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Rosetting *Plasmodium falciparum*-Infected Erythrocytes Express Unique Strain-Specific Antigens on Their Surface

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Spontaneous binding of uninfected erythrocytes to *Plasmodium falciparum*-infected erythrocytes (rosetting) has been suggested to have a critical role in the induction of cerebral malaria. We report here that rosetting can be mediated by several molecular mechanisms involving parasite polypeptides with M_rs of 22,000 or 28,000, termed rosettins. Antibodies to either polypeptide disrupt rosettes in a strain-specific fashion. Rosettes of five of the seven isolates examined thus far are more easily disrupted by anti-22,000-M_r rosettin antibodies than by anti-28,000-M_r rosettin antibodies. Polyclonal anti-22,000-M_r rosettin antibodies raised in mice or rabbits strongly and strain specifically stain the surface of nonfixed erythrocytes infected with late asexual stages of rosetting *P. falciparum*. Simultaneous antibody staining and rosetting are seen when the anti-22,000-M_r rosettin antiserum is diluted so that only partial disruption of rosettes is obtained, confirming that the fluorescence-labelled infected erythrocytes are involved in rosetting. The 22,000-M_r rosettin is accessible for surface iodination on erythrocytes infected with strains of rosetting parasites sensitive to anti-22,000-M_r rosettin antibodies, whereas no labelling occurred on either normal erythrocytes or nonrosetting-*P. falciparum*-infected erythrocytes. Purified anti-22,000-M_r rosettin serum immunoglobulin G immunoprecipitated three parasite-derived polypeptides with M_rs of 22,000, 45,000 (doublet), and 50,000 from lysates of [^{35}S]methionine-labelled, parasite-infected erythrocytes. Our results suggest that rosetting is mediated by strain-specific, antigenically distinct, *P. falciparum*-derived polypeptides.

Erythrocytes infected with mature asexual stages of the human malaria-causing parasite *Plasmodium falciparum* do not circulate but are selectively bound in capillaries and venules. This erythrocyte sequestration is believed to be due to adhesion of infected erythrocytes to the vascular endothelium (21, 22, 29, 32) and to rosetting, i.e., the binding of uninfected erythrocytes to infected erythrocytes (6, 10, 33, 34). It may represent a selective advantage for the parasite, which thereby escapes splenic clearance, but can also cause serious pathological consequences for the host. Thus, excessive localization of both normal and parasitized erythrocytes in the brain, the lungs, the liver, or the kidneys is correlated with severe clinical symptoms from the affected organ due to obstruction of the local blood flow and impaired oxygen delivery (1, 3, 9, 13, 17, 24–26).

The relative importance of rosetting and/or endothelial binding in inducing the pathology of the disease remains largely unknown, but the obstruction of the blood flow in ex vivo vessels was much more pronounced with rosetting and cytoadherent parasites than with parasites that were cytoadherent only (14). Moreover, the forces holding rosettes together are strong and are approximately five times greater than that binding parasitized cells to the vascular endothelium (23). Rosetting has also been suggested to play an important role in inducing cerebral malaria because *P. falciparum* isolates obtained from unconscious patients all formed rosettes and/or expressed a significantly higher mean rosetting rate than isolates from patients with uncomplicated malaria (4, 11, 30). Furthermore, sera from children with cerebral malaria are devoid of antirosetting activity, whereas sera from patients with a mild form of the disease carry high levels of antirosetting antibodies (4, 30). These findings, taken together with those mentioned above, support the hypothesis that erythrocyte rosetting contributes to the pathogenesis of cerebral malaria and suggest that antirosetting antibodies protect against cerebral disease.

We have previously observed that the rosettes of a cloned strain of *P. falciparum* (PAR$^+$1) can be disrupted by antibodies which react with a 28,000-M_r, parasite-derived polypeptide in immunoprecipitation and weakly stain a 90,000-M_r polypeptide in immunoblotting (5). However, rosettes of some but not all wild Gambian isolates are disrupted by the antibodies (4). We describe here the presence of a novel strain-specific rosette-associated polypeptide with a M_r of 22,000 which is strongly labelled on the surface of live infected erythrocytes by radioiodination and specific antibodies.

