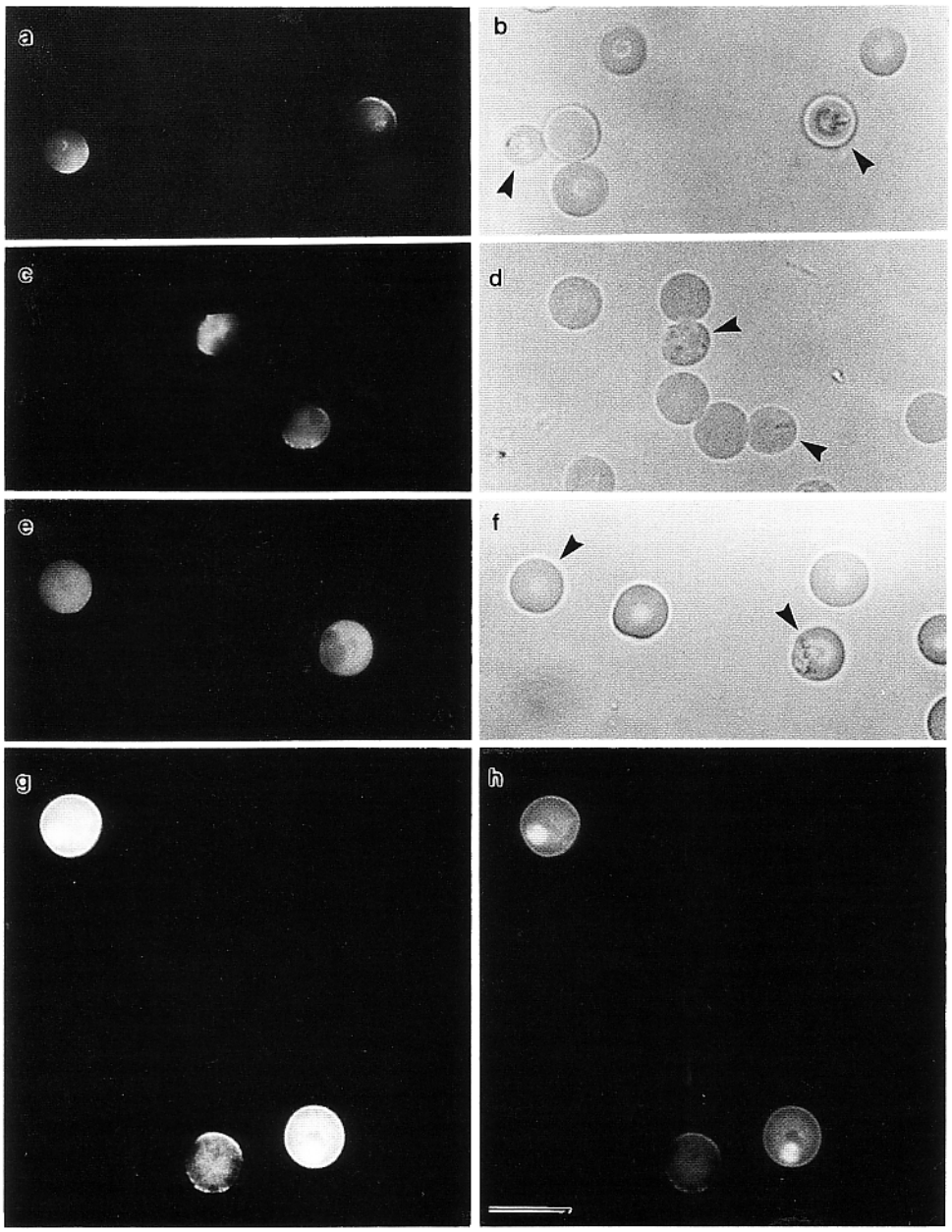


**Fig. 1 a–h.** Erythrocyte membrane immunofluorescence. *Plasmodium berghei*-infected (**a, b, e–h**) or *P. chabaudi*-infected (**c, d**) erythrocytes were fixed with 0.025% glutaraldehyde, incubated with either mAb-16.3 (**a, b**), mAb-13.5 (**c, d**), or anti-spectrin (**e–h**) and analyzed by immunofluorescence as described in Materials and methods (also see Table 1). Shown are the immunofluorescence as viewed under UV illumination (**a, c, e**) and the corresponding fields as viewed under direct light (**b, d, f**). *Arrowheads* indicate the erythrocytes that are fluorescing. In the case of staining with anti-spectrin, it was not always clear that all of the fluorescing cells were infected; therefore, the parasites were counterstained with ethidium bromide (**g, h**) and the pictures were developed such that either the immunofluorescence (green in the original) was most apparent (**g**) or the ethidium bromide staining (orange in the original) was most apparent (**h**). Bar = 10 μm

grids were incubated for 2 h with either mAb-I2.6 or mAb-W3.5 (undiluted conditioned media from hybridoma cultures), thoroughly washed, incubated for 1 h with 5-nm protein A-colloidal gold (Janssen Biotech), and washed again. Supernatant media from unfused myelomas were used as negative controls.

