Involvement of CD36 on Erythrocytes as a Rosetting Receptor for Plasmodium falciparum-Infected Erythrocytes

By Shiroma M. Handunnetti, Marie Rose van Schravendijk, Thomas Hasler, John W. Barnwell, Dale E. Greenwald, and Russell J. Howard

Plasmodium falciparum-infected erythrocytes (parasitized red blood cells [PRBCs]) can adhere to uninfected erythrocytes (RBCs) to form rosettes, and adhere to the endothelial cell (EC) surface antigen CD36. These adherence phenomena have previously been considered quite different. We show that anti-CD36 monoclonal antibodies (MoAbs) reverse rosetting of PRBCs from both a culture-adapted line (Malayan Camp [MC] strain) and a natural isolate, GAM425. Three MoAbs that block adherence of PRBCs to ECs or C32 melanoma cells also reversed rosetting by greater than 50% at levels of less than 1 μg/mL (OKM5, OKM8, and 8A6). Two other MoAbs that react with purified CD36 (1D3 and 1B1), but do not react with the surface of C32 cells, failed to reverse rosetting. When rosettes were disrupted and the RBCs and PRBCs were pretreated separately with antibodies before mixing to allow rosette reformation, only pretreatment of RBCs had an effect. MoAb 8A6 pretreatment of RBCs blocked rosette reformation, while MoAb 1B1 pretreatment did not. Rosetting was also reversed by purified human platelet CD36. In conjunction with evidence that CD36 is expressed on normal human erythrocytes (van Schravendijk et al, Blood 80:2105, 1992), we conclude that this CD36 is able to act as a host receptor for rosetting in the MC strain and some natural isolates of P. falciparum.

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Human erythrocytes infected with mature asexual stages of the malaria parasite Plasmodium falciparum (parasitized red blood cells [PRBCs]) attach to several host cells, including endothelial cells (ECs), monocytes, and platelets. In addition, with some parasites, uninfected erythrocytes (RBCs) attach to PRBCs, forming a rosette of up to 10 attached RBCs. Attachment of PRBCs to ECs lining postcapillary venules allows them to sequester from the peripheral blood and avoid destruction in the spleen. While the advantages conferred to the parasite by RBC rosetting around PRBCs remain speculative, some results suggest that when rosetting occurs in natural infections it contributes to the pathology of acute cerebral malaria.

Attachment of PRBCs to monocytes and C32 human amelanotic melanoma cells has been shown to involve the CD36 glycoprotein (also called GPIIIb or GPIV). PRBC attachment to human umbilical vein ECs involves CD36 or intercellular adhesion molecule-1 (ICAM-1) with some parasites. PRBCs also attach specifically to purified CD36 immobilized on plastic, to COS7 cells transiently transfected with CD36 cDNA, and to stable transfectants of Chinese hamster ovary (CHO) cells expressing cell surface CD36 (Hasler et al, submitted). Liquid-phase CD36 can also bind to PRBCs. Anti-CD36 monoclonal antibodies (MoAbs) such as OKM5, OKM8, and 8A6 can inhibit adherence of PRBCs to C32 melanoma cells, monocytes, COS-CD36 cells, and CD36 on plastic. These results have led to the concept that P. falciparum-PRBCs express a surface receptor for CD36 and that this participates in attachment to ECs.

The mechanisms of attachment of PRBCs to ECs (or to C32 melanoma cells) and to RBCs in rosettes have previously been thought to be quite different. Attachment of strain C+ PA PRBCs to C32 cells is reversed by the human IgM MoAb 33G2, but this MoAb has no effect on rosetting by the cloned line R+ PA1. Murine MoAb 89, which reacts with malarial histidine-rich protein HRP1, reverses rosettes of clones R+ PA1 and R+ TM180, but has no effect on attachment of those parasites to C32 cells. Treatment of rosettes with heparin disrupts them, whereas heparin has no effect on PRBC attachment to C32 cells.

