Characterization of a Modified Red Cell Membrane Protein Expressed on Erythrocytes Infected with the Human Malaria Parasite \textit{Plasmodium falciparum}: Possible Role as a Cytoadherent Mediating Protein

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\textbf{Abstract.} Infections with the human malaria \textit{Plasmodium falciparum} are characterized by the retention of parasitized erythrocytes in tissue capillaries and venules. Erythrocytes containing trophozoites and schizonts attach to the endothelial cells that line these vessels by means of structurally identifiable excrescences present on the surface of the infected cell. Such excrescences, commonly called knobs, are visible by means of scanning or transmission electron microscopy. The biochemical mechanisms responsible for erythrocyte adherence to the endothelial cell are still undefined. In an attempt to identify the cytoadhesive molecule on the surface of the infected cell, we have prepared monoclonal antibodies to knob-bearing erythrocytes infected with the FCR-3 strain of \textit{P. falciparum}. One of these monoclonal antibodies, designed 4A3, is an IgM that reacts (by means of immunofluorescence) with the surface of unfixed erythrocytes bearing mature parasites of the knobby line; it does not react with knobless lines or uninfected erythrocytes. By immunoelectron microscopy the monoclonal antibody 4A3 was localized to the knob region. In an in vitro cytoadherence assay, the monoclonal antibody partially blocked the binding of knob-bearing cells (FCR-3 strain) to formalin-fixed amelanotic melanoma cells. The monoclonal antibody was used to immunoprecipitate a protein from extracts of knobby erythrocytes that had been previously surface iodinated. By a two-dimensional peptide mapping technique, the antigen recognized by the monoclonal antibody was found to be structurally related to band 3 protein, the human erythrocyte anion transporter.

Infections with the human malaria parasite \textit{Plasmodium falciparum} are characterized by the absence of mature forms of the parasite (i.e., trophozoites and schizonts) in the peripheral circulation. This phenomenon, called sequestration, is a consequence of the binding of trophozoite- and schizont-infected erythrocytes to the venular endothelium of various organs such as the heart, muscle, adipose tissue, and, in the case of acute infections, the brain. It has been hypothesized (Udeinya et al., 1981) that, by sequestration, infected erythrocytes avoid passage through the spleen, the major organ of host defense against a malaria infection.

Sequestration of parasitized erythrocytes has been ascribed to the presence of surface excrescences or knobs (Miller, 1972). Knob formation is related to parasite development (Vernot-Hernandez and Heidrich, 1985), and, by transmission electron microscopy, the knob consists of a conical elevation of surface membrane below which an electron-dense material is located (Aikawa, 1977).

Several workers have shown that the knobby regions of \textit{P. falciparum}–infected erythrocytes are antigenically different from other regions of the host membrane. Kilejian et al. (1977) and Langreth et al. (1979) showed that sera from immunized animals reacted with the knobby regions of infected cells. Similarly, Sherman and Greenan (1986) found a preferential association of some lectins and cationized ferritin over the knobby regions. More recently, Winograd et al. (1987) showed that a modified host cell antigen was associated with these membrane elevations.

The molecular mechanisms underlying cytoadherence of \textit{P. falciparum}–infected erythrocytes to the venular endothelium remain largely unknown. Knob formation in knobby variants has been correlated with expression of a histidine-rich protein (Kilejian, 1979). More recently this protein was immunolocalized under these membrane excrescences in association with the red cell skeleton, and, since the protein was shown not to be exposed to the extracellular environment (Ardeshir et al., 1987; Culvenor et al., 1987; Pologe et al., 1987; Taylor et al., 1987), its direct participation in cytoadherence becomes very unlikely. Leech et al. (1984) and Aley et al. (1984) found that a ∼300-kD malarial protein was correlated with cytoadherence of infected erythrocytes to melanoma cells in vitro.

The present report describes the production and characterization of a cytoadherence-inhibiting monoclonal antibody that recognizes a modified erythrocyte membrane protein.
Materials and Methods

Materials

Biotin-conjugated goat anti-mouse IgG (heavy and light chain specific) and FITC-conjugated avidin were purchased from Calbiochem-Behring Corp. (San Diego, CA). Rabbit anti-mouse IgM was from Miles Scientific Div. Miles Laboratories Inc. (Naperville, IL). Acidic/colloidal gold was obtained from E. Y. Laboratories, Inc. (San Mateo, CA). TEPC 183 ascites fluid was from Sigma Chemical Co., (St. Louis, MO). Sepharose CL-6B, protein A-Sepharose, and activated thiol-Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden). α-Chymotrypsin was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Na₂51 was from Amersham International (Arlington, UK). Iodogen was from Pierce Chemical Co. (Rockland, IL). All other chemicals were purchased from Sigma Chemical Co.

