

CYTOADHERENCE BY *PLASMODIUM FALCIPARUM*-INFECTED
ERYTHROCYTES IS CORRELATED WITH THE EXPRESSION
OF A FAMILY OF VARIABLE PROTEINS ON
INFECTED ERYTHROCYTES

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Erythrocytes infected with trophozoites and schizonts of the human malaria parasite *Plasmodium falciparum* develop surface protrusions (knobs) (1) by which the infected erythrocytes (IRBCs)¹ adhere specifically to venular endothelium in vivo (2, 3) and to human endothelial cells (4) and some lines of melanoma cells (5) in vitro. Cytoadherence between IRBCs and venular endothelium has a critical role in the pathogenesis of falciparum malaria, since it permits the mature parasites to evade spleen-dependent immune mechanisms (6) and since the sequestered parasites may occlude blood flow, as seen in cerebral malaria (7). Antibody in immune serum reacts with a strain-specific parasite-determined antigen on IRBCs and inhibits cytoadherence in vitro (8) and in vivo (9). The inhibition of cytoadherence by antibody may protect the host from the clinical consequences of falciparum malaria.

Both cytoadherence and the IRBC surface antigen were shown to be destroyed by incubating IRBCs with proteases (9, 10), suggesting that the two properties are determined by proteins on the IRBC surface. In addition, the cytoadherence phenotype of *P. falciparum* parasites and the expression of the IRBC surface antigen were modulated together by the spleen in a monkey model of falciparum malaria (9, 10), suggesting that the two properties are linked and perhaps determined by the same protein. A family of potential cytoadherence proteins was identified in studies with IRBCs from Aotus monkeys (11). The members of the protein family differed in antigenicity and molecular size among strains of *P. falciparum*, but had in common several biochemical properties, including their accessibility to surface radioiodination, detergent solubility, and cleavage by the same concentration of trypsin that inhibited cytoadherence (11).

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¹ *Abbreviations used in this paper:* IRBC, infected erythrocyte; KAHRP, knob-associated histidine-rich protein; TEM, transmission EM.

To investigate the potential role of this protein family in cytoadherence, we developed *in vitro* techniques for modulating the cytoadherence phenotype of cloned parasites, and we examined the expression of the proteins on IRBCs with different cytoadherence phenotypes. Our results demonstrate that expression of the family of radioiodinatable surface proteins by cloned parasites is variable and that changes in cytoadherence phenotype are correlated with changes in the expression or the molecular size of the proteins, suggesting that the members of this protein family have a role in mediating cytoadherence.

Materials and Methods

Parasites. The D6 clone of parasites was from a patient who was infected in Sierra Leone; the W2 clone was from a patient infected in Indochina. D6 and W2 parasites were cloned by micromanipulation at the Walter Reed Army Institute of Research (Wash. DC) and were a gift from Dr. Wil Milhouse. The ITG2F6 clone was from a patient infected in Brazil. The parasites were cloned twice by limiting dilution and were obtained from the Laboratory of Parasitic Diseases, National Institutes of Health (Bethesda, MD). B11 parasites were from a patient infected in Uganda and were cloned by limiting dilution at the Division of Tropical Medicine, Harvard Medical School (Boston, MA). They were obtained from Dr. Peter David.

Culture and Cloning. Parasites were cultured as previously described (12). We used cloned parasites to eliminate the possibility that changes in the cytoadherence or protein phenotypes were due to the selection of preexisting subpopulations of parasites. ITG2F6 parasites were cloned again by us by limiting dilution (13) in 96-well tissue culture plates (Micro Well III; Becton Dickinson & Co., Oxnard, CA) at a density of 0.2 IRBCs per well. Serum and erythrocytes were from the same donor. The wells were screened between days 21 and 28 by examination of Giemsa-stained blood smears. A Poisson distribution was assumed to calculate the probability that a positive well contained the progeny of a single parasite using the formula $u e^{-u} / 1 - e^{-u}$, where u is the average density of parasites in the inoculum; $u = \ln[1 + (\text{number of positive wells} / \text{number of negative wells})]$ (13, 14).

Selection of Knobbed (K^+) Parasites for different Cytoadherence Phenotypes. To facilitate our studies of the molecular mechanisms involved in cytoadherence, we developed techniques for reversibly modulating the cytoadherence phenotypes of cloned K^+ parasites *in vitro*. Previous studies have shown that knobs are necessary for cytoadherence (3, 15) and that expression of a knob-associated histidine-rich protein (KAHRP) is correlated with the K^+ phenotype (16). Since knobs may be lost during long term culture (17), the K^+ phenotype of the parasites used in this study was maintained by frequent incubation of synchronized (18) IRBCs containing trophozoites with 0.5% (wt/vol) gelatin/RPMI 1640 as previously described (14). K^+ IRBCs were collected from the supernatant, and the cells that sedimented were discarded. Selection was repeated every 4 or 6 d. The knob phenotype of IRBCs was assessed by indirect immunofluorescence assay on acetone-fixed IRBCs with an mAb (mAb 89) to the KAHRP (19) and by transmission EM.