MATERIALS AND METHODS

Parasites. *P. falciparum* parasites were cultured in vitro at a hematocrit of 5% according to the method of Trager and Jensen in human group O$^+$ erythrocytes (28). Rosettes were enriched on Ficoll-Paque according to the method of Udomsangpetch et al. (33). The following strains were used. The rosetting parasites were Ugandan strain Palo Alto (clone PAR$^+$1, 80% rosettes) and Brazilian strain ITO4 (clone R29, 45% rosettes), which had been kept in continuous culture for various periods of time. Cultures of isolates TM180 (80% rosettes), TM284 (80% rosettes), TM280 (50% rosettes), and TM177 (30% rosettes) were established from malaria patients infected in Thailand, whereas the isolate BR1 (50% rosettes) was from a malaria patient infected in Burma (isolates kindly provided by S. Thaitong, Bangkok, Thailand). The IPC/RAY isolate (75% rosettes) was fresh and

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cultivated directly from the blood of a squirrel monkey. The nonrosetting parasites were Brazilian strain IMTM22 (clone 7G8) and Gambian strain FCR-3, which had been propagated extensively in vitro.

Extraction of *P. falciparum* RNA and Northern (RNA) blotting. For extraction of RNA, parasites were harvested from asynchronous cultures when parasitemias were between 10 and 20%, at about 12 to 15 h after merozoite invasion. Cytoplasmic RNA was extracted by the method described by Maniatis et al. (18). Total RNA was electrophoresed in a 1% formaldehyde–agarose gel, transferred to a nylon membrane, and hybridized according to standard procedures with an oligonucleotide probe (5'GAAACCTC CACCTGGTCTTGCTTCCCTG3') complementary to the coding strand of the *P. falciparum* HRP1 gene (7). The filter was washed for 2 h in 0.5× SSC (75 mM NaCl, 7.5 mM Na citrate) at 42°C prior to autoradiography.

Immunization of mice and rabbits. To raise anti-22,000-M_r polypeptide sera, the animals were immunized with a small gel slice excised from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel carrying the 22,000-M_r polypeptide that was allowed to rehydrate in phosphate-buffered saline (PBS) followed by homogenization to form a smooth suspension. The mixture was incorporated into Freund's complete adjuvant and injected intramuscularly into New Zealand White rabbits or intraperitoneally and subcutaneously into BALB/c mice. The animals were boosted after three weeks with antigen in Freund's incomplete adjuvant and bled one week later. Immunoglobulin G (IgG) was prepared from the sera by purification on protein A-Sepharose (Pharmacia, Uppsala, Sweden).

Reversal of rosette formation. Studies were performed on cultures with a hematocrit of 5% and 5 to 10% parasitemia at the late trophozoite stage. Aliquots (20 μl each) of the parasite culture were mixed with 20-μl dilutions of the mouse or rabbit antibody to give a final dilution of 1:2 to 1:20, and the mixtures were incubated in a 96-well microtiter plate (Flow, McLean, Va.), covered with a lid, at 37°C for 60 min prior to assessment of rosetting. The rosetting rate of each well was compared with that of a preimmune serum control and a medium control.

Indirect fluorescence on live infected erythrocytes. Aliquots (50 μl) of rosetting *P. falciparum*-infected erythrocytes (5% hematocrit) were washed twice in PBS and incubated with 50 μl of rabbit serum (diluted to give a final dilution of 1/10 in PBS) at room temperature for 1 h, followed by incubation with biotinylated goat anti-rabbit IgG and subsequently fluorescein isothiocyanate-avidin for 30 min (Vector Laboratories, Burlingame, Calif.). The erythrocytes were washed three times in PBS between each incubation. The suspended, live infected erythrocytes were counterstained with ethidium bromide, and the fluorescence was examined directly by microscopy in incident UV light (Leitz Laborlux).

Identification of surface-radioiodinated proteins of infected erythrocytes. *P. falciparum* parasites were surface iodinated with ^{125}I by the lactoperoxidase method (16) using late-trophozoite-stage-infected cultures at a parasitemia of about 20 to 25%. Occasionally, under optimal culture conditions, parasitemias could reach up to 70 to 75% without any enrichment step of the infected erythrocyte. The labelled cultures were extracted in 1% Triton X-100 containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1% saturated ε-amino-*n*-caproic acid) at 4°C for 1 h. A non-Triton X-100-soluble pellet was obtained by centrifugation of the culture in an Eppendorf centrifuge at maximum speed for 15 min and removal of the Triton X-100-soluble supernatant. The insoluble pellet was extracted in 2% SDS with protease inhibitors and separated on a 7.5 to 17.5% linear gradient SDS-PAGE gel by the method of Laemmli (15). Gels containing ^{125}I-labelled material were analyzed directly by autoradiography.