Parasites

The Gambian FCR-3 strain of *P. falciparum* (kindly provided by L. H. Miller, National Institutes of Health, Bethesda, MD) was cultured according to the method of Trager and Jensen (1976). Cultures were synchronized at the ring stage by sorbitol lysis of mature forms (Lambros and Vanderberg, 1980). Mature forms of the parasite were concentrated by the method of Gruenberg and Sherman (1983).

Immunizations and Monoclonal Antibody Production

Techniques for the production of monoclonal hybridoma antibodies were according to Zola and Brooks (1982). Briefly, 1-2 × 10⁷ erythrocytes infected (50% parasitemia) with mature forms of the parasite (trophozoites and schizonts) were injected intraperitoneally into a BALB/c mouse every week for a total of three injections. A final boost, with the same number of parasites, was given intravenously 72 h before killing the mouse. Spleen cells were fused with the mouse P3-X63-Ag8.653 plasmacytoma cell line and selection of hybridomas in hypoxanthine-aminopterin-thymidine medium was as previously described (Kohler and Milstein, 1975). Growing colonies were screened for the production of antibodies against cell surface antigens by an indirect immunofluorescence assay described below. Erythrocytes infected with a knobless strain as well as uninfected erythrocytes were used as negative controls. Positive hybrids were subcloned twice by limiting dilution, and immunoglobulin class and subclass determined by an ELISA kit purchased from Calbiochem-Behring Corp. For the purification of the monoclonal antibody (4A3 IgM), ascites fluid was produced by injecting 4 × 10⁶ hybrid cells into the peritoneal cavity of a Pristane (2, 6, 10, 14-tetramethylpentadecane) primed BALB/c mouse; the antibody was purified by gel filtration on a Sepharose CL-6B column.

Immunofluorescence

20 μl of a 10% (vol/vol) suspension of erythrocytes containing mature forms of *P. falciparum* (FCR-3 strain) were incubated with 100 μl of hybridoma supernatants for 30 min at room temperature. Cells were then washed twice in PBS (10 mM sodium phosphate buffer, pH 7.4/145 mM sodium chloride), and incubated for 30 min at room temperature with 20 μg/ml of biotin-conjugated goat anti-mouse IgG followed by 10 μg/ml of FITC-conjugated avidin. The cells were washed three to four times in PBS, mounted in 90% fluid diluted 1:50 (vol/vol) glycerol in PBS. Cells were then washed three times in PBS, and 50 μl of 20 μg/ml biotin-conjugated goat anti-mouse IgG were added, and incubation was carried out for 30 min at room temperature. After three PBS washes, cells were incubated in 0.36 μg/ml of avidin/colloidal gold. Cells were fixed and processed for electron microscopy as previously described (Winograd et al., 1987). Negative controls included ascites fluid from the TEPC 183 tumor cell line secreting a mouse IgM, omission of monoclonal antibody, or presaturation of biotin binding sites with unlabeled avidin before the avidin/colloidal gold incubation.

Cell Surface Labeling

Erythrocyte membrane proteins were surface radiolabeled with Na₂51 (carrier-free) using Iodogen by the method of Markwell and Fox (1978). Briefly, 250 μl of a 10% (vol/vol) erythrocyte suspension was placed in a glass scintillation vial previously coated with 100 μg Iodogen. The iodination reaction was initiated by the addition of 500 μCi Na₂51 and allowed to proceed for 12 min at room temperature. Cells were transferred to a test tube containing 15 ml PBS plus 5 mg/ml BSA and 5 mM potassium iodide, and centrifuged for 5 min at 700 g. The supernatant was removed, and the cells were washed four times in the same buffer.