Knobs and KAHRP are not sufficient for cytoadherence since some K^+ parasites maintained *in vitro* rapidly lose the ability to bind to endothelial cells and melanoma cells (20). To select for cytoadherent (C^+) parasites, IRBCs (1.5 cc) from the 0.5% gelatin/RPMI 1640 supernatant were collected as described above and added to a monolayer of unfixed C32 melanoma cells (American Type Culture Collection, Rockville, MD) in a 25-cm² tissue culture flask (Corning Glass Works, Corning, NY) and incubated for 1 h at 37°C with gentle rocking by hand every 10–15 min. The monolayer was washed with RPMI lacking sodium bicarbonate until all nonadherent cells were removed. Fresh complete medium and erythrocytes were added, and the culture was incubated overnight. The adherent parasites underwent schizogony, ruptured host erythrocytes, and released merozoites that invaded erythrocytes in the overlying suspension. IRBCs containing ring stage parasites do not bind to melanoma cells (5), and these cells (containing the progeny of C^+ parasites) were collected for continued culture.

To select for noncytoadherent (C^-) parasites, K^+ IRBCs from the gelatin supernatant were added to a monolayer of C32 melanoma cells as described above, and the nonbinding cells

were collected and incubated with successive monolayers of melanoma cells until no binding of IRBCs to the melanoma cells was observed (usually after the fourth incubation). These nonadherent IRBCs were washed, added to fresh media and erythrocytes, and cultured. Selection was repeated every 4 or 6 d, and the cytoadherence phenotype was monitored by cytoadherence assays as described below.

Cytoadherence Assays. Cytoadherence assays were performed with fixed C32 melanoma cells (21) or with fresh human umbilical vein endothelial cells (4) cultured on Thermanox tissue culture coverslips in Lux 8 well multiplates (both from Miles Laboratories, Naperville, IL). Parasitemias were adjusted to 2–6% on the day before the assay by the addition of uninfected erythrocytes to cultures with higher parasitemias. The pH of the medium used for cytoadherence assays was 7.3, and sodium bicarbonate was omitted to prevent alkalization of the media during the assay. IRBCs in 0.65 cc of medium (2% hematocrit) containing 10% serum were incubated with endothelial cells or melanoma cells for 1 h at 37°C with gentle rocking by hand every 15 min. The coverslips were gently washed in the same medium, fixed with 2% (vol/vol) glutaraldehyde (Eastman Kodak Co., Rochester, NY) in PBS, and stained with Giemsa. The number of IRBCs bound to 100 melanoma cells was determined by light microscopy.

EM. IRBCs were washed and then fixed for 1 h in a solution of 3% (vol/vol) glutaraldehyde, 50 mM L-lysine in 100 mM cacodylate (22). After fixation, the cells were washed, post-fixed with 2% osmium tetroxide, 2% ferricyanide, held overnight at 4°C in 2% uranyl acetate, dehydrated with acetone, and embedded in Epox. Blocks were sectioned in order to sample material through the depth of the pellet.

Identification of Surface Radioiodinated Proteins of IRBCs. Synchronized cultures containing trophozoites were used for surface radioiodination. K⁺ IRBCs were enriched with 0.5% gelatin/RPMI 1640 before labeling, as described above. Uninfected control RBCs were also cultured and incubated with 0.5% gelatin/RPMI before labeling. Cells were washed twice with RPMI 1640, twice with PBS, and surface radioiodinated using the lactoperoxidase technique (23). 2.5–5.0 × 10⁸ cells were labeled in 0.5 ml of PBS containing 0.125 mg lactoperoxidase (Worthington Biochemical Corp., Freehold, NJ), 1 μM potassium iodide, and 0.5 mCi Na¹²⁵I (IMS.300, 350–600 mCi/ml, Amersham Corp., Arlington Heights, IL). Hydrogen peroxide (0.03%) (Sigma Chemical Co., St. Louis, MO) was added as follows: 12 μl initially and 6 μl every minute for 3 min. In some experiments, 0.0003% hydrogen peroxide was used with identical results.

After labeling, the cells were washed five times in RPMI 1640. Trypsin treatment of intact IRBCs was performed by incubating the cells with Trypsin-TPCK (Worthington Biochemical Corp.) (10 μg/ml) with and without 100 μg/ml of soybean trypsin inhibitor (Worthington Biochemical Corp.) in PBS for 5 min at room temperature followed by the addition of soybean trypsin inhibitor (100 μg/ml). The cells were washed with PBS and extracted directly with electrophoresis sample buffer containing 5% (wt/vol) SDS and 5% (vol/vol) 2-ME or sequentially with 1% (wt/vol) Triton X-100 followed by centrifugation (13,000 g for 5 min) and extraction of the insoluble residue with electrophoresis sample buffer as previously described (11). Samples were boiled for 1 min, and the proteins were separated by electrophoresis in a 5–7.5% polyacrylamide gel using the discontinuous buffer system of Laemmli and Favre (24). ¹²⁵I proteins were identified by autoradiography.