Metabolic labelling and immunoprecipitation of *P. falciparum* polypeptides. Metabolic labelling was performed with [^{35}S]L-methionine (Amersham, Buckinghamshire, United Kingdom) (100 μCi/ml of medium; 1 mCi = 37 MBq) added to cultures at the early ring stage (parasitemia, 25%) in methionine-depleted medium, in which the parasites were allowed to develop until the late trophozoite stage (28 to 36 h). Either the parasites were extracted with Triton X-100, after which the insoluble pellets were reextracted in 2% SDS, or they were extracted directly in 2% SDS–1% Triton X-100. All extraction solutions contained 100 μg of leupeptin and 100 μg of chymostatin per ml (Sigma Chemical Co., St. Louis, Mo.) as protease inhibitors. Immunoprecipitations were performed as follows. Antibody (50 μg) was incubated for 1 h with 100 μl of extract, 400 μl of NETT buffer (150 mM NaCl, 5 mM disodium EDTA, 50 mM Tris-base, 0.02% Na azide, 0.5% Triton X-100, pH 7.4), and 1% high-quality bovine serum albumin (Sigma). Seventy-five microliters of a 50% suspension of protein A-Sepharose beads in NETT buffer was added to each immunoprecipitation solution, and the mixture was incubated for 1 h at room temperature. After several washings of the beads with NETT buffer, the beads were boiled for 2 min in the presence of sample buffer containing 5% SDS and 5% 2-mercaptoethanol. The extracts and the immunoprecipitates were separated by SDS-PAGE as described above. Gels containing ^{35}S-labelled material were treated with Amplify (Amersham) prior to fluorography.

RESULTS AND DISCUSSION

We have previously reported that antibodies to the *P. falciparum* histidine-rich protein 1 (HRP1) (M_r, 85,000 to 105,000), an antigen associated with knoblike structures on the surface of *P. falciparum*-infected erythrocytes, disrupt rosettes formed by a cloned knobless parasite (PAR^+1 [5, 33]) and immunoprecipitate a 28,000-M_r antigen from the same parasite (5). Precipitation of only a single 28,000-M_r polypeptide from the rosetting strain PAR^+1 (5) may be due to the lack of knobs on the surface of this parasite because the transcript for the *P. falciparum* HRP1 gene is undetectable (PAR^+1) (Fig. 1A, lane 3). The 28,000-M_r rosettin therefore seems to be the target of the apparently cross-reacting *P. falciparum* HRP1 antibodies. Further evidence that the 28,000-M_r antigen is a rosettin is its absence in nonrosetting strains (data not shown). The 28,000-M_r rosettin can be metabolically labelled with [^3H]histidine (5) but proved to be difficult to detect by indirect immunofluorescence or by surface iodination labelling techniques on live rosetting infected erythrocytes (5) (Fig. 1B, lane 5). This inability to label may be due to low expression of the molecule on the surface of the infected erythrocytes.

Rosettes formed by parasites insensitive to the anti-28,000-M_r polypeptide antibodies were enriched to identify alternative rosetting mechanisms. Cultures of highly rosetting infected erythrocytes (80 to 95% rosettes, 20 to 75% parasitemia) were radioiodinated by the lactoperoxidase technique, and the extracted polypeptides were separated by SDS-PAGE. As is shown in Fig. 1B (lanes 1 and 2), the autoradiography revealed a strongly labelled polypeptide