Immunoprecipitation of Cell Surface Antigens

Surface-labeled erythrocytes were hypotonically lysed in 5 mM sodium phosphate, pH 7.0 (Fairbanks et al., 1971). A membrane protein extract was prepared by incubating 5-8 × 10⁵ hypotonically lysed infected erythrocytes in 1 ml 1% (vol/vol) Triton buffer (5 mM sodium phosphate, pH 8.0, 0.15 mM NaCl, 1 mM diethiothreitol, 1% (vol/vol) Triton X-100 containing a cocktail of protease inhibitors: 1 mM EDTA, 0.5 μg/ml leupeptin, 20 μg/ml PMSF, 4 μg/ml pepstatin A, and 5 μg/ml aprotinin); after 30 min at 0°C, the extract was clarified by centrifugation at 13,000 g for 15 min in a microfuge (Beckman Instruments, Inc., Fullerton, CA). Alternatively, membrane proteins were extracted with Triton X-100 as described above and the sample centrifuged at 13,000 g for 15 min. The Triton-insoluble pellet was incubated for 30 min at room temperature in 0.1 ml SDS-PBS (1% (vol/vol) SDS in PBS, 1 mM EDTA, plus protease inhibitors). The sample was centrifuged at 13,000 g for 15 min, and the supernatant dialyzed 20 times in ice cold 2% Triton buffer (same as the 1% Triton buffer but containing two times the amount of Triton X-100). For isolation of antigens, extracts (1 × 10⁷ cpm) were incubated overnight at 4°C with 2 μl of antibodies fluid. 5 μl of protein A-Sepharose previously coated with rabbit anti-mouse IgM was then added, and incubation was carried out with constant shaking for 1 h at 4°C. The Sepharose beads were centrifuged and washed four times in 5 mM PBS, pH 8.0, containing 1% (vol/vol) Triton X-100. The beads were then extracted in sample buffer, and antigens were analyzed by autoradiography containing 10-15% linear gradient SDS-polyacrylamide gels (Laemmli, 1970), containing 2-mercaptoethanol.

Isolation of Band 3 and Band 3 Fragments

Intact band 3 and its cytoplasmic domain (TR-41) were isolated from fresh uninfected erythrocytes following the methods of Drickamer (1976) and Appell and Low (1981), respectively. Chymotryptic fragments of 17,000- (CH-17) and 35,000-D (CH-35) fragments were isolated on an activated thiol-Sepharose column as described by Fukuda et al. (1978).

Two-dimensional Peptide Mapping

Proteins of interest were cut out of 10% acrylamide-SDS gels and radiiodinated according to the protocol of Elder et al. (1977). Two-dimensional peptide maps of the proteolytic fragments of each protein were made according to Markowitz and Marchesi (1981).

Cytodherence Assay

The in vitro cytodherence inhibition assay of Udeinya et al. (1983) was used to test the ability of the 4A3 monoclonal antibody to prevent the cytodherence of parasitized erythrocytes (*P. falciparum*, FCR-3 strain) to formalin-fixed melanoma cells. Briefly, 1 ml of infected erythrocytes (5% [vol/vol] cell suspension) in RPMI/sodium bicarbonate, pH 7.4, was placed on top of the melanoma cells in the presence of either 30 μl 4A3, TEPC 183 IgM ascites fluid, or PBS, and incubation was carried out for 90 min at 37°C with occasional stirring. Coverslips were washed, stained, and the inhibition by the monoclonal antibody was determined as described previously (Udeinya et al., 1983).

Results

4A3 Monoclonal Antibody

Monoclonal antibodies were used in the present study to identify newly exposed membrane antigens present on the
membranes of erythrocytes infected with the human malaria parasite, *P. falciparum*. After screening ~150 growing hybridoma clones, a cell line secreting an IgM monoclonal antibody, designated 4A3, was isolated. This monoclonal antibody was the only one that reacted against the surface of intact live knobby (K+) infected erythrocytes of the FCR-3 strain as shown by an indirect immunofluorescence assay (Fig. 1). Furthermore, 4A3 did not react with erythrocytes infected with a knobless (K-) strain (not shown), uninfected red cells, nor with human red cells infected with the ItG2 (Brazilian) strain (not shown).

**Immunoprecipitation Experiments**

The molecular specificity of 4A3 was tested by reacting this monoclonal antibody with 125I-labeled membrane proteins extracted with Triton X-100 or SDS from surface-iodinated red cells (Fig. 2). Incubation of 4A3 monoclonal antibody with a Triton X-100 extract resulted in immunoprecipitation of band 3. The reactivity of 4A3 towards band 3 is nonspecific because addition of TEPC 183 to the Triton X-100 also resulted in band 3 precipitation. This finding is in agreement with a previous study that showed the tendency of band 3 to precipitate nonspecifically (Gahmberg, 1982). In an earlier

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*Figure 1. Specificity of 4A3 monoclonal antibody. Binding of 4A3 monoclonal antibody to erythrocytes infected with a knobby variant of the FCR-3 strain of *P. falciparum* by means of an immunofluorescence assay. (A) Phase-contrast microscopy. (B) Same field under fluorescent light. Note that only the two malaria-infected erythrocytes fluoresce. Bars, 80 μm.*
these membrane proteins was possible only after the addition of SDS. (However, it was necessary to add excess Triton X-100 to the SDS extract before carrying out the immunoprecipitation to avoid denaturation of antibodies.) When 4A3 monoclonal antibody was incubated with an SDS-Triton extract (Fig. 2) precipitation of an 85-kD antigen resulted; however, addition of TEPC 183 to this same extract did not result in precipitation of this protein.