Identification of Other Membrane-associated Proteins. mAb 89 to the KAHRP and mAb 8B7.4 to MESA-PfEMP2 (a parasite protein associated with the membrane of K⁺ and K-IRBCs (25, 26) were used to detect the proteins by immunoblotting as previously described (27). Blots were incubated with ¹²⁵I protein A (Amersham Corp.) and antigen-antibody complexes were identified by autoradiography. mAb 89 was a gift from Dr. D. W. Taylor, Department of Biology, Georgetown University, Washington, DC. mAb 8B7.4 was a gift from Dr. R. J. Howard, DNAX Inc., Palo Alto, CA.

Results

Cytoadherence Studies with Cloned Parasites before In Vitro Selection. We determined the knob and cytoadherence phenotypes of four culture-adapted clones of *P. falciparum*.

IRBCs containing B11 and W2 parasites were K⁻ by three criteria: transmission electron microscopy (TEM), failure to react with mAb 89, and lack of enrichment in the 0.5% gelatin/RPMI supernatant. Very few B11 or W2 IRBCs bound to C32 melanoma cells (Table I). Conversely, IRBCs containing D6 and ITG2F6 parasites were K⁺ by TEM, reacted with mAb 89, and were enriched in the 0.5% gelatin/RPMI 1640 supernatant. Significantly more K⁺ IRBCs than K⁻ IRBCs bound to C32 melanoma cells (Table I), but the numbers of IRBCs per 100 melanoma cells were low compared with IRBCs from infected humans (20) or Aotus monkeys (4, 5) and are not sufficient for studies of the molecular basis of cytoadherence.

Selection of K⁺ Parasites for Increased or Decreased Cytoadherence. Parasites of the ITG2F6 and D6 clones were subjected to repeated rounds of selection for the K⁺C⁺ phenotype by gelatin enrichment and binding to C32 melanoma cells as described in Materials and Methods. With ITG2F6 parasites, a marked and consistent increase in the binding of IRBCs to C32 melanoma cells was observed after 11 selections over 7 wk (Table II). With D6 parasites, there was a smaller but persistent increase in cytoadherence. Selection for the K⁺ phenotype alone did not affect cytoadherence. D6 parasites that were maintained in vitro without selection for 6 mo became K⁻, while D6 parasites continually selected for the K⁺C⁺ phenotype remained K⁺ and continued to bind to melanoma cells. Thus, the selective technique that we employed resulted in increased cytoadherence by two different culture adapted clones of *P. falciparum*. The cytoadherence assay results with the selected ITG2F6 parasites were similar to results obtained with parasites from infected humans and Aotus monkeys.

To determine whether changes in the cytoadherence phenotype were reversible, we began with K⁺C⁺ ITG2F6 parasites (1,078 IRBCs bound per 100 melanoma cells) and repeatedly selected for the K⁺ and the C⁻ phenotypes (Fig. 1). Cytoadherence decreased to <200 IRBCs per 100 melanoma cells after 18 selections over 12 wk (Table III). The difference in cytoadherence between K⁺C⁺ and K⁺C⁻ parasites was observed with both melanoma cells and endothelial cells, indicating that the selected parasites bound to the same receptor on both cell types. The IRBCs that were selected for the K⁺C⁻ phenotype reacted with mAb 89, were K⁺ by TEM, and were enriched in the supernatant of 0.5% gelatin/RPMI 1640, confirming that they were indeed K⁺. The K⁺C⁻ parasites were cloned (4 positive wells, 188 negative wells, probability of 0.99 that the parasites of each well were the progeny

TABLE I
*Knob and Cytoadherence Phenotypes of Cloned Parasites before
In Vitro Selection*

Clone	Knobs	KAHRP*	Cytoadherence [†]	<i>n</i>
B11	K ⁻	No	4.4 (0-13)	(5)
W2	K ⁻	No	0.5 (0-2)	(4)
D6	K ⁺	Yes	29 (0-87)	(5)
ITG2F6	K ⁺	Yes	77 (6-116)	(3)

* Knob-associated histidine-rich protein.

† Results presented as mean (range) (number of infected erythrocytes per 100 melanoma cells). *n*, number of experiments. Parasitemias were 2-6%.

TABLE II
*Selection of ITG2F6 and D6 Parasites for the K⁺ Phenotype and
 the K⁺C⁺ Phenotype*

Clone	Selection	Cytoadherence*	
			<i>n</i>
ITG2F6	None	77 (6-116)	(3)
ITG2F6	K ⁺	130 (13-277)	(10)
ITG2F6	K ⁺ C ⁺	1,092 (702-1,512)	(8)
D6	None	29 (0-87)	(5)
D6	K ⁺	20.3 (17-24)	(3)
D6	K ⁺ C ⁺	106 (27-273)	(8)

* See Fig. 1. Parasitemias were 2-6%.

of a single parasite). Each clone was also K⁺ and had a cytoadherence phenotype that was similar to that of the parent K⁺C⁻ IRBCs (Table III). The two clones with the fewest IRBCs per 100 melanoma cells in cytoadherence assays (ITG2F6-D5 and ITG2F6-E8) were then selected for the K⁺ and C⁺ phenotypes. After eight selections over 5 wk, the parasites of both clones bound to C32 melanoma cells much better (more IRBCs per 100 melanoma cells) than the K⁺C⁻ parasites (Table IV). Thus, the cytoadherence phenotype of cloned parasites was modulated sequentially from K⁺C⁺ to K⁺C⁻ and, after subcloning, back to K⁺C⁺.

