KNOB-INDEPENDENT CYTOADHERENCE OF
PLASMODIUM FALCIPARUM TO THE LEUKOCYTE
DIFFERENTIATION ANTIGEN CD36

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Erythrocytes infected with mature Plasmodium falciparum parasites sequester within the capillaries and postcapillary venules of organs such as the heart, placenta, and brain, thereby avoiding circulation through the spleen and subsequent destruction by host immune defences (1, 2). The sequestration of infected erythrocytes in the cerebral circulation, however, may result in the death of the host from cerebral malaria. It has previously been determined from autopsy studies that cytoadherence occurs at least in part at thickened areas of the erythrocyte membrane called "knobs" (3-5). A major structural protein in knobs is the knob-associated histidine-rich protein (KAHRP) (6-8). Studies of cytoadherence in vitro using umbilical vein endothelium or amelanotic melanoma cells also showed a correlation between binding and the presence of knobs (9-11). Culture-adapted isolates of P. falciparum often lose the ability to cytoadhere in vitro. This occurs independently of whether the knob-positive (K+) phenotype is retained or lost (K-), and therefore knobs were previously considered necessary but not sufficient for cytoadherence in vitro (12).

A major host cell receptor for cytoadherence to C32 melanoma cells is the Mr 88-kD leukocyte differentiation antigen, CD36, which is defined by the mAbs OKM5 and CI Mega I (13-17). This antigen is expressed in variable amounts on the surface of umbilical vein endothelium, C32 melanoma cells, monocytes, and platelets, and may serve as a receptor for the platelet protein, thrombospondin (TSP) (18). Immobilized, calcium-replete TSP is also able to bind infected erythrocytes, although some melanoma cells that express this protein do not bind infected erythrocytes (19, 20). Some short- and long-term cultured isolates of P. falciparum bind to both CD36 and TSP (15, 19), and, recently, another endothelial cell surface molecule, the inter-
cellular adhesion molecule 1 (ICAM-1), has been shown to be involved in cytoadherence in vitro (21).

Although knob-independent cytoadherence has been documented for immature gametocytes of *P. falciparum* (22) and other malaria species, such as *P. knowlesi* (23), *P. berghei* (24), and *P. chabaudi* (25), it has only recently been described for the asexual stages of *P. falciparum* (26, 27). In both cases described, K⁺ cytoadherent clones were obtained by repeated selection for a minor population of K⁻-infected erythrocytes that bound to melanoma cells. Knob-independent cytoadherence may occur via a similar mechanism to that of K⁺ lines, as Udomsangpetch et al. (27) showed that cytoadherence of their K⁻ line was inhibited by mAb OKM5.

We have investigated the cytoadherence of a K⁻ clone (B8C⁻) of *P. falciparum* to establish the characteristics of knob-independent cytoadherence. The cytoadherence of B8C⁻ to host cell proteins CD36, TSP, and ICAM-1, and the trypsin sensitivity, surface labeling, and immunoprecipitation of the parasite molecules involved in this process have been examined.

### Materials and Methods

**Selection of a K⁻ Cytoadherent Clone (B8C⁻).** The cytoadherence of an initially noncytoadherent, knobless IgG2F6 clone, B8, was enhanced by repeatedly selecting and culturing those few infected erythrocytes that bound to C32 melanoma cells (American Type Culture Collection, Rockville, MD) (26). Briefly, cytoadherence assays were performed as previously described (10, 28), except that, after washing away all unbound cells, complete medium with 10% AB human serum and uninfected erythrocytes at 2% hematocrit were added. Cultures were transferred to fresh dishes after schizont rupture when erythrocytes infected with ring-stage parasites were no longer cytoadherent. This selection process was repeated on six occasions until B8 was strongly cytoadherent (designated B8C⁻). Assays were performed using sterile conditions, and incubation steps were carried out in the same gaseous environment used for routine malaria culture (O₂, 5%; CO₂, 5%; N₂, 90%). Parasites were maintained in culture as previously described (29, 30).