To confirm the results obtained with protein A-gold, immunostaining was repeated using ferritin-conjugated goat anti-mouse IgG (Cappel Laboratories) diluted 1:10 (v/v) with PBS as the secondary antibody. Gold and ferritin markers were stabilized by fixation with 2.5% glutaraldehyde in wash buffer. Thin sections on copper grids were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-1200EX II electron microscope.

The gold particles distributed over the different subcellular fractions were quantitated by counting the numbers of gold particles found over uninfected erythrocytes, the cytoplasm of infected erythrocytes, the parasite, and the area between erythrocytes (i.e., plastic) and dividing these counts by the total square inches making up these four fractions. The area of the fractions was determined by cutting out these subcellular fractions and weighing the photographs as compared with a known area of developed photographic paper.

## Results

MAb-16.3 and mAb-13.5 specifically recognize *P. berghei* and *P. chabaudi* acidic phosphoproteins associated with the erythrocyte membrane (Wiser et al. 1988). Both of these mAbs recognize the erythrocyte surface of infected cells fixed with 0.025% glutaraldehyde as determined by indirect immunofluorescence (Fig. 1 a–d). MAb-16.8 and mAb-17.4 did not react in this assay (Table 1). This fluorescence is not observed if the infected erythrocytes are unfixed or fixed with less than 0.025%

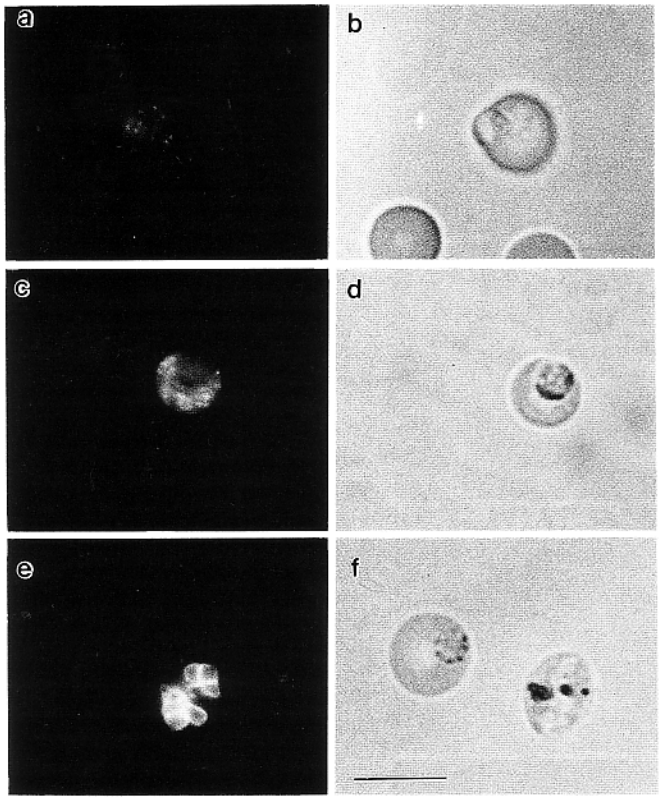


**Fig. 2a–f.** Immunofluorescence following detergent extraction. *P. berghei*-infected erythrocytes were fixed with 0.025% glutaraldehyde and analyzed for indirect immunofluorescence using mAb-I2.6 (**a, b**). Alternatively, the infected erythrocytes were extracted with 0.1% Triton X-100 following glutaraldehyde fixation and analyzed for immunofluorescence with mAb-I2.6 (**c, d**) or mAb-F4.4 (**e, f**). Immunofluorescent images as viewed under UV illumination are shown in the *left-hand panels* and the corresponding images as viewed under direct light are shown on the *right*. Bar = 10 μm

glutaraldehyde. At increasing glutaraldehyde concentrations, detection of FITC fluorescence is greatly diminished due to the increasing levels of glutaraldehyde-induced autofluorescence. At concentrations of glutaraldehyde exceeding 0.25%, no specific FITC fluorescence is detectable above the autofluorescence (data not shown). Paraformaldehyde was also tried as a fixative, but this resulted in lysis of the erythrocytes (data not shown).