Identification of Trypsin-sensitive Radioiodinatable Proteins with Cytoadherent IRBCs. IRBCs were radioiodinated by the lactoperoxidase technique and incubated with trypsin (10 µg/ml for 5 min) or trypsin plus an excess of soybean trypsin inhibitor. This concentration of trypsin was selected since the incubation of IRBCs with 10 µg/ml of trypsin prevented cytoadherence. The radioiodinated IRBCs were extracted sequentially with 1% Triton X-100 and 5% SDS electrophoresis sample buffer to obtain Triton X-100-insoluble proteins soluble in 5% SDS. Cytoadherent (K⁺C⁺) IRBCs had ¹²⁵I proteins with *M_r* > 2.4 × 10⁵ that were not detected with uninfected RBCs and that were cleaved by trypsin (Fig. 2 A). These proteins were detected with IRBCs extracted with 5% SDS electrophoresis sample buffer, but not with the Triton X-

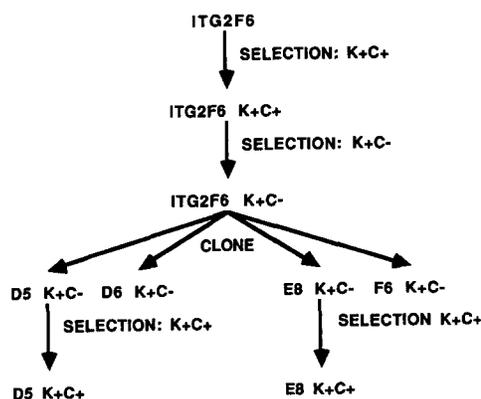


FIGURE 1. In vitro selection of K⁺ ITG2F6 parasites for sequential changes in cytoadherence phenotype.

TABLE III
Selection of K⁺C⁺ Parasites for the K⁺C⁻ Phenotype

Clone	Selection	KAHRP	Cytoadherence*	<i>n</i>
ITG2F6	K ⁺ C ⁺	Yes	1,078 (482-1,892)	(19)
ITG2F6	K ⁺ C ⁻	Yes	73.6 (11-169)	(3)
ITG2F6-D5†		Yes	55 (2-134)	(6)
ITG2F6-D6†		Yes	92 (31-173)	(6)
ITG2F6-E8†		Yes	78 (19-151)	(6)
ITG2F6-F6†		Yes	147 (33-282)	(6)

* See Table I.

† ITG2F6-D5, -D6, -E8, -F6 are subclones of the K⁺C⁻ ITG2F6 parasites (Fig. 1).

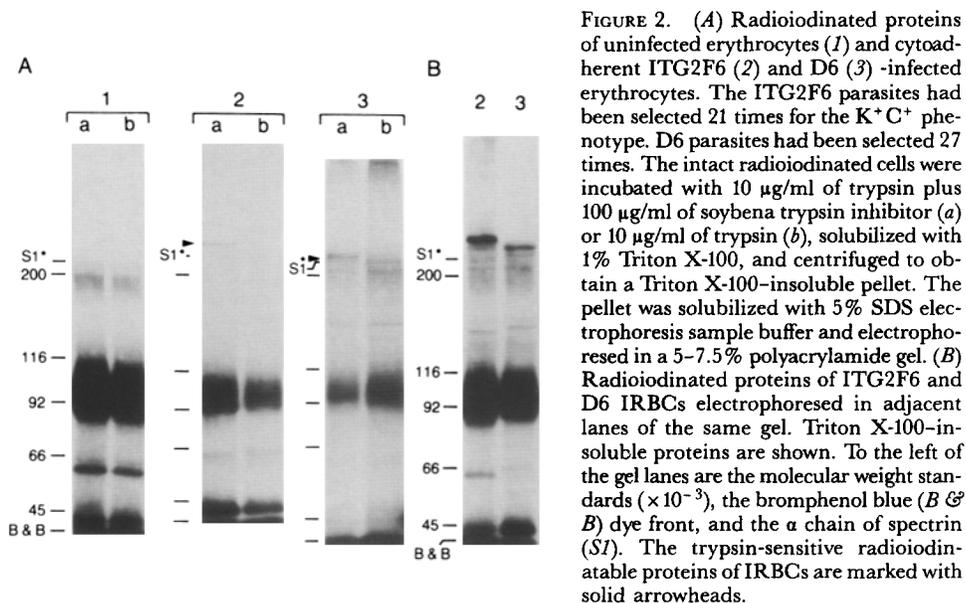
100-soluble extract (not shown). The M_r of the proteins was 2.6×10^5 for ITG2F6 and 2.4×10^5 for D6 (Fig. 2 B). Identical results were obtained in five experiments with each clone. The trypsin-sensitive ¹²⁵I proteins were not detected and were apparently lost after long-term culture without selection of ITG2F6 parasites (Fig. 3). Other labeled proteins included band 3, the major transmembrane protein of RBCs, which was detected with uninfected RBCs and with IRBCs; several ¹²⁵I proteins with M_r 1.2-1.6 $\times 10^5$; and ¹²⁵I proteins that comigrated with spectrin. Although these proteins were detected with IRBCs, they were not cleaved by trypsin (Fig. 2 A).