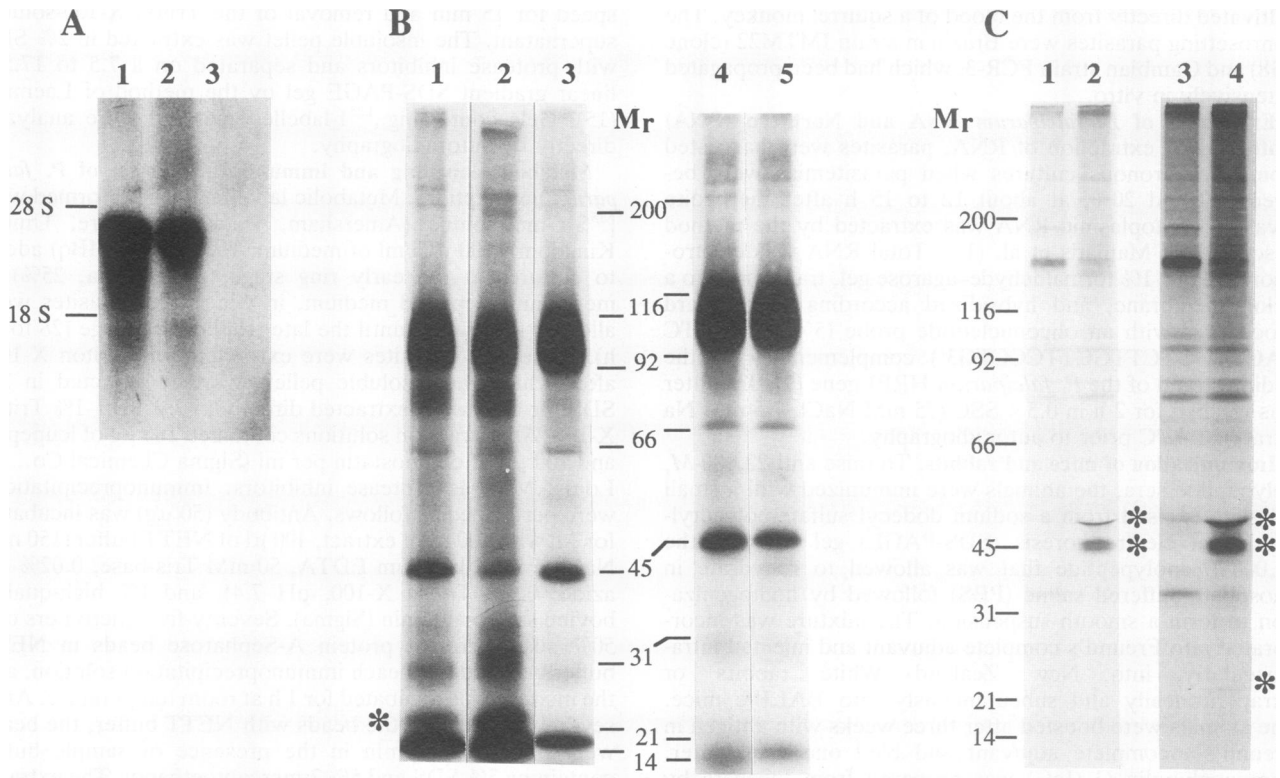

FIG. 1. (A) Autoradiogram of Northern blot analysis of *P. falciparum* HRP1 gene expression. Four micrograms of total RNA of *P. falciparum* 7G8 (K^+R^-) (lane 1), FCR-3 (K^+R^-) (lane 2), and PAR^+1 (K^-R^+) (lane 3) was separated by electrophoresis and hybridized to an oligonucleotide probe specific for the *P. falciparum* HRP1 gene. The markers depicted are the large and small rRNAs of *P. falciparum* (3.72 and 1.97 kb, respectively). (B) Analysis of surface-located polypeptides on *P. falciparum*-infected erythrocytes after ^{125}I surface labelling. Autoradiogram of non-Triton X-100-soluble pellets (SDS extract) of ^{125}I surface-labelled *P. falciparum*-infected erythrocytes. The polypeptides were separated on 7.5 to 17.5% linear gradient SDS-polyacrylamide gels under reducing conditions. The parasite strains $TM180R^+$ (lane 1), $TM284R^+$ (lane 2), $7G8R^-$ (lane 3), $FCR\text{-}3R^-$ (lane 4), and PAR^+1 (lane 5) are shown. The asterisk indicates the 22,000-M_r polypeptide. No labelling of hemoglobin was obtained (data not shown), indicating that mainly surface components were labelled; however, both the alpha- and beta-spectrin sometimes incorporated radioactivity, as frequently occurs with *P. falciparum*-infected erythrocytes (16). The molecular weight markers are indicated (in thousands) (Bio-Rad, Laboratories, Richmond, Calif.). (C) Fluorogram of immunoprecipitation of solubilized metabolically labelled ([^{35}S]methionine) rosetting parasites. Triton X-100–SDS extract of the rosetting strain $TM180R^+$ precipitated with preimmune rabbit IgG (lanes 1 and 3) and anti-22,000-M_r polypeptide rabbit IgG (R143; lanes 2 and 4). Lanes 3 and 4 are the same fluorogram as lanes 1 and 2 after prolonged exposure of the gel. Asterisks indicate the 22,000-, 45,000-, and 50,000-M_r polypeptides. The molecular weight markers are indicated (in thousands) (Bio-Rad).

with an M_r of 22,000 in addition to normally exposed erythrocyte components. In contrast, no labelling was obtained in this molecular weight range with nonrosetting infected erythrocytes or anti-28,000-M_r polypeptide antibody-sensitive rosetting infected erythrocytes (Fig. 1B, lanes 3 to 5). With some strains of parasites, a weakly labelled polypeptide with an M_r of ca. 33,000 was sometimes seen (Fig. 1B, lane 2). The 22,000-M_r polypeptide was degraded by trypsin at a concentration similar to that which disrupted the rosettes (100% inhibition at 100 μg of trypsin [data not shown]), suggesting that the molecule is involved in rosetting.