The acidic phosphoproteins are localized to the cytoplasmic face of the erythrocyte membrane (Wiser et al. 1988), indicating that the mild glutaraldehyde fixation is making the internal face of the erythrocyte membrane accessible to antibody. To confirm this, antibodies against proteins that are definitely localized exclusively to the cytoplasmic face of the erythrocyte membrane were also tested in this liquid-phase immunofluorescence assay. Antibodies against spectrin (Fig. 1e–h), ankyrin and band 4.1 (data not shown) all result in a strong membrane-associated fluorescence of infected erythrocytes, confirming that the fixation method is exposing the internal face of the erythrocyte membrane. Interestingly, the fluorescence due to anti-spectrin (and anti-ankyrin and anti-band 4.1) antibodies is almost exclusively associated with infected erythrocytes, suggesting that the accessibility to the cytoplasmic face is a result of both the fixation procedure and parasite infection.

However, as confirmed by counterstaining with ethidium bromide, a few uninfected erythrocytes do exhibit a membrane fluorescence with anti-spectrin antibodies. In the data presented (Fig. 1g, h), two of three fluorescing erythrocytes exhibit both a parasite-associated ethidium bromide fluorescence and an erythrocyte membrane-associated FITC fluorescence. The third erythrocyte exhibits only an FITC fluorescence. The number of uninfected erythrocytes that fluoresced varied from experiment to experiment, but generally, 1%–2% of the uninfected erythrocytes fluoresce and the fluorescence associated with uninfected erythrocytes is generally less intense than that of infected erythrocytes as demonstrated in Fig. 1g. All of the infected erythrocytes react with anti-spectrin, and no apparent difference in the intensity of labeling is observed between ring-, trophozoite-, or schizont-infected erythrocytes.

MAb-I2.6, recognizing a 31-kDa *P. berghei* antigen associated with undefined structures within the cytoplasm of the infected erythrocyte (Wiser 1986), was also tested in this liquid-phase immunofluorescence assay. A barely detectable fluorescence is observed with mAb-I2.6 (Fig. 2a, b). Attempts to enhance the permeability of the fixed erythrocytes to antibody were carried out by extracting them in detergent. Triton X-100, saponin, and sodium docecyl sulfate (SDS) were all tested at concentrations ranging from 0.01% to 1.0%. The reactivity of mAb-I2.6 was greatly enhanced by extracting the fixed erythrocytes with all of the detergents, but 0.1% Triton X-100 was optimal (data not shown). Brightly fluorescing aggregates within the cytoplasm of the infected erythrocyte are readily detected after permeabilization of the samples with detergent (Fig. 2c, d). This pattern of fluorescence is similar to that previously reported for acetone-fixed thin blood smears (Wiser 1986). MAb-W3.5, recognizing a 13-kDa antigen, gave results identical to those obtained with mAb-I2.6 (data not shown). MAb-T4.3 did not react in the immunofluorescence assay (Table 1).

Similarly mAb-F4.4, recognizing MSP1 on the surface of schizonts, is reactive only with the glutaraldehyde-fixed erythrocytes after Triton X-100 permeabilization (Fig. 2e, f). The pattern of immunofluorescence is typical for this antigen (Holder 1988), with the surface of the budding merozoites being clearly visible. An immature parasite not expressing MSP1 is also apparent in the same field. Extraction with Triton X-100 also enhances the reactivity of mAb-16.3, but the increase in fluorescence is not nearly as great as in the cases of mAb-I2.6 and mAb-F4.4 (data not shown). Detergent extraction resulted in all erythrocytes fluorescing when anti-spectrin was used (data not shown).

The nature of the aggregates, or inclusion bodies, recognized by mAb-I2.6 and mAb-W3.5 was further investigated by immunoelectron microscopy. The antigen recognized by mAb-I2.6 is localized to the erythrocyte cytoplasm (Fig. 3). Quantitating the numbers of gold