Appearance of Additional IRBC Surface Proteins after Repeated Selections for the K⁺C⁺ Phenotype. ITG2F6 parasites that were subjected to 21 selections for the K⁺C⁺ phenotype contained a single predominant trypsin-sensitive ¹²⁵I protein with M_r 2.6×10^5 . However, after continued selection for the K⁺C⁺ phenotype, two additional ¹²⁵I proteins with M_r 2.9-3.2 $\times 10^5$ were detected (Fig. 4 A). During the course of selection, the amount of radioactivity that could be introduced into the M_r $2.6 \times$

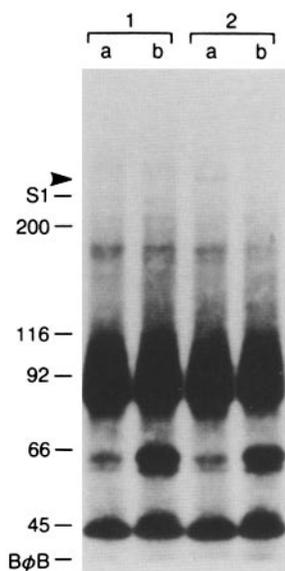
TABLE IV
Selection of Subcloned K⁺C⁻ Parasites for the K⁺C⁺ Phenotype

Parasites	Exp.	Selected phenotype	Parasitemia %	Cytoadherence*
ITG2F6-D5	1	K ⁺ C ⁻	2.4	191
		K ⁺ C ⁺	2.0	1,097
	2	K ⁺ C ⁻	7.4	80
		K ⁺ C ⁺	3.4	4,650
ITG2F6-E8	1	K ⁺ C ⁻	10.6	47
		K ⁺ C ⁺	7.2	350
	2	K ⁺ C ⁻	3.0	144
		K ⁺ C ⁺	3.2	1,494
	3	K ⁺ C ⁻	9.2	51
		K ⁺ C ⁺	5.5	800

* Results presented as the mean number of infected erythrocytes per 100 melanoma cells with each experiment performed in duplicate.



10^5 protein by radioiodination of IRBCs gradually decreased, and after 37 selections, the $M_r 2.9 \times 10^5$ protein became the predominant trypsin-sensitive ^{125}I protein (not shown). We hypothesized that new subpopulations of parasites having different IRBC surface proteins had arisen during repeated selection for the K^+C^+ phenotype. However, each of two subclones prepared from the parasite population with three ^{125}I proteins contained multiple trypsin-sensitive ^{125}I proteins (Fig. 4 B). Sur-



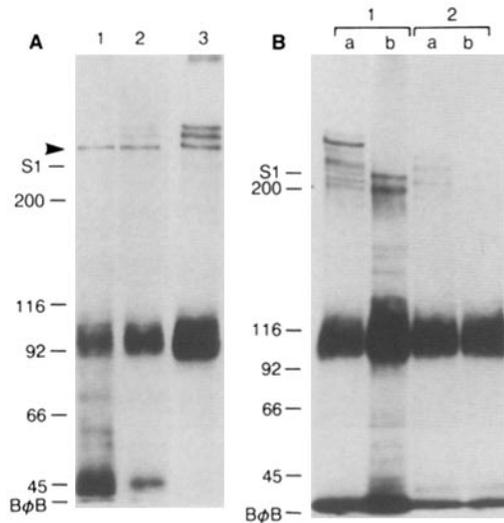


FIGURE 4. (A) Radiiodinated proteins of IRBCs containing ITG2F6 parasites that were selected for the K^+C^+ phenotype 25 times (1), 29 times (2), and 31 times (3). Triton X-100-insoluble proteins are shown. The parasites were cultured, the cells were labeled in parallel, and the proteins were subjected to electrophoresis in adjacent lanes of the same gel. (B) Radiiodinated proteins of the two clones (1 and 2) obtained by subcloning parasites selected 31 times for the K^+C^+ phenotype. The cells were incubated with trypsin plus inhibitor (a) or trypsin (b). Triton X-100-insoluble proteins are shown. Multiple trypsin-sensitive proteins were identified between $M_r 2.2 \times 10^5$ and 3.5×10^5 . The notations to the left of the figures are the same as in Fig. 2. The solid arrowhead marks the $M_r 2.6 \times 10^5$ ^{125}I protein. All lanes of Fig. 3 A are from the same gel, and all lanes of Fig. 3 B are from the same gel.

face radioiodination was performed 5 wk after cloning, indicating that the parasites rapidly produced polymorphic forms of the trypsin-sensitive radioiodinatable protein family.