**Trypsin Sensitivity of B8C⁻ Cytoadherence.** The trypsin sensitivity of B8C⁻ cytoadherence to C32 melanoma cells was compared with that of a K⁺ IgG2F6 sibling clone, FAC8, using a modification of the published method (31). Parasite cultures were washed three times in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) buffered with Hepes (BDH Chemicals Ltd., Poole, England). The cell pellets were resuspended in RPMI-Hepes containing trypsin (Sigma Chemical Co., St. Louis, MO) at a final concentration of 10, 1, or 0.1 μg/ml. Soybean trypsin inhibitor (100 μg/ml; Sigma Chemical Co.) was added simultaneously with trypsin (10 μg/ml) to controls. Tubes were incubated at 37°C for 30 min, and trypsin inhibitor was added to each tube to a final concentration of 10 times that of trypsin. 10% human serum in RPMI-Hepes was added to each sample to achieve a final volume of 2 ml. Cytoadherence assays were performed and results expressed as the number of infected erythrocytes bound to 100 melanoma cells.

**Cytoadherence to CD36 and TSP.** B8C⁻ and a K⁺ sibling clone, GBC6, were tested for cytoadherence to CD36 and TSP as described (15, 19). TSP was purified from platelets by affinity chromatography on heparin Sepharose and shown to migrate as an *M*₅₅~480-kD protein by SDS-PAGE (32). CD36 antigen was purified from Triton X-100 detergent lysates of platelets by affinity chromatography using mAb IA7 coupled to Sepharose 4B. The column was washed with 10 mM Tris, pH 7.6, 0.1% Triton X-100, and the protein was eluted with 10 mM diethylamine, pH 12, 0.01% Triton X-100. The purified sample was analyzed by SDS-PAGE with known standards, and silver stained. A single band of *M*₅₅~88 kD was evident. The concentration of this protein was estimated by reference to the standards. The concentration and purity of CD36 were confirmed by protein sequencing (Wilkinson, D., R. Aebersold, and A. W. Boyd, unpublished results). To test for cytoadherence to CD36, tissue culture cover-
slips (Thermanox; Miles Scientific, Naperville, IL) contained in the wells of a 24-well plate (Linbro Chemical Co., Hamden, CT) were incubated with 10 µl of soluble CD36 (1 µg/ml) overnight at 4°C. This was followed by incubation with 1% BSA in PBS for 1 h at room temperature. The coverslips were washed twice with RPMI-Hepes, and then 0.5 ml B8C' infected erythrocytes (2% hematocrit, 5-10% trophozoites) in 10% human serum in RPMI-Hepes was added to each well. The plates were rocked gently at 37°C for 1 h, and unbound erythrocytes were washed away with RPMI-Hepes. Bound erythrocytes were fixed in 2% glutaraldehyde and stained with 10% giemsa. Cytoadherence to TSP was assayed similarly except that TSP was diluted to 50 µg/ml in 1 mM CaCl₂, 10 mM Tris, 100 mM NaCl, pH 7.4. For blocking, BSA (1%) in 50 mM Tris, 5 mM CaCl₂, and 0.1 mM PMSF was used. In both types of assay, PBS and fibronectin (50 µg/ml) were used as negative controls. Results are expressed as the number of trophozoite-infected erythrocytes bound per mm².

**Cytoadherence to Human Umbilical Vein Endothelial Cells (HUVEC).** Cytoadherence assays were performed as described previously. HUVEC were shown by immunofluorescence with an anti–ICAM 1 mAb, WEHI-CAMI (33), and two anti-CD36 mAbs, IA7 and IE8, to express large amounts of ICAM 1, apparently on the cell surface, but little or no cell surface CD36. mAbs IA7 and IE8 were raised against CD36 that had been affinity purified on Cl Mega 1 antibody. Both IA7 and IE8 reacted with CD36 on the surface of platelets, monocytes, and C32 melanoma cells. Results are expressed as the number of infected erythrocytes bound to 100 endothelial cells. Two sibling K1G2F6 clones, FAF6 and GBC6, were included in the same experiment as positive and negative controls.