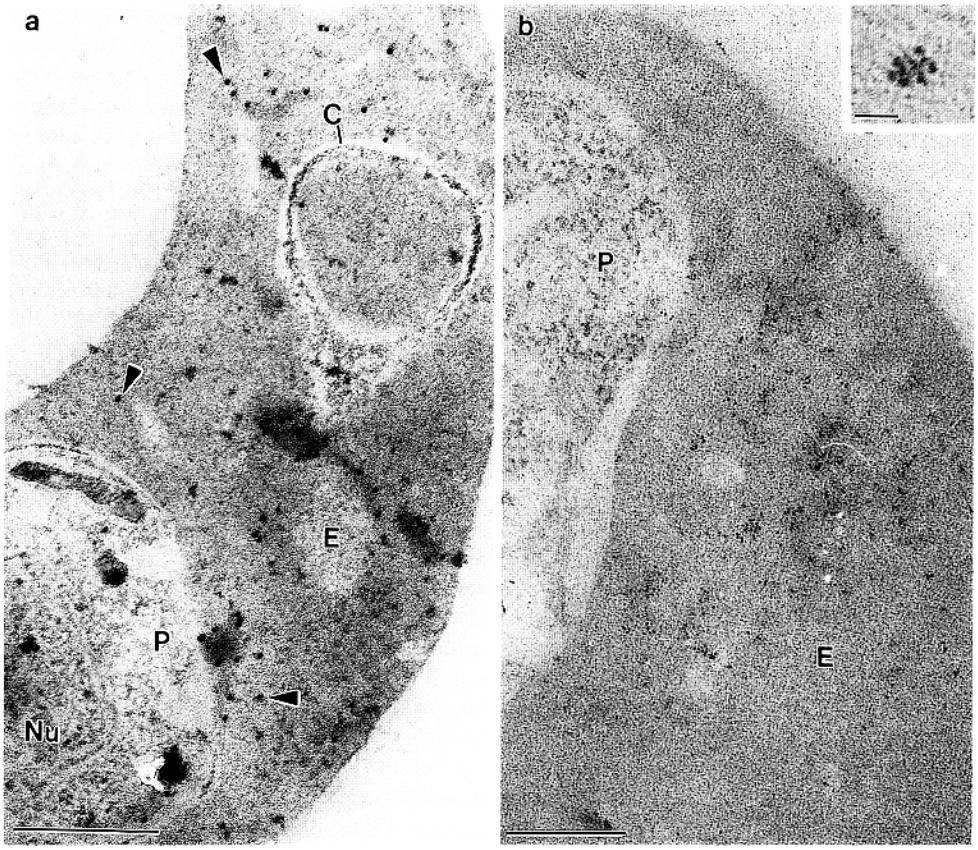


**Fig. 3a, b.** Localization of Ag-I2.6 by immunoelectron microscopy. **a** A *P. berghei*-infected erythrocyte poststained with protein A-gold, demonstrating the distribution of Ag-I2.6. The label (*arrowheads*) is found primarily in the host cell cytoplasm (*E*) and not associated with the parasite (*P*). No label is associated with a membrane-bound cleft (*C*) within the host cytoplasm. *Nu*, Parasite nucleus. Bar = 0.5 μm. **b** A *P. berghei*-infected erythrocyte poststained with ferritin-conjugated anti-mouse IgG, demonstrating the distribution of Ag-I2.6. The tendency for the label to be found in clusters within the erythrocyte cytoplasm (*E*) is more evident in this case than with the protein A-gold label. Bar = 250 nm. *Inset:* Higher magnification of a cluster of ferritin particles. Bar = 25 nm

**Table 2.** Quantitative analysis of the distribution of gold particles

| | Erythrocyte cytoplasm | Parasite | Uninfected erythrocytes | Plastic |
|---|---|---|---|---|
| Total gold particles | 275 | 131 | 19 | 59 |
| Total area | 87 | 73 | 29 | 146 |
| Gold particles per sq. inch | 3.24 | 1.79 | 0.66 | 0.40 |

The numbers of gold particles associated with the erythrocyte cytoplasm of infected cells, with the parasite, and with uninfected erythrocytes as well as those localized between cells (i.e., plastic) were quantitated over defined areas

particles localized over various subcellular compartments confirms that the majority of the label is localized to the erytrhocytic cytoplasm (Table 2). Few gold particles are found associated with uninfected erythrocytes or between cells on the same grid, indicating that the immunolabeling is specific. In addition, virtually no gold particle is associated with either erythrocytes or parasites if no primary antibody is used (data not shown).

In agreement with the immunofluorescence data, the distribution of Ag-I2.6 is not diffuse but appears patchy with areas of higher concentrations of label. This is most evident with the ferritin label, which resulted in a much denser labeling than was achieved with protein A-colloidal gold (Fig. 3b). The antigen is apparently not associated with membrane-bound vesicles or clefts, although membrane-bound clefts are detectable in the cytoplasm of *P. berghei*-infected erythrocytes, as has also previously been noted (Mackenstedt et al. 1989). Similar results were also obtained with mAb-W3.5 (data not shown).

The mild glutaraldehyde fixation and detergent extraction procedure was used for the analysis of infected erythrocytes by two-color flow cytometry (Fig. 4). Uninfected erythrocytes were used to set quadrant values to determine the percentage of infected erythrocytes (ethidium bromide-positive) and the percentage of erythrocytes labeled with mAbs. The results of the two-color flow cytometry indicate that the mAbs react only with infected erythrocytes. Furthermore, the analyses are in agreement with what is known about these antigens in terms of stage of expression and localization. For example, Pb(em)65 (recognized by mAb-16.3) is expressed early in the trophozoite stage (Wiser et al. 1988), whereas