Finally, PRBCs from some laboratory-derived or natural isolates attach to CD36, but do not rosette.

Specifically, the surface receptor for CD36 on PRBCs has previously been discounted as relevant to the RBC rosetting phenomenon. In one study, the anti-CD36 MoAb OKM5 was added to malaria cultures containing synchronous ring stages of the R+ PA1 clone before expression of the rosetting phenotype, and no effect on rosetting was detected after overnight parasite growth to the rosetting trophozoite stage. Furthermore, CD36 was not detected on adult erythrocytes in a comparative study on CD36 expression by different erythroid cells.

We report here that antibodies against CD36, including OKM5 and OKM8, reverse rosetting, and that only those anti-CD36 MoAbs capable of blocking PRBC adherence to CD36-bearing cells are able to reverse rosetting. Liquid-phase CD36 is shown to reverse rosetting, while a CD36 analogue from bovine milk does not. We have recently shown expression of low levels of CD36 antigen on the surface of RBCs in normal human blood. We conclude that attachment to RBC CD36 via the PRBC surface receptor for this molecule does contribute to the cell adhesion processes responsible for rosetting. These results

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have implications for malaria pathogenesis, including relevance to cerebral malaria, and for the phenotypic diversity of PRBC receptors.

MATERIALS AND METHODS

Antibodies. The mouse IgG1 MoAbs OKM5 and OKM8 were generous gifts of Dr P. Rao (Ortho Pharmaceuticals, Raritan, NJ). The properties of these antibodies against CD36 have been described.21 Other mouse IgG1 MoAbs specific for human CD36, 8A6, 1D3, and 1B1 were produced and characterized as described elsewhere15 and were used as protein A-Sepharose-purified antibodies. Mouse IgG MoAbs SB7,4 and 89, specific for the P falciparum proteins PTEMP2 and PHRP1 located under the PRBC outer membrane at knobs,22,23 were used as controls. The human IgM MoAb 33G215,16 was kindly provided by Dr K. Berzins (University of Stockholm, Sweden).

Assays for properties of anti-CD36 antibodies. The reactivity of anti-CD36 antibodies with human platelet-derived CD36 was assessed by several techniques. CD36 that had been electrophoresed on reducing or nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore Corp, Bedford, MA; no. IPVH-151-50) was probed in standard Western blotting protocols using anti-CD36 MoAbs at 10 μg/mL. For detection of murine IgG MoAbs bound to the membrane, we used a 1:1,000 dilution of alkaline phosphatase-labeled Go anti-MuIgG (H + L) (Cappel/Organon Teknika Corp, Durham, NC; no. 59296) followed by BioRad substrate kit no. 179-6432 (5-bromo-4-chloro-3-indolylphosphate and p-nitroblue-tetrazolium chloride; BioRad, Richmond, CA). Reactivity of antibodies with CD36 coated on plastic was measured by standard enzyme-linked immunosorbent assay (ELISA) protocols, coating 50 μL CD36/well at 1 μg/mL at 4°C overnight on flexible Microtiter plates (Dynatech Labs Inc, Chantilly, VA; no. 001-010-2401) and probing with 0.1 to 10 μg/mL anti-CD36 MoAbs and 1/1,000 dilution of alkaline phosphatase-labeled Go anti-MuIgG (H + L) (Cappel) and BioRad substrate kit no. 172-1063 (p-nitrophenyl phosphate and diethanolamine buffer). Methods for immunoprecipitation and for blockade of adherence to C32 cells have been described.9,12,24 Reactivity of antibodies with the surface of nonfixed C32 cells and CHO-CD36 cells (stable transformants we generated that express human CD36) was measured by fluorescence-activated cell sorting (FACS) analysis.