Size Alteration of IRBC Surface Proteins Is Correlated with Selection for Increased or Decreased Cytoadherence. When *in vitro* selection for decreased cytoadherence was applied to the cytoadherent ITG2F6 parasites, the pattern of ^{125}I proteins on the resulting K^+C^- IRBCs changed. Compared with their K^+C^+ progenitors, the K^+C^- IRBCs did not have the $M_r 2.6 \times 10^5$ protein, but had a ^{125}I protein with $M_r 2.4 \times 10^5$ (Fig. 5 A). Two subclones of the K^+C^- population (D5 and E8) also lacked the $M_r 2.6 \times 10^5$ protein and had ^{125}I proteins with $M_r 2.4 \times 10^5$ (Fig. 5 B). Back selection for the K^+C^+ phenotype from these subclones was accompanied by loss of the $M_r 2.4 \times 10^5$ proteins and appearance of ^{125}I proteins with $M_r \geq 2.6 \times 10^5$ (Fig. 5 C).

The Molecular Size of KAHRP and MESA-PfEMP2 Does not Vary among ITG2F6 Parasites with Different Cytoadherence Phenotypes. KAHRP and MESA-PfEMP2 are parasite proteins that are associated with knobs and the erythrocyte membrane (19, 25, 26, 28). Both have been shown to vary in molecular size among parasites from different isolates (25, 26, 28). To confirm the clonal nature of ITG2F6 parasites having different cytoadherence and IRBC surface protein phenotypes, we examined the expression and molecular size of KAHRP and MESA-PfEMP2 by each of the phenotypically different populations of the parasites. There was no size variation of either protein among the phenotypically different ITG2F6 parasites (Fig. 6).

Discussion

We report that the capacity for cytoadherence by *P. falciparum*-infected erythrocytes is correlated with the expression of a family of parasite-determined proteins on IRBCs. Cytoadherent IRBCs from two cloned lines of culture-adapted parasites had large, radioiodinatable proteins that were not detected with uninfected erythro-

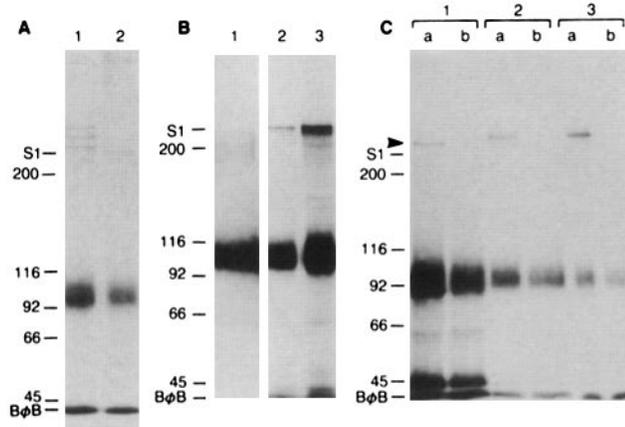


FIGURE 5. (A) Radioiodinated proteins of IRBCs containing parasites repeatedly selected for increased cytoadherence (K^+C^+) (1) or decreased cytoadherence (K^+C^-) (2). (B) Radioiodinated proteins of uninfected erythrocytes (1) and IRBCs containing the ITG2F6-D5 (2) and -E8 (3) subclones of K^+C^- ITG6F6 parasites. (C) Radioiodinated proteins of the IRBCs containing ITG2F6 parasites selected 21 times for the cytoadherent phenotype (1), ITG2F6-D5 (2) and -E8 (3) parasites after back selection for the K^+C^+ phenotype. The intact radioiodinated cells were incubated with trypsin plus inhibitor (a) or trypsin (b) as described for Fig. 2. See Fig. 1 for the selection protocol and Table IV for the results of cytoadherence assays with the parasites. The notations to the left of the figures are the same as in Fig. 2. The solid arrowhead marks the M_r 2.6×10^5 protein.

cytes or with nonadherent IRBCs containing parasites cultured for many months without selection. The radioiodinatable proteins that we detected with ITG2F6 and D6 parasites differed in molecular size, but were cleaved by the same concentration of trypsin that inhibited cytoadherence ($10 \mu\text{g/ml}$) and were not soluble in Triton X-100. The proteins that we identified on cytoadherent human erythrocytes containing culture-adapted parasites appear to be identical to the proteins with similar properties that were previously identified with IRBCs from Aotus monkeys (11). Because of their shared biochemical properties of accessibility to surface radioiodination, trypsin sensitivity, and detergent solubility, we argue that these polymorphic proteins constitute a family of parasite proteins expressed on the host erythrocyte. Other studies with cultured parasites have not detected similar proteins (29), but the cytoadherence phenotype of the parasites was not described and may have been negative since cultured parasites frequently become nonadherent (20, and this study).