The 22,000-M_r polypeptide band was excised from SDS-polyacrylamide gels and incorporated into Freund's adjuvant to immunize mice and rabbits. Significant levels of rosette-disrupting activity were readily detectable in the sera after two injections, and the levels of disruptive antibodies increased following a booster injection (data not shown). The antisera against the 22,000- and 28,000-M_r polypeptides revealed that there are different types of rosettes among different strains or isolates of human parasites. Indeed, the rosettes of five of the seven strains or isolates studied were highly sensitive to the anti-22,000-M_r polypeptide antiserum, whereas rosettes of two 28,000-M_r polypeptide-dependent strains generally remained unaffected (Fig. 2A). Low dilutions of the anti-22,000- and the anti-28,000-M_r polypeptide antisera occasionally yielded disruption of rosettes expressing a heterologous rosetting mechanism, indicating a cross-reactivity between the rosettins or a simultaneous expression of the two rosetting mechanisms (Fig. 2A and B). In summary, the polypeptides involved in rosetting seem to be strain specific, roughly dividing the rosettes into 22,000- or 28,000-M_r rosettin-dependent strains.

To determine whether the 22,000-M_r rosettin was parasite derived, rosetting parasites were metabolically labelled with [^{35}S]methionine, extracted in SDS–Triton X-100, and reacted with the anti-22,000-M_r rosettin antiserum (Fig. 1C). A 22,000-M_r polypeptide was precipitated by the antiserum, but it was the minor polypeptide of the three specifically recognized polypeptides (22,000-, 45,000 [doublet]-, and 50,000-M_r polypeptides; Fig. 1C, lanes 2 and 4) and it appeared only after prolonged fluorography of the gel (Fig.

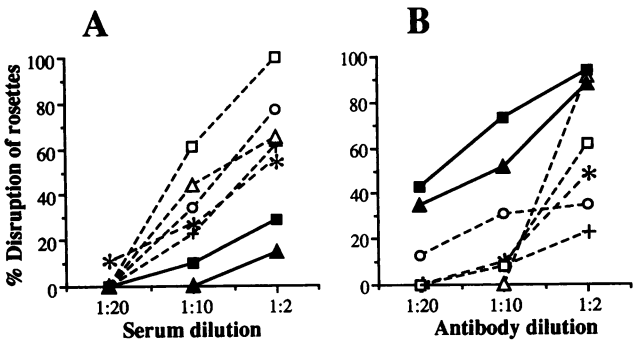

FIG. 2. Disruption of rosettes of different parasite strains with an anti-22,000-M_r polypeptide mouse antiserum (A) and an anti-28,000-M_r polypeptide antibody (monoclonal antibody 89, ascites) (see references 5 and 27) (B). The following strains were used. ■, PAR$^+$1; ▲, R29; *, TM180; +, TM284; □, TM280; ○, TM177; △, BR1. Rosette reversion was performed as described in Materials and Methods.

1C, lane 4). None of the polypeptides was precipitated from extracts of nonrosetting parasites, and preimmune sera or sera raised to a control band (M_r, 25,000), prepared in an identical fashion from SDS-PAGE gels, did not disrupt rosettes or specifically precipitate any of these polypeptides (data not shown). The anti-22,000-M_r polypeptide antiserum did not react in immunoblotting with a parasite extract, although the 22,000-M_r polypeptide was satisfactorily transferred to the blotting membrane, nor did it react with normal erythrocyte components (data not shown). Taken together, these results show that the 22,000-M_r rosettin originates from the parasite but do not indicate whether the polypeptides precipitated by the anti-22,000-M_r polypeptide antiserum are dimers of the 22,000-M_r rosettin, represent separate molecules with cross-reactive epitopes, or are immunologically unrelated but coprecipitated. Although the 22,000-/28,000-M_r rosettins seem antigenically distinct, it remains to be proven that they are genetically related.