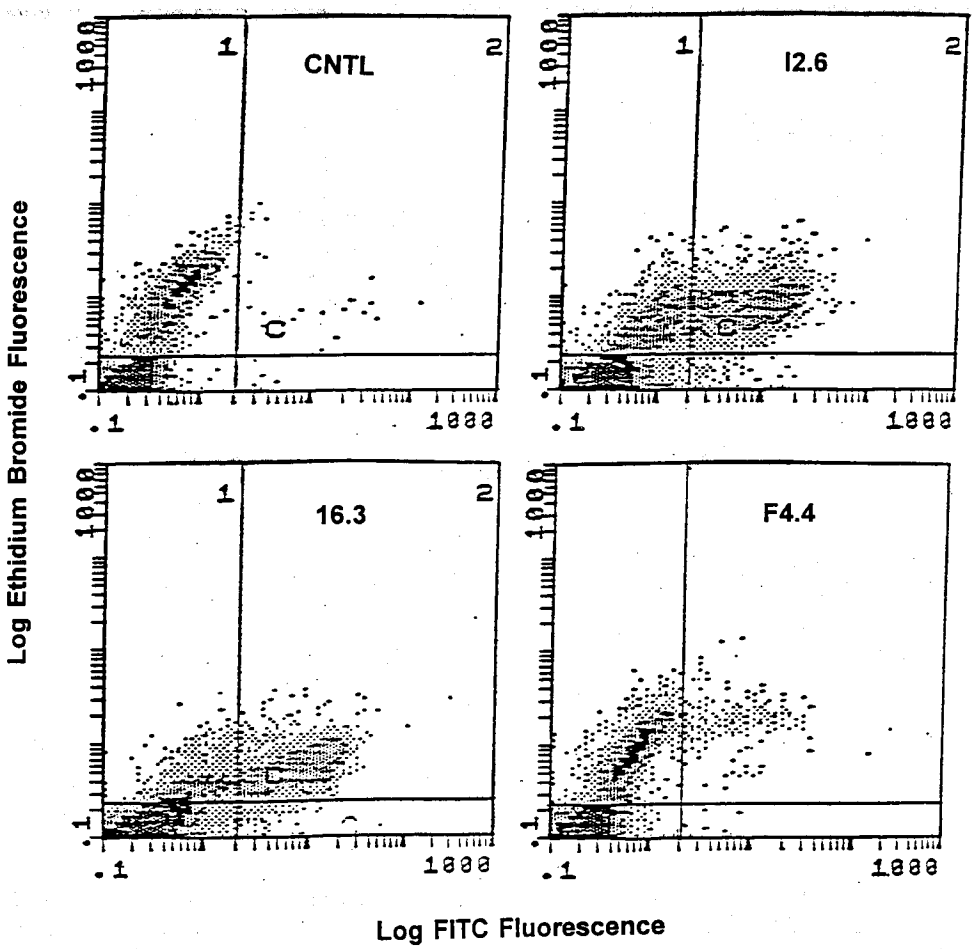


**Fig. 4.** Flow cytometry analysis. *P. berghei*-infected erythrocytes were fixed with 0.025% glutaraldehyde and extracted with 0.1% Triton X-100. The samples were treated with either no primary antibody (*CNTL*). mAb-16.3, mAb-I2.6, or mAb-F4.4 as indicated, followed by an incubation with FITC-conjugated anti-mouse IgG. Parasites were stained with 10 μg ethidium bromide/ml and analyzed for fluorescence as described in Materials and methods. Shown are scatter plots with the FITC fluorescence on the x-axis and the ethidium bromide fluorescence on the y-axis. Uninfected erythrocytes were used to set boundaries for positive ethidium bromide fluorescence (*quadrants 1, 2*) and FITC staining (*quadrants 2, 4*) as indicated by the *horizontal and vertical lines*, respectively, on each scatter plot. The dots in quadrants 1 (*upper left*) represent infected erythrocytes that are negative for antibody staining, and those in quadrant 2 (*upper right*) represent infected erythrocytes that are positive for antibody staining

Ag-I2.6 and Ag-W3.5 are expressed later in the trophozoite stage (Wiser 1989) and MSP1 (recognized by Mab-F4.4) is expressed in the schizont stage (Holder 1988). The percentage of infected cells that label with mAb-16.3, mAb-I2.6, and mAb-F4.4 is 62%, 53%, and 20%, respectively. The lower than expected value for mAb-16.3 may be due to the observation that Pb(em)65 is not as abundant as the other proteins and many double-positive cells may be missed due to the FITC fluorescence intensity being below the cutoff value. mAb-W3.5 exhibited a pattern very similar to that shown for mAb-I2.6, with 51% of the infected erythrocytes also being positive for the FITC label (data not shown ). MAb-T4.3 exhibited the same pattern as the negative control (data not shown).