**Inhibition of Cytoadherence to Melanoma Cells and CD36.** Melanoma cells or CD36-coated coverslips were preincubated for 1 h at 37°C with RPMI-Hepes containing either rabbit antiserum to CD36, a mAb to CD36, FA6-152 (1/100 dilution of ascites; reference 34), soluble TSP (50 µg/ml), or a tetrapeptide known to be important in TSP-cell adhesion, asparagine, glycine, aspartic acid, and serine (RGDS: 500 µg/ml). The mAb FA6-152, unlike IA7 and IE8, had been previously shown to inhibit cytoadherence of infected erythrocytes to melanoma cells (unpublished data). A rabbit antiserum was raised against purified platelet CD36. This rabbit antiserum was shown to be specific for CD36 by immunoprecipitation of a single protein of Mr ~88 kD from 125I-labeled platelets and also by immunofluorescence analysis of CD36⁺ and CD36⁻ cell lines. Preimmune rabbit serum was used as a control.

**Labeling and Immunoprecipitation.** Synchronized cultures containing trophozoites were used for surface radioiodination. The infected erythrocytes were purified to >99% parasitemia by centrifugation through a Percoll gradient (36) before labeling. Alternatively, the cells were incubated with a monolayer of C32 melanoma cells in 75-cm² tissue culture flasks. Uninfected erythrocytes and nonadherent infected erythrocytes were removed by washing, and the adherent infected erythrocytes were released from the melanoma cells by striking the bottom of the flask briskly with the back of the hand. Purified infected erythrocytes were surface radioiodinated using the lactoperoxidase technique (37). For metabolic labeling, cultures were synchronized at the ring stage and cultured for 24 h with 35S methionine (100 µCi/ml; Amersham Corp., Arlington Heights, IL) until the parasites were trophozoites. The infected erythrocytes were purified as described above.

Surface-labeled or metabolically labeled infected erythrocytes were incubated for 5 min at 18°C with trypsin-α-tosylamide-2-phenylglycine methyl ketone (TPCK) ( Worthington Biochemical Corp., Freehold, NJ) at concentrations of 0.1, 1.0, or 10 µg/ml in PBS or with trypsin-TPCK (10 µg/ml) and soybean trypsin inhibitor (100 µg/ml). An excess of soybean trypsin inhibitor (100 µg/ml) was added, the cells were washed with PBS, and then extracted sequentially with 1% (wt/wt) Triton X-100 followed by 2% (wt/wt) SDS, as previously described (37).

Immunoprecipitation analysis was performed using pooled human sera collected from adults residing in areas endemic for P. falciparum malaria and a mAb to the mature erythrocyte surface antigen (MESA), mAb 8B7.4, that was kindly provided by Dr. Russell Howard, DNAX Corp., Palo Alto, CA. The Triton X-100 and SDS extracts were diluted with equal volumes of 10% Triton X-100 and 3 vol of 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.5% (wt/vol) Triton X-100, pH 7.4, and incubated with antibody for 18 h at 4°C. Immune complexes were collected with protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), and the
beads were washed as described (38). Samples were boiled for 1 min and proteins separated by electrophoresis in a 6% polyacrylamide gel. Labeled proteins were identified by autoradiography.

Results

Cytoadherence to CD36 and TSP. The C32 melanoma cells used in these studies were shown by immunofluorescence to express high levels of CD36 and ICAM 1, but very little TSP (data not shown). To determine which of these receptors were important in the cytoadherence of B8C+, we performed experiments to test cytoadherence to purified TSP and CD36 immobilized on plastic coverslips, and to HUVEC expressing ICAM 1 but little or no surface CD36. Soluble fibronectin was used as a negative control. We found that B8C+ bound strongly to CD36 but showed minimal binding to TSP or fibronectin (Table I). The number of B8C+ trophozoite-infected erythrocytes binding to CD36 varied from 4,910 to 7,470 per mm². Binding of the K+ sibling clone, GBC6, varied from 5,200 to 14,170 infected erythrocytes per mm² in three different experiments. The binding of B8C+ to TSP was 20-50-fold lower than that of GBC6. In three different experiments, 90, 50, and 715 B8C+-infected erythrocytes bound per mm², compared with 1,965, 2,530, and 5,505 GBC6-infected erythrocytes per mm².