Fluorescence staining with the anti-22,000-M_r polypeptide serum gave a strong, uniform fluorescence around the entire surface of nonfixed rosetting infected erythrocytes. However, a more speckled pattern was occasionally observed (Fig. 3A and B). The surface staining was restricted to erythrocytes which form rosettes, i.e., mature infected erythrocytes (trophozoites and schizonts) (Fig. 3A to C). No antibody binding was seen on immature, ring-infected, or uninfected erythrocytes (Fig. 3D), on anti-28,000-M_r polypeptide-dependent rosetting strains, or on strains of nonrosetting infected erythrocytes (data not shown). Simultaneous antibody staining and rosetting could be seen when the anti-22,000-M_r polypeptide antiserum was diluted so that only partial disruption of rosettes was obtained, confirming that the 22,000-M_r polypeptide fluorescence-labelled erythrocytes were involved in rosetting (Fig. 3A). Fixation of the erythrocytes with acetone and/or by air drying, which leads to partial denaturation of some antigens, completely abrogated surface staining, suggesting that the epitopes recognized by the anti-22,000-M_r polypeptide antibodies are highly dependent on the tertiary structure of the rosettin for binding. This theory is further supported by the ability of the serum to react with the native 22,000-M_r polypeptide in immunoprecipitation but not to bind to the antigen after denaturation in immunoblotting.

Our recent data from The Gambia suggest that the rosettins contain regions of variability since some sera that were devoid of autologous rosette-disrupting activity could disrupt rosettes formed by other wild isolates (30). Previously, several other investigators have reported on the existence of antigenic variability of the surface of the *P. falciparum*-infected erythrocyte as seen by indirect immunofluorescence, antibody-mediated agglutination, surface iodination, or cytoadherence inhibition (2, 8, 12, 19, 20, 31). In light of this, it seems reasonable to suggest that the 22,000-/28,000-M_r rosettins may have been responsible in part for this activity, perhaps in combination with other surface-located molecules (2, 8, 12, 19, 20, 33, 35). Whether the rosettins are also involved in endothelial cytoadherence is more uncertain, as we have found no inhibitory effect on such binding with our anti-22,000-M_r polypeptide antibodies (unpublished data).

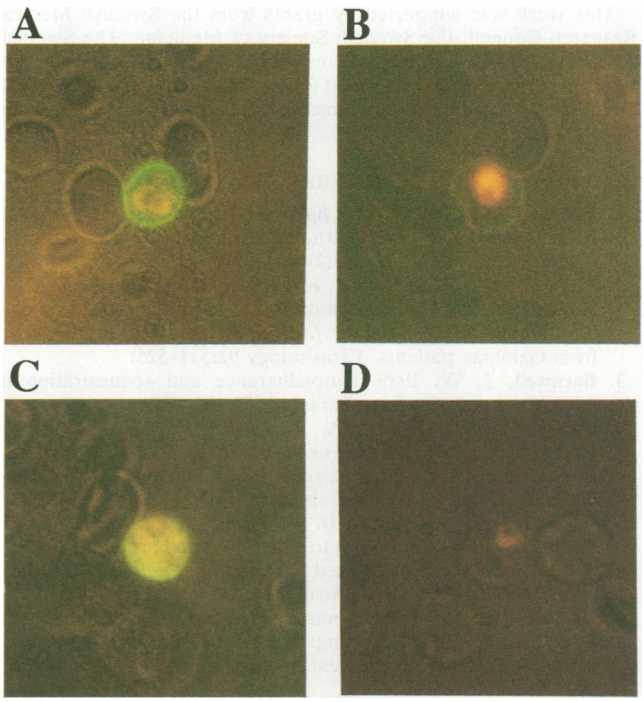

FIG. 3. Indirect surface immunofluorescence of live, unfixed trophozoite- or schizont-infected erythrocytes of rosetting strain TM180 with a rabbit anti-22,000-M_r polypeptide antiserum. The internal labelling is due to counterstaining with ethidium bromide. The development stages were as follows: trophozoite, partially rosette forming, showing uniform staining (A); trophozoite showing speckled fluorescence (B); schizont (C); and ring stage (D).

The identification and isolation of the unique polypeptides believed to be involved in rosetting are potentially important in elucidating the molecular basis of parasite sequestration. Monoclonal antibodies able to abrogate rosetting and endothelial cytoadherence and specific for the phenotypes present in nature may prove to be useful therapeutically for reversal of erythrocyte sequestration in cerebral malaria.

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