Parasites and cells. The Malayan Camp (MC) strain of P falciparum adapted to growth in spleen-intact Aotus monkeys was subsequently adapted to in vitro growth in human RBCs.9 After repeated selection for expression of knobs and the capacity to form rosettes, a parasite line was derived that was knob-positive (K+) and rosetting-positive (R+) with 80% to 95% of mature PRBCs in rosettes.6,17 Although rosetted PRBCs adhere to C32 cells or CD36 markedly less than K+R- PRBCs derived from the same strain, purified PRBCs from disrupted rosettes adhere strongly to C32 cells and CD36.25 The K+R- parasite was cryopreserved at the ring stage, thawed, and cultured in vitro by standard methods, except for the use of fetal calf serum (FCS) in the culture medium.7,16 These in vitro cultures were maintained for up to 3 weeks after thaw and were used as the source of parasite material for experiments. In other experiments, an isolate of P falciparum cryopreserved at the ring stage from a Gambian malaria patient (sample 425) was thawed and cultured for 30 hours in vitro in the donor's original RBCs (blood group O), under standard conditions.26 The PRBCs in this isolate were then in mature asexual stages, and the cytoadherence properties of these PRBCs have been described elsewhere.18 With the patient's own RBCs, less than 5% of PRBCs were in rosettes,18 but when O+ RBCs from healthy adult donors were added, 50% of PRBCs formed rosettes, and all rosetting experiments with these PRBCs were performed with normal O+ RBCs. Normal O+ human RBCs were obtained from healthy adult donors and were depleted of leukocytes before use by passage through a RC50 transfusion grade leukocyte removal filter (Pall Biomedical Products Co, East Hills, NY).

Purification of PRBCs from rosettes. Cultures of MC strain K+R+ parasites were selected at a time of maximum parasitemia of mature, pigmented PRBCs (usually 1% to 5%). Between 80% and 95% of pigmented PRBCs were in rosettes. For studies on the reversal of rosettes by antibodies or proteins, the cultured cells were washed twice by centrifugation (500 g for 5 minutes at 20°C) in RPMI without sodium bicarbonate, containing additional glucose (2 g/L), 30 mmol/L HEPES, pH 6.8, gentamycin (100 μg/mL), and 10% fetal bovine serum (binding medium) and the samples used directly. The omission of bicarbonate and addition of HEPES ensures stable pH in the region of maximal rosetting and cytoadherence.16,27 For studies on antibody-mediated blockade of rosette reformation, the PRBCs were purified as follows. When greater than 90% of mature PRBCs were in rosettes, the sample was subjected directly to disruption of rosettes by heparin treatment followed by separation of PRBCs by the gelatin-flotation method.17,28 In some experiments, an additional gelatin step was included before the disruption of rosettes to remove nonrosetting parasites and thereby enrich the rosetting parasites in the culture. In this way, the results with purified PRBCs would reflect the properties of cells that were originally in rosettes.

Assay for rosetting. RBs attached to mature PRBCs were enumerated by light microscopy.8 Mature PRBCs were detected by the presence of refractile malaria pigment. Triplicate measurements of 100 mature PRBCs were made, enumerating PRBCs with 0, 1, 2, or greater than 2 attached RBCs. We calculate the percentage of PRBCs in the number of PRBCs with greater than 2 attached RBCs. Conventional light microscopy and Giemsa staining was performed on all control samples to ensure that the percentage of rosetting PRBCs was consistent with the percentage of pigmented, mature PRBCs.

Antibody- and CD36-mediated reversal of rosetting. Trophozoite- or schizont-infected RBCs from cultures (1% to 5% parasitemia) were washed twice with binding medium and resuspended to a 50% hematocrit (Hct). Ten microliters of this suspension was added to 50 μL of antibody dilution or CD36 preparation, mixed gently, and incubated at 37°C for 1 hour. Binding medium was used to prepare cell suspensions and dilutions of antibodies and proteins. Rosetting was assessed as described above.

Antibody-mediated blockade of rosetting. Uninfected RBCs and PRBCs (purified to 50% to 80% parasitemia by gelatin enrichment as described above) were incubated (10% Hct) with anti-CD36 antibodies at 4°C for 30 minutes in binding medium. Cells were washed twice with 1 mL of ice-cold binding medium. Five microliters of RBCs and 5 × 10^8 PRBCs were mixed in 50 μL binding medium and incubated at 37°C and assessed for rosette reformation after 2 hours.