Essential to this study was the development of *in vitro* techniques for modulating the cytoadherence phenotype of cloned parasites. In initial experiments, culture-adapted parasites were found to be less cytoadherent than parasites from infected humans or monkeys. Furthermore, cultured parasites frequently lost the capacity for cytoadherence altogether. Selection for the K^+ phenotype alone did not increase cytoadherence, but a coupled selection for the K^+ and C^+ phenotypes by gelatin enrichment followed by binding to C32 melanoma cells resulted in large increases in cytoadherence by ITG2F6 parasites (~ 15 fold) and smaller increases in cytoadherence by D6 parasites (~ 3.5 fold). The biological significance and the molecular

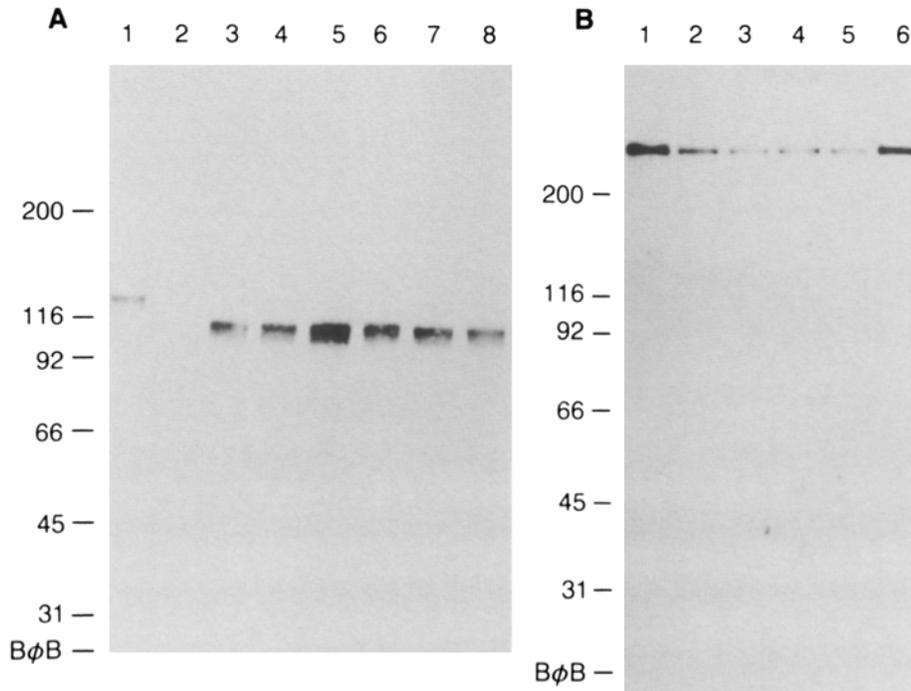


FIGURE 6. Autoradiographs of immunoblots probed with mAb 89 against KAHRP (A) and mAb 8B7.4 against MESA-PfEMP2 (B) followed by incubation of the blots with ^{125}I protein A. IRBCs containing trophozoites were purified (to >95% parasitemia) by centrifugation on a Percoll gradient as described (30). The IRBCs were solubilized with 5% electrophoresis sample buffer and subjected to electrophoresis in 5–15% polyacrylamide gels. (A) The lanes are as follows: 1, K^+C^+ D6 parasites; 2, K^-C^- D6 parasites that appeared after long term culture without selection; 3, ITG2F6 parasites selected 21 times for the K^+C^+ phenotype; 4, K^+C^- ITG2F6-E8 parasites; 5, K^+C^+ ITG2F6-E8 parasites; 6, K^+C^- ITG2F6-D5 parasites; 7, K^+C^+ ITG2F6-D5 parasites; 8, ITG2F6 parasites selected 31 times for the K^+C^+ phenotype. (B) The lanes are: 1, K^+C^- ITG2F6-E8 parasites; 2, K^+C^+ ITG2F6-E8 parasites; 3, K^+C^- ITG2F6-D5 parasites; 4, K^+C^+ ITG2F6-D5 parasites; 5, ITG2F6 parasites selected 31 times for the K^+C^+ phenotype; 6, ITG2F6 parasites selected 21 times for the K^+C^+ phenotype. The notations to the left of the figures are the same as for Fig. 2.

basis of such a quantitative difference in cytoadherence phenotype is unknown. However, the cytoadherent D6 parasites had no detectable MESA-PfEMP2 (Petersen, C., unpublished observation), suggesting that the protein, although apparently not necessary for knobs or cytoadherence, may increase the capacity for cytoadherence.