85-kD Antigen Is Specific for Knobby Erythrocytes

Fig. 3 shows an autoradiograph of the membrane proteins extracted with the nonionic detergent Triton X-100 from surface-iodinated knobby (K⁺) and knobless (K⁻) infected erythrocytes. The 85-kD protein was insoluble in Triton X-100 and was associated with membranes of erythrocytes infected with the knobby (K⁺) variant (Fig. 3). A faint band with the same Mr was also observed in preparations made from erythrocytes infected with the knobless variant; however, this band is probably different from the 85-kD antigen since the 4A3 monoclonal antibody did not immunoprecipitate an 85-kD protein in extracts made from erythrocytes infected with the knobless (K⁻) variant. The 85-kD antigen became Triton soluble only when knobby infected erythrocytes were incubated for 15 min at 37°C in 1.0 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0, a treatment reported to elute spectrin from the human erythrocyte membrane (Marchesi et al., 1970).

Identification of the 85-kD Antigen

Because our attempts to metabolically label parasite proteins using several radioactively labeled amino acids ([35S]methionine, [3H]isoleucine, [3H]histidine) failed to label the 85-kD antigen, it was concluded that this antigen was not a parasite protein but instead was a modified red cell membrane.

Figure 2. Autoradiographic analysis of immunoprecipitates generated by 4A3 monoclonal antibody. The molecular specificity of 4A3 was studied by incubating the monoclonal antibody with 125I-labeled membrane proteins extracted with Triton X-100 or SDS from surface-iodinated red cells. Isolation of immune complexes was carried out by means of protein A-Sepharose previously coated with anti-mouse IgM (see Materials and Methods). Antigens were analyzed by running 10-15% linear gradient SDS-acrylamide gels containing mercaptoethanol followed by autoradiography. (Lane A) Triton extract of surface-iodinated infected red cells; (lane B) SDS extract of surface-iodinated infected red cells; (lanes C and D) immunoprecipitates of the Triton X-100 extract using 4A3 monoclonal antibody and TEPC 183, respectively; (lanes E and F) immunoprecipitates of the SDS extract using 4A3 monoclonal antibody and TEPC 183, respectively. Note that only the 4A3 monoclonal antibody recognized the 85-kD antigen.

Figure 3. The 85-kD antigen is specific for knobby infected erythrocytes. Membrane proteins were extracted with Triton X-100 from surface-iodinated knobby (k⁺) and knobless (k⁻) erythrocytes. After centrifugation, a soluble extract was obtained. The pellet from this incubation (labeled in the figure as TRITON INSOLUBLE) was washed once in Triton extraction buffer, and solubilized in sample buffer before application to a 10% acrylamide-SDS gel under reducing conditions. The antigen was visualized by autoradiography.
protein. Based on our observation that 4A3 monoclonal antibody recognized band 3 in Triton X-100 extracts made from noninfected erythrocytes and because of the binding of concanavalin A to the 85-kD antigen (Winograd, E., and I. W. Sherman, unpublished observations) the possibility that the antigen might be structurally related to the human red cell anion transporter, band 3, was explored. Comparisons between these two proteins was carried out by means of two-dimensional peptide maps (Fig. 4). Examination of the maps revealed a striking similarity between these two proteins despite the reduced number (40%) of spots for the 85-kD protein. All of the spots in the 85-kD map had a correspondent spot in the band 3 map. This result strongly suggested that the 85 kD was either derived from band 3, or that its amino acid sequence was very similar to that of band 3. To gain a better understanding of the structural differences between the 85-kD antigen and band 3, each of the major band 3 domains—cytoplasmic (TR-41) and the two membrane-associated domains (CH-17 and CH-35)—fragments were isolated, and two-dimensional peptide maps of each were prepared (Fig. 5). This information was then used to determine the correspondence of each spot in the band 3 map to each band 3 fragment; i.e., TR-41, CH-35, or CH-17. With few exceptions, all of the spots could be related to one of the band 3 domains (Fig. 5 B). Comparison of the band 3 peptide map with the 85-kD map showed that most or all of the TR-41 cytoplasmic domain was absent from the 85-kD antigen; two spots that corresponded to the CH-35 fragment map were also missing; therefore, it appeared that a small deletion at the carboxy-terminal end of band 3 may have occurred.