Purification of human platelet CD36 and bovine milk CD36. Human platelet CD36 was partially purified as previously described.29 Briefly, a membrane fraction was prepared from frozen human platelets, followed by TX114 phase partitioning, anion exchange chromatography, and gel filtration. The peak fraction of CD36 (purification no. 7, pool of fractions 45-47, denoted 7.45-47) showed one major band at 88 Kd on SDS-PAGE after silver staining and several minor bands of lower molecular weight. CD36 from bovine milk was purified by cation exchange chromatography, as previously described,30 and was pure as judged by SDS-PAGE.

For rosetting reversal, detergent was removed from the protein
samples as far as possible. Different fractions of human platelet
CD36 from the gel filtration step (0.1 mL each; 7.43-44 pool;
7.45-47 pool; fraction 7.49; all containing 0.1% Lubrol) and bovine
milk CD36 (30 µL, 0.5 mg/mL, in TBS containing 0.1% TX100)
were passed through a 0.1 mL Extractigel (Pierce, Rockford, IL)
column equilibrated with phosphate-buffered saline (PBS). Total
protein concentration was assayed with the bicinchoninic acid
(BCA) assay (Pierce) relative to bovine serum albumin (BSA) as
standard and CD36 concentration was estimated by silver-stained
SDS-PAGE gels relative to a purified CD36 standard. The proteins
were diluted with binding medium for the rosette reversal assays. They
were also tested for cytoadherence activity by spotting 10-µL
aliquots of dilutions in PBS ranging from 1/20 to 1/640 on to
bacteriologic petri dishes and performing a standard cytoadher-
ce assay using the parafilm method* with MC R+ parasites at
7.5% parasitemia and with 60% of PRBCs in rosettes.

RESULTS

Properties of anti-CD36 MoAbs. The panel of anti-CD36
antibodies was tested for reactivity with CD36 in several
assays, including assays using purified CD36 and assays in
which antibody binding to CD36 was tested on unfixed cells
expressing the native protein (Table 1). MoAbs 8A6, OKM5, and
OKM8 react with CD36 immobilized on plastic in ELISA, and with CD36
expressed on C32 melanoma cells or stable transformants of CHO cells expressing CD36. The same antibodies also block attachment of PRBCs to
C32 cells** (Barnwell, unpublished results). The two anti-CD36 MoAbs 1B1 and 1D3 did not react with CD36 expressed on C32 cells or on CHO cell transformants. This pair of MoAbs also failed to affect PRBC attachment to
C32 melanoma cells** (Barnwell, unpublished results). Both of these MoAbs did react, however, with CD36 in
ELISA, immunoprecipitation, and after Western Blotting under nonreducing conditions (Barnwell, unpublished
results), suggesting that the epitopes they recognize are not
accessible on native CD36 protein as expressed on the
surface of a cell.

Reversal of rosettes by anti-CD36 antibodies. The panel
of murine IgG anti-CD36 MoAbs was tested for their
capacity to reverse *P. falciparum rosettes at 10 µg/mL final
concentration (Fig 1 and Table 2). OKM5, OKM8, and 8A6
reversed rosetting by greater than 95%. The 1D3 and 1B1
antibodies did not affect rosetting at this antibody concen-
tration (Fig 1 and Table 2). From the differential enumeration
of PRBCs with 0, 1, 2, or greater than 2 adherent RBCs
(Table 2), it is evident that the antibodies that disrupted
rosettes with greater than 2 RBCs adherent per PRBC did not
simply generate very small rosettes of 1 or 2 adherent
RBCs per PRBC. In the presence of 10 µg/mL of OKM5,
OKM8, or 8A6, 90% or more of the PRBCs did not have
any adherent RBCs.
The effect of MoAb concentration (0.001 to 100 µg/mL)
on reversal of rosetting was tested (Fig 1). Only the same
three anti-CD36 MoAbs reversed rosetting. Fifty percent
reversal of rosetting was obtained using concentrations 0.08
µg/mL, 0.2 µg/mL, and 0.5 µg/mL for 8A6, OKM8, and
OKM5, respectively. The 1D3 and 1B1 MoAbs had no
effect over this concentration range. The IgG MoAbs SB7.4
and 89, specific for malarial parasites located under the
PRBC membrane, also failed to reverse rosettes (Fig 1).
The human IgM antibody 33G2, which others have shown
to reverse adherence of PRBCs of the uncloned C+ PA
strain to C32 cells, but not to affect rosetting by
the R+ PAI clone, was also without effect on rosetting by the R+ PRBCs used here (Fig 1).