Cytoadherence to HUVEC Expressing ICAM 1. Both B8C+ and GBC6 failed to bind to HUVEC (0, 2, and 3; and 10, 10, and 2 infected erythrocytes per 100 endothelial cells in three different experiments) that were shown to express large amounts of surface ICAM 1 (data not shown). Another K+ sibling clone, FAF6, which was included as a positive control, bound 276 infected erythrocytes per 100 endothelial cells.

Inhibition of Cytoadherence to CD36 and Melanoma Cells by Antibody. The ability of polyclonal and monoclonal antibodies raised against CD36 to block cytoadherence was tested in order to determine if CD36 was the major receptor involved in the cytoadherence of B8C+ to C32 melanoma cells (see Table II). The cytoadherence of B8C+ erythrocytes to CD36 was reduced 94% by the anti-CD36 mAb, FA6-152.
TABLE II

Inhibition of cytoadherence of B8C⁺ to CD36 and C32 Melanoma Cells

<table>
<thead>
<tr>
<th>Preincubation with:</th>
<th>CD36</th>
<th>C32 melanoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune rabbit sera*</td>
<td>1:100 dilution</td>
<td>4,030¹</td>
</tr>
<tr>
<td>Rabbit antibody to CD36</td>
<td>1:10 dilution</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:100 dilution</td>
<td>140 (96%)</td>
</tr>
<tr>
<td></td>
<td>1:1,000 dilution</td>
<td>ND</td>
</tr>
<tr>
<td>RPMI-Hepes*</td>
<td>1:100 dilution</td>
<td>4,505</td>
</tr>
<tr>
<td>mAb FA6-152</td>
<td>1:10 dilution</td>
<td>0 (100%)</td>
</tr>
<tr>
<td></td>
<td>1:100 dilution</td>
<td>295 (94%)</td>
</tr>
<tr>
<td></td>
<td>1:1,000 dilution</td>
<td>1,435 (68%)</td>
</tr>
<tr>
<td></td>
<td>1:10,000 dilution</td>
<td>4,470</td>
</tr>
<tr>
<td>RPMI-Hepes*</td>
<td>1:100 dilution</td>
<td>5,746</td>
</tr>
<tr>
<td>Soluble TSP (50 μg/ml)</td>
<td></td>
<td>1,900 (57%)</td>
</tr>
<tr>
<td>RPMI-Hepes*</td>
<td></td>
<td>4,700</td>
</tr>
<tr>
<td>RGDS peptide (0.5 mg/ml)</td>
<td></td>
<td>4,145</td>
</tr>
</tbody>
</table>

Results from one representative experiment. Assays were performed in duplicate and the mean result is shown.
* CD36 or C32 melanoma cells were preincubated with preimmune rabbit sera or RPMI-Hepes as a positive control for each experiment.
¹ The results of cytoadherence to immobilized CD36 (1 μg/ml) are expressed as the number of infected erythrocytes bound per mm².
⁵ The results of cytoadherence to C32 melanoma cells are expressed as the number of infected erythrocytes bound per 100 melanoma cells.
Figures in parentheses represent the percentage inhibition of cytoadherence when compared with the positive control for that experiment.

at a dilution of 1:100, and 96% by a rabbit antiserum raised against purified CD36. Similarly, cytoadherence to C32 melanoma cells was inhibited by FA6-152 (96%), and the rabbit antiserum (88%) at dilution 1:100. Other experiments performed using different dilutions of antibody gave comparable results (data not shown).

Effect of RGDS and Soluble TSP on Cytoadherence. To determine if the molecule responsible for the cytoadherence of B8C⁺ had a TSP-like moiety, we attempted to inhibit cytoadherence to CD36 with the peptide RGDS, which is known to be important in TSP-cell adhesion, and with soluble TSP (50 μg/ml). Even at high concentration (500 μg/ml), RGDS failed to inhibit cytoadherence of B8C⁺ to CD36 (Table II). Soluble TSP inhibited cytoadherence of B8C⁺ to CD36 (67%) but did not inhibit cytoadherence to C32 melanoma cells.