We observed that the cytoadherence phenotype of cloned, K^+ ITG2F6 parasites could be reversibly modulated *in vitro* by incubating IRBCs with C32 melanoma cells and selecting adherent or nonadherent cells for culture. Using this technique, we produced a sequence of changes in the cytoadherence phenotype (Fig. 1). The change from increased to decreased cytoadherence was correlated with a decrease in the molecular size of the trypsin-sensitive radioiodinatable protein (from M_r 2.6×10^5 to M_r 2.4×10^5), and the change from decreased cytoadherence to increased cytoadherence was correlated with an increase in the molecular size of the protein (from M_r 2.4×10^5 to M_r 2.6×10^5 and larger proteins). In contrast to these results, the molecular size of two other membrane associated proteins (KAHRP and MESA-

PfEMP2) did not change during selection for different cytoadherence phenotypes. Thus, the varied expression of the trypsin-sensitive, radioiodinatable protein appears to be uniquely and closely linked to the cytoadherence phenotype of IRBCs, and the correlation of the larger protein with increased cytoadherence and the smaller protein with decreased cytoadherence suggests that the larger proteins may contain domains that mediate or promote cytoadherence. Previous studies showed that the protein was detected with K⁺ but not with K-IRBCs (30) and that it was cleaved by the same concentration of trypsin that prevented cytoadherence (11). Taken together, the results suggest that members of this protein family have a role in mediating cytoadherence to endothelial cells and melanoma cells.

With continued selection of ITG2F6 parasites for the cytoadherent phenotype, we observed additional trypsin-sensitive, radioiodinatable proteins having larger molecular sizes. In contrast, cytoadherent parasites cultured and processed in parallel, but without the additional selections, continued to express only the M_r 2.6×10^5 protein. Although we suspected that the appearance of additional proteins was due to new populations of parasites, this was not confirmed by subcloning the parasites since the clones had a multiplicity of trypsin-sensitive, radioiodinatable proteins with M_r from 2.2 – 3.5×10^5 . Thus, the parasites had the capacity to produce rapid changes in the size of the protein, and newly cloned parasites produced multiple polymorphic forms of the protein. The synthesis of multiple polymorphic forms of the protein by newly cloned parasites could be due to the rapid production of new parasite populations, each having a different IRBC surface protein. The rapid appearance of multiple populations expressing different forms of the IRBC surface proteins might be due to rapid intragenic recombination and unequal crossing over as postulated for other variable malarial proteins such as the S-antigen (31) and the circumsporozoite protein (32). Alternatively, we cannot exclude the possibility that individual parasites may be capable of synthesizing multiple forms of the protein. Additional studies will be required to determine the molecular basis of this size polymorphism and whether the size polymorphism is accompanied by antigenic polymorphism.

We note an interesting parallel between our in vitro results and the reported in vivo modulation of cytoadherence and IRBC surface antigenicity by the spleen. In studies with both *P. falciparum* (9, 10) and *P. fragile* (33) (*P. fragile* is a knobbed, sequestering parasite of macaque monkeys), the expression of both the cytoadherence phenotype and the serologically detected IRBC surface antigen were linked. IRBCs from monkeys with spleens were cytoadherent and had the IRBC surface antigen; IRBCs from monkeys without spleens were not cytoadherent and had no detectable surface antigen. The variation in the family of trypsin-sensitive radioiodinatable proteins that we observed was also linked to changes in the cytoadherence phenotype and is consistent with the hypothesis that the protein is the surface antigen on IRBCs. The linked expression of the cytoadherence phenotype, the IRBC surface antigen, and the family of IRBC surface proteins suggests that changes in all three properties are initiated by the same genetic mechanism.

Summary

Plasmodium falciparum-infected erythrocytes (IRBCs) adhere specifically to venular endothelium and thereby evade spleen-dependent immune mechanisms. We have investigated the molecular basis of cytoadherence. We report here that the capacity

for cytoadherence of IRBCs is correlated with the expression of a family of variable proteins on the surface of IRBCs. Essential to these studies was the use of in vitro techniques for modulating the cytoadherence phenotype of cloned parasites. In initial studies, we found culture-adapted parasites to be poorly cytoadherent or noncytoadherent. To select for cytoadherent parasites, we incubated knobbed IRBCs with C32 melanoma cells and cultured the adherent cells. Repeated rounds of selection produced parasites with increased cytoadherence. To select for noncytoadherent parasites, we cultured the cells that did not adhere to C32 melanoma cells. Cytoadherent IRBCs from two different cloned isolates had large ($M_r > 2.4 \times 10^5$) radioiodinatable proteins that differed in size between the isolates but had in common the biochemical properties of trypsin sensitivity and insolubility with Triton X-100. The proteins were not detected with uninfected erythrocytes, indicating that they were parasite determined, nor were they detected with IRBCs containing parasites cultured for many months without selection. With continued selection for the cytoadherent phenotype, additional IRBC surface proteins with larger molecular sizes (M_r 2.9×10^5 and 3.2×10^5) appeared. A sequence of reversible changes in the cytoadherence phenotype of cloned parasites was accompanied by variation in the molecular size of the IRBC surface protein. Increased cytoadherence was correlated with expression of larger proteins and decreased cytoadherence was correlated with expression of smaller proteins; there was no change in the molecular size of two other parasite proteins associated with the IRBC membrane. The results indicate that the expression of this family of proteins is closely linked to the cytoadherence phenotype of the parasites, suggesting that the members of the protein family have a role in mediating cytoadherence between IRBCs and endothelial cells.

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