**Immunoelectron Microscopy Using 4A3**

The specificity of the monoclonal antibody towards eryth-
Binding of monoclonal antibody to *P. falciparum*-infected erythrocytes (FCR-3 strain) was detected by means of biotin-conjugated anti-mouse immunoglobulin followed by avidin/colloidal gold. Gold particles were preferentially associated with the membrane excrescences of erythrocytes infected with the knobby variant. Bar, 0.2 μm.

*Binding Assay*

Having established the specificity of 4A3 toward the knobby regions of infected erythrocytes, an in vitro cytoadherence assay (Udeinya et al., 1983) was used to determine whether this monoclonal antibody inhibited binding of parasitized erythrocytes (FCR-3 strain) to melanoma cells. The degree
of inhibition caused by 4A3 monoclonal antibody was compared to an IgM secreted by the TEPC 183 tumor cell line. In three different experiments (Table I), the mean percentage inhibition caused by 4A3 was 47% (range, 33–78%). The reasons underlying this variability are unknown. These results indicate that the monoclonal antibody 4A3 binds at or near the site responsible for in vitro cytoadherence of knobby infected erythrocytes (FCR-3 strain) to amelanotic melanoma cells.

Discussion

Erythrocytes infected with malaria parasites become structurally and antigenically altered as a result of the interaction between parasite and host cell. Attachment of parasitized erythrocytes to cells of the venular endothelium is presumed to be mediated by some of these newly exposed antigens. Since it has been hypothesized that through sequestration the malaria parasite escapes host-mediated destruction (by avoiding passage through the spleen), identification of such cytoadherent molecules becomes critical to the development of a malaria vaccine.

Several laboratories have provided evidence (for review see Howard and Barnwell, 1984) that neoantigen exposure to the extracellular environment is the result of parasite-encoded proteins that are translocated and inserted into the infected erythrocyte membrane by an uncharacterized transport mechanism. In contrast, we previously demonstrated (Winograd et al., 1987) modification of existing host membrane proteins to explain the appearance of some of these new membrane antigens.

In the present study, we isolated a monoclonal antibody that reacted against a newly identified 85-kD membrane protein. The antigen appears to be structurally related to band 3, the human erythrocyte anion transporter, based on the following evidence: (a) two-dimensional peptide maps, (b) cross-reactivity of naturally occurring anti-band 3 antibodies for the 85-kD antigen (Winograd, E., and I. W. Sherman, manuscript in preparation), (c) concanavalin A binding to the 85-kD antigen (Winograd, E., and I. W. Sherman, unpublished results), and (d) the binding of 4A3 to band 3 in Triton X-100 extracts made from noninfected erythrocytes (Winograd, E., and I. W. Sherman, unpublished results). Assuming that the molecular mass of band 3 protein is 90 kD, then cleavage of the 41-kD cytoplasmic domain from band 3 would be insufficient to explain the formation of an 85-kD protein. It is possible, therefore, that the 85-kD antigen is derived from the covalent nondisulfide bonding between two band 3 monomers, each of which lacks the cytoplasmic domain. This would explain our inability to detect reactivity (by immunoprecipitation) of anti-band 3 antiserum specific for the cytoplasmic domain of band 3 (Low, 1986) towards the 85-kD antigen (Winograd, E., and I. W. Sherman, unpublished observations).

Although the monoclonal antibody 4A3 was partially effective as an inhibitor of cytoadherence of the FCR-3 strain of Plasmodium falciparum, membrane proteins other than 85 kD could be involved in binding of infected erythrocytes since the inhibition of cytoadherence could be a consequence of a blocking of the actual binding site or of steric hindrance.

It should be noted that the monoclonal antibody 4A3 reacted, by immunofluorescence, only with the erythrocyte surface of the knobby FCR-3 strain of P. falciparum, and not with the Brazilian ITG2 strain (data not shown). Consequently, it is unlikely that this antibody would have any effect on the cytoadherence of ITG2.

Special thanks to Dr. R. Zidovetzki for his helpful suggestions throughout this work. Monoclonal antibodies were produced with the assistance of Dr. D. J. Gumpf and Mr. J. Diaz. Cytoadherence assays were performed by E. L. Valdez. Preparation of samples for electron microscopy was done by Ms. J. Greenan. Polyclonal antibodies specific for the cytoplasmic domain of the band 3 molecule were kindly by P. S. Low, Purdue University, West Lafayette, Indiana.

This work was supported by research grants from the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, and the National Institutes of Health (AI-21251).

Received for publication 13 July 1988, and in revised form 8 September 1988.

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