Trypsin Sensitivity of K⁺ Cytoadherence. The trypsin sensitivity of proteins involved in the cytoadherence of the K⁺ cytoadherent clone B8C⁺ was compared with that of a sibling K⁺ ItG2F6 clone. Binding of B8C⁺ was exquisitely sensitive to treatment with trypsin at 0.1, 1.0, and 10 μg/ml, reducing cytoadherence by 56, 92, and 96%, respectively. This response was similar to the effect of trypsin on the K⁺ clone, FAC8, for which cytoadherence was reduced by 31, 86, and 94%, respectively (see Table III). Other experiments performed using different concentrations of trypsin and different K⁺ ItG2F6 clones as controls gave similar results (data not shown).
TABLE III
Trypsin Sensitivity of B8C+ and a K+ Sibling Clone, FAC8

<table>
<thead>
<tr>
<th>Trypsin concentration (µg/ml)</th>
<th>B8C+</th>
<th>FAC8*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>35 (96)</td>
<td>70 (94)</td>
</tr>
<tr>
<td>1</td>
<td>76 (92)</td>
<td>164 (86)</td>
</tr>
<tr>
<td>0.1</td>
<td>392 (56)</td>
<td>825 (31)</td>
</tr>
<tr>
<td>Control*</td>
<td>900</td>
<td>1,192</td>
</tr>
</tbody>
</table>

Results from one representative experiment. Assays were performed in duplicate and the mean result is shown.

* Results are expressed as the number of infected erythrocytes bound to 100 melanoma cells.
1 The percent reduction in cytoadherence is shown in parentheses.
5 Trypsin (10 µg/ml) and trypsin inhibitor (100 µg/ml) were added simultaneously to controls.

Surface Labeling of Infected Cells. In previous studies, a surface radioiodinatable, trypsin-sensitive, Triton X-100-insoluble protein (P. falciparum erythrocyte membrane protein 1; PfEMP1) was identified on K+ C+ -infected erythrocytes. By virtue of its surface localization and trypsin sensitivity, PfEMP1 has been implicated as a potential cytoadherence protein (31, 39). To determine whether B8C+ -infected erythrocytes expressed PfEMP1, we surface radioiodinated infected erythrocytes containing trophozoites. An Mr ~300-kDa radioiodinated protein doublet was identified, which like PfEMP1, was cleaved by incubating the cells with trypsin (Fig. 1 A). In contrast to the PfEMP1 of K+ C+ -infected erythrocytes, the trypsin-sensitive radioiodinated protein doublet on B8C+ cells was present in both Triton X-100-soluble and -insoluble proteins.
soluble fractions. It was immunoprecipitated by antibody from pooled human immune sera (Fig. 1 B, lane 1), but not by the anti-MESA mAb 8B7.4 (Fig. 1 B, lane 2). A high $M_r$ trypsin-sensitive protein was immunoprecipitated from K$^+$ clones GBC6 and FAF6, although this protein was not found in the Triton X-100-soluble fraction (data not shown).

Discussion

The selection of a K$^-$ clone, B8C$^+$, for strong cytoadherence to C32 melanoma cells has enabled us to show that K$^-$-infected erythrocytes can bind to the leukocyte differentiation antigen CD36. Cytoadherence to C32 melanoma cells was inhibited by both an anti-CD36 polyclonal rabbit antiserum and mAb FA6-152, suggesting that CD36 is the major receptor involved in this type of cytoadherence. Furthermore, B8C$^+$ did not bind to HUVEC that were shown to express high levels of ICAM 1 but little CD36 on the cell surface, suggesting that ICAM 1 is unlikely to be an alternative receptor for knob-independent cytoadherence of this clone. FAF6, another P. falciparum clone derived from ITG2F6, did cytoadhere to endothelial cells, but it has not been established whether this binding is via ICAM 1 or another endothelial cell surface molecule.