## Discussion

The results of this study demonstrate that mild glutaraldehyde fixation exposes the cytoplasmic face of the erythrocyte membrane of infected cells but, in general, not that of uninfected erythrocytes. Interestingly, all of the infected erythrocytes and only 1%–2% of the uninfected erythrocytes fluoresce in the immunofluorescence experiments using anti-spectrin. These results further emphasize that the erythrocyte membrane of infected cells is different from that of uninfected cells. It is not clear why only a portion of the uninfected erythrocytes fluoresce when antibodies against spectrin and other erythrocyte membrane proteins are used. One possibility is that the fluorescene is related to the age of the erythrocyte. In this regard, Winograd et al. (1987) report that infection with *Plasmodium falciparum* results in the expression of senescence-related antigens on the surface of erythrocytes, and Turrini et al. (1992) have suggested that *P. falciparum* infection modifies erythrocytes in a fashion similar to that of senescence or oxidant stress.

In addition, the results of this study should serve as a caveat about claims concerning the erythrocyte surface location of antigens following fixation. For example, Baruch et al. (1989, 1991) report that modulation of the lipid composition of the lipid bilayer will expose otherwise cryptic antigens. However, their samples were fixed with 0.25% glutaraldehyde before analysis. The results obtained in the present study with antibodies against the acidic phosphoproteins and erythrocyte-membrane skeletal proteins demonstrate that glutaraldehyde fixation exposes the cytoplasmic face of the membrane. A mAb against spectrin was used as a control for the exposure of the cytoplasmic face in the previous study (Baruch and Cabantchik 1989), but no reactivity was de-

tected. One possible explanation for this lack of reactivity is that the epitope recognized by this particular antispectrin mAb is sensitive to glutaraldehyde fixation. Alternatively, the differences between the study reported herein and the previous investigation (Baruch and Cabantchik 1989) could be explained by mouse erythrocytes behaving differently from human erythrocytes during the fixation procedure. Regardless of the explanation, extreme care should be taken in the interpretation of data describing parasite antigens on the erythrocyte surface.

Problems with other methods for the identification of surface antigens such as iodination with lactoperoxidase have also been reported. For example, despite its cytoplasmic disposition, Pf155/RESA (Berzins 1991) has been heavily labelled with $^{125}I$ by the lactoperoxidase method (Saul et al. 1988). Infected erythrocytes tend to be more fragile and the amount of radioisotope incorporated into hemoglobin and spectrin needs to be carefully monitored (Howard et al. 1982). In addition, the concentration of erythrocytes, iodine, and other components can also result in labeling of intraerythrocytic proteins (Howard et al. 1979). In regard to this report, the 93-kDa *P. chabaudi* protein is not iodinated with lactoperoxidase (Wunderlich et al. 1988). Treatment of intact erythrocytes with proteases is another method to identify surface proteins. However, Vernot-Hernandez and Heidrich (1985) have observed that treatment of intact infected erythrocytes results in some loss of spectrin along with a 92-kDa *P. falciparum* protein associated with knob formation. In summary, multiple methods and adequate controls are necessary before malarial antigens can be unambiguously localized to the surface of the infected erythrocyte.

The fixation/permeabilization protocol described herein is useful for the analysis and characterization of malarial antigens by immunofluorescence and flow cytometry. The use of 0.025% glutaraldehyde eliminates the autofluorescence that interferes with immunofluorescence and flow cytometry analyses. In addition, the ability to carry out the immunofluorescence in the fluid phase as opposed to thin smears fixed on microsocpe slides has some advantages. For example, in the cases of Ag-I2.6 and Ag-W3.5, the ability to view the immunofluorescence patterns in three dimensions confirmed the earlier conclusions that these antigens were associated with inclusions within the erythrocyte cytoplasm (Wiser 1986). One cannot unambiguously conclude that these antigens are not associated with the erythrocyte membrane solely on the basis of the immunofluorescence patterns previously reported for acetone-fixed thin smears (Wiser 1986). Similar patterns of immunofluorescence have in the past been attributed to malarial antigens associated with the host erythrocyte membrane (Koenen et al. 1984), only to be shown later to be associated with cytoplasmic granules adjacent to the parasitophorous vacuolar membrane (Scherf et al. 1992). Similarly, the immunofluorescence obtained with mAb-F4.4 in this fluid-phase assay gives a clearer picture of the three-dimensional morphology of the segmenting schizont than does the use of acetone-fixed thin smears (Wiser 1986).

The nature of the structures that are revealed by mAbs-I2.6 and -W3.5 is not completely understood at this time. Immunofluorescence indicates that the antigens are associated with aggregates within the cytoplasm of the host erythrocyte, and electron microscopy indicates that they are not associated with membrane-bound vesicles or clefts. Ag-I2.6 and Ag-W3.5 exhibit a strong surface hydrophobicity as evidenced by the inability to elute them from hydrophobic columns (Wiser, unpublished results). One speculation is that the surface hydrophobicity of these proteins promotes an aggregation and assembly into these undefined structures within the host cytoplasm. The acidic phosphoproteins have recently been proposed to be secreted from the parasite as soluble proteins (Wiser and Lanners 1992). It is possible that Ag-I2.6 and Ag-W3.5 are also secreted from the parasite as soluble proteins and then assemble into the non-membrane-bound aggregates.

PfHRPII has also been described to be localized to non-membrane-bound aggregates within the host cytoplasm (Howard et al. 1986). The function of PfHRPII is unknown at this time, but the protein is secreted from the infected erythrocyte. The aggregate structures to which PfHRPII localizes have been proposed to be involved in the extraparasite transport of this protein through the host cytoplasm. Ag-I2.6 and Ag-W.35 are also released from the infected erythrocyte into the culture medium (Wiser 1989) and may also be transported through the host cytoplasm by these aggregate structures. These non-membrane-bound structures probably represent a different compartment and, possibly, a different extraparasite transport mechanism than do the vesicles and membrane-bound clefts (Stenzel and Kara 1989; Barnwell 1990). In agreement with this, Gormley et al. (1992) have recently demonstrated that several *P. falciparum* antigens are associated with protein aggregates within the host cytoplasm but are definitely not associated with lipids.

In addition to providing a more qualitative analysis of malarial antigens in terms of immunofluorescene, the fixation/permeabilization protocol can also be used in quantitative analyses such as two-color flow cytometry. These studies extend those of Pattanapanyasat et al. (1992), in which antibodies against decay-accelerating factor were used to show that two-color analysis of *Plasmodium*-infected erythrocytes is possible. In addition, permeabilization of the infected erythrocytes with low concentrations of detergent allows for the detection and characterization of other malarial antigens besides those localized to the erythrocyte membrane.

*Acknowledgements.* These experiments were supported in part by Biomedical Research Support Grant Funds from Tulane University and from the Louisiana Board of Regents through the Louisiana Education Quality Support Fund, contract number LEQSF(1990-91)-RD-A-23 (to M.F.W.). The electron microscopy segment of this work was supported by NIH grants RR 00164, Division of Research Resources, and R22 AI26189, NIAID (to H.N.L.). The skillful technical help of Maryjane Dodd with the electron microscopy and of Ed Benes with the flow cytometry is also gratefully acknowledged.

## References


Aikawa M, Atkinson CT (1990) Immunoelectron microscopy of parasites. Adv Parasitol 29:151–214

Atkinson CT, Aikawa M (1990) Ultrastructure of malaria-infected erythrocytes. Blood Cells 16:351–368

Barnwell JW (1990) Vesicle-mediated transport of membrane and proteins in malaria-infected erythrocytes. Blood Cells 16:379–395

Baruch D, Cabantchik ZI (1989) Passive modulation of antigenic expression in the surface of normal and malaria-infected erythrocytes. Mol Biochem Parasitol 36:127–138

Baruch D, Glickstein H, Cabantchik ZI (1991) *Plasmodium falciparum*: modulation of surface antigenic expression of infected erythrocytes as revealed by cell fluorescence ELISA. Exp Parasitol 73:440–450

Berzins K (1991) Pf155/RESA is not a surface antigen of *Plasmodium falciparum*-infected erythrocytes. Parasitol Today 7:193–194

Biggs BA, Anders RF, Dillon HE, Davern KM, Martin M, Petersen C, Brown GV (1992) Adherence of infected erythrocytes to venular endothelium selects for antigenic variants of *Plasmodium falciparum*. J Immunol 149:2047–2054

Coppel RL, Cowman AF, Anders RF, Bianco AE, Saint RB, Lingelbach KR, Kemp DJ, Brown GV (1984) Immune sera recognize on erythrocytes a *Plasmodium falciparum* antigen composed of repeated amino acid sequences. Nature 310:789–792

Ginsberg H (1990) Alterations caused by the intraerythrocytic malaria parasite in the permeability of its host cell membrane. Comp Biochem Physiol [A] 95:31–39

Gormley JA, Howard RJ, Taraschi TF (1992) Trafficking of malarial proteins to the host cell cytoplasm and erythrocyte surface membrane involves multiple pathways. J Cell Biol 119:1481–1495

Holder AA (1988) The precursor to major merozoite surface antigens: structure and role in immunity. Prog Allergy 41:72–97

Howard RJ, Smith PM, Mitchell GF (1979) Optimal conditions for lactoperoxidase catalyzed radioiodination of external proteins on mouse erythrocytes. Aust J Exp Biol Med Sci 57:355–368

Howard RJ, Barnwell JW, Kao V, Daniel WA, Aley SB (1982) Radioiodination of new protein antigens on the surface of *Plasmodium knowlesi* schizont-infected erythrocytes. Mol Biochem Parasitol 6:343–367