In contrast to K$^+$ clones, B8C$^+$ showed very low levels of adherence to TSP. This raises the possibility that knobs or the expression of the KAHRP may be important in the cytoadherence of infected cells to TSP. It is also possible that the selection of B8C$^+$ for cytoadherence to C32 melanoma cells, which are known to secrete only small amounts of TSP (unpublished data), resulted in the selection of a parasite expressing large amounts of a protein that specifically binds to CD36. Alternatively, different domains in a single parasite molecule may mediate binding to CD36 and TSP. If this is the case, the B8C$^+$ cytoadherence molecule must have undergone a modification that prevented cytoadherence to TSP. The observation that cytoadherence of B8C$^+$ to melanoma cells was abolished by the same low concentration of trypsin (1 $\mu$g/ml) that is required to abolish cytoadherence of a number of other lines favors the hypothesis that a single protein is involved in the cytoadherence of both K$^+$ and K$^-$ clones.

We identified a large radioiodinatable protein doublet on the surface of B8C$^+$-infected erythrocytes. Like the putative cytoadherence molecule PfEMP1 (31, 37), these proteins were cleaved by incubating infected erythrocytes with the same low concentration of trypsin that abolishes cytoadherence. Unlike PfEMP1, however, the proteins were partially soluble in 1% Triton X-100. These radioiodinated proteins were shown to differ from MESA by immunoprecipitation analysis with mAb 8B7.4. This anti-MESA mAb immunoprecipitated an $M_r$ ~300-kD metabolically labeled protein, but not the trypsin-sensitive, radioiodinated protein doublet. We suggest that the $M_r$ ~300-kD radioiodinated protein doublet of B8C$^+$-infected erythrocytes is PfEMP1, and that its solubility with Triton X-100 is due to differences in the interaction of the proteins with the erythrocyte membrane and membrane skeleton in K$^-$/C$^+$ cells compared with K$^+/C^+$ cells. PfEMP1 has not been detected previously in K$^-$ lines (36). Our observation of a PIEMP1-like molecule associated with K$^-$/C$^+$-infected erythrocytes is consistent with a role for PIEMP1 in cytoadherence to CD36 and C32 melanoma cells, and indicates that the expression of PIEMP1 on the surface, and cytoadherence to CD36, can occur independently of the presence of knobs.
Although cytoadherence of the asexual stages of \textit{P. falciparum} to CD36 may occur in the absence of knobs, this may not be relevant to malaria in vivo. Previous reports suggest a good correlation between the presence of knobs, sequestration, and virulence in vivo \citep{MacPherson1985, Howard1988}. It is conceivable that the expression of KAHRP and/or knobs on the erythrocyte surface allows initial low affinity attachment to TSP present at the endothelial cell surface. High affinity attachment may then occur via CD36 and perhaps other host cell receptors, such as ICAM 1. The knob-independent mechanism of cytoadherence to CD36 may be of primary importance in the cytoadherence to endothelial cells of other malaria species and of the immature sexual stages of \textit{P. falciparum}.

### Summary

The survival of \textit{Plasmodium falciparum}-infected erythrocytes is enhanced by the sequestration of mature trophozoites and schizonts from the peripheral circulation. Cytoadherence of infected erythrocytes in vivo is associated with the presence of knobs on the erythrocyte surface \citep{Howard1988}, but we and others have shown recently that cytoadherence to C32 melanoma cells may occur in vitro in the absence of knobs \citep{Langreth1978, Trager1966}. We show here that a knobless clone of \textit{P. falciparum} adheres to the leukocyte differentiation antigen, CD36, suggesting that binding to CD36 is independent of the presence of knobs on the surface of the infected erythrocyte. This clone showed little cytoadherence to immobilized thrombospondin or to endothelial cells expressing the intercellular adhesion molecule 1. Furthermore, an \(M_r\approx300\text{-kD}\) trypsin-sensitive protein doublet was immunoprecipitated from knobless trophozoite-infected erythrocytes. Finding a \textit{P. falciparum} erythrocyte membrane protein 1 (PfEMP1)-like molecule on these infected erythrocytes is consistent with a role for PfEMP1 in cytoadherence to CD36 and C32 melanoma cells.

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### References

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