Howard RJ, Uni S, Aikawa A, Aley SB, Leech JH, Lew AM, Wellems TE, Rener J, Taylor DW (1986) Secretion of a malarial histidine-rich protein (PfHRPII) from *Plasmodium falciparum*-infected erythrocytes. J Cell Biol 103:1269–1277

Howard RJ, Barnwell JW, Rock EP, Neequaye J, Ofori-Adjei D, Maloy WL, Lyon JA, Saul A (1988) Two approximately 300 kilodalton *Plasmodium falciparum* proteins at the surface membrane of infected erythrocytes. Mol Biochem Parasitol 27:207–224

Koenen M, Scherf A, Mercereau O, Langsley G, Sibilli L, Dubois P, Silva LP da, Müller-Hill B (1984) Human antisera detect a *Plasmodium falciparum* genomic clone encoding a nonapeptide repeat. Nature 311:382–385

Leech JH, Barnwell JW, Miller LH, Howard RJ (1984) Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. J Exp Med 159:1567–1575

Mackenstedt U, Brockelman CR, Mehlhorn H, Raether W (1989) Comparative morphology of human and animal malaria parasites. 1. Host-parasite interface. Parasitol Res 75:528–535

Miller LH, Howard RJ, Carter R, Good M, Nussenzweig RS, Nussenzweig V (1986) Research towards malarial vaccines. Science 284:1349–1356

Pattanapanyasat K, Webster HK, Udomsangpetch R, Wanachiwanawin W, Yongvanitchit K (1992) Flow cytometric two-color staining technique for simultaneous determination of human erythrocyte membrane antigen and intracellular DNA. Cytometry 13:182–187

Pattanapanyasat K, Udomsangpetch R, Webster HK (1993) Two-color flow cytometric analysis of intraerythrocytic malaria parasite DNA and surface membrane-associated antigen in erythrocytes infected with *Plasmodium falciparum*. Cytometry 14:449–454

Saul A, Maloy WL, Rock EP, Howard RJ (1988) A portion of the Pf155/RESA antigen of *Plasmodium falciparum* is accessible on the surface of infected erythrocytes. Immunol Cell Biol 66:269–276

Scherf A, Carter R, Petersen C, Alano P, Nelson R, Aikawa M, Mattei D, Silva LP da, Leech J (1992) Gene activation of Pf11-1 of *Plasmodium falciparum* by chromosome breakage and healing: identification of a gametocyte-specific protein with a potential role in gametogenesis. EMBO J 11:2293–2301

Stenzel DJ, Kara UAK (1989) Sorting of malarial antigens into vesicular compartments within the host cell cytoplasm as demonstrated by immunoelectron microscopy. Eur J Cell Biol 49:311–318

Turrini T, Ginsburg H, Bussolino F, Pescarmona GP, Serra MV, Arese P (1992) Phagocytosis of *Plasmodium falciparum*-infected human red blood cells by human monocytes: involvement of immune and non-immune determinants and dependence on parasite developmental stage. Blood 80:801–808

Vernot-Hernandez JP, Heidrich HG (1985) The relationship to knobs of the 92,000 D protein specific for knobby strains of *Plasmodium falciparum*. Z Parasitenkd 71:41–51

Winograd E, Greenan JRT, Sherman IW (1987) Expression of senescent antigen on erythrocytes infected with a knobby variant of the human malaria parasite *Plasmodium falciparum*. Proc Natl Acad Sci USA 84:1931–1935

Wiser MF (1986) Characterization of monoclonal antibodies directed against erythrocytic stage antigens of *Plasmodium berghei*. Eur J Cell Biol 42:45–51

Wiser MF (1989) *Plasmodium* antigens external to the parasite but within the infected erythrocyte. Parasitol Res 75:206–211

Wiser MF, Lanners HN (1992) Rapid transport of the acidic phosphoproteins of *Plasmodium berghei* and *P. chabaudi* from the intraerythrocytic parasite to the host membrane using a miniaturized fractionation procedure. Parasitol Res 78:193–200

Wiser MF, Schweiger HG (1985) Cytosolic protein kinase activity associated with the maturation of the malaria parasite *Plasmodium berghei*. Mol Biochem Parasitol 17:179–189

Wiser MF, Leible MB, Plitt B (1988) Acidic phosphoproteins associated with the host erythrocyte membrane of erythrocytes infected with *Plasmodium berghei* and *P. chabaudi*. Mol Biochem Parasitol 27:11–22

Wunderlich F, Helwig M, Schillinger G, Speth V, Wiser MF (1988) Expression of the parasite protein Pc90 in plasma membranes of erythrocytes infected with *Plasmodium chabaudi*. Eur J Cell Biol 47:157–164


### Note added in proof

Pattanapanyasat et al. (1993) have recently described two-color flow cytometry of *Plasmodium falciparum*-infected erythrocytes following fixation with 0.025% glutaraldehyde and permeabilization with saponin.