Pretreatment of RBCs, but not PRBCs, with anti-CD36
antibodies blocks rosette reformation. For these experi-
ments, PRBCs were purified from cultures of R+ MC as
defined in Materials and Methods. The purified MC R+ PRBCs and fresh RBCs were treated separately by incuba-
tion with anti-CD36 or control antibodies. Antibody-
treated cells were washed and mixed with cells treated with
the same or other antibodies, or control untreated cells, and
the extent of rosette reformation in the different combinations
measured. The concentration of antibodies used was
10 µg/mL. With the K+R+ MC strain parasites, 80% of the
PRBCs reformed rosettes when both the RBCs and PRBCs
were preincubated without antibodies (Fig 2A). Pretreat-
ment of PRBCs with anti-CD36 MoAbs 8A6 or 1B1 had no
effect on rosette reformation when the RBCs were pre-
treated with MoAb 1B1 or no antibody. In contrast,

<table>
<thead>
<tr>
<th>MoAb and Isotype</th>
<th>Western Blot*</th>
<th>Western Blot†</th>
<th>ELISA‡</th>
<th>Immunoprecipitation</th>
<th>Blockade of Adherence to C32 Cells</th>
<th>Fluorescence Staining of Cells</th>
<th>Reversal of Rosetting†</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKM5 (IgG 1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OKM8 (IgG 1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8A6 (IgG1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1D3 (IgG1)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>#</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>1B1 (IgG1)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>#</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: +, positive reactivity in the assay; ±, weak reactivity in some experiments; -, no reactivity in the assay; ND, not determined.

*After electrophoresis by SDS-PAGE under reducing conditions.
†After electrophoresis by SDS-PAGE under nonreducing conditions.
‡ELISA reactivity with purified CD36 adsorbed onto plastic.
§FACScan analysis.
|
| Reversal of Rosetting measured at 10 µg/mL antibody. +, > 95% reversal; -, < 20% reversal.
|<refResults from references 9, 12, 21, 24, and 29.
#These results were obtained with [3H]-labeled CD36 from C32 cells. Different results may be obtained with other forms of CD36.
Fig 1. The effect of anti-CD36 MoAbs on rosetting of RBCs around P. falciparum PRBCs of the K+R+ MC strain. Various concentrations of anti-CD36 MoAbs (OKM5 and OKM8 in [A]; 8A6, 1D3, and 1B1 in [B]) or control MoAbs (887.4 and 3362 in [A]; 89 in [B]) were added to a K+R+ MC culture containing 80% of PRBCs in rosettes. After incubation for 1 hour at 37°C, the number of rosettes (PRBCs carrying >2 RBCs) was counted and expressed as a percentage of the number of rosettes in samples without antibody. Mean values ± sample SD are shown of triplicate counts. (A): (—□—) OKM5; (—○—) OKM8; (—■—) 8B7.4; (—△—) 33G2. (B): (—■—) 8A6; (—△—) 1D3; (—□—) 1B1; (—○—) 89.

Table 2. Reversal of Rosettes by Anti-CD36 Antibodies

<table>
<thead>
<tr>
<th>PRBC With No. Adherent RBCs (%) of total PRBCs</th>
<th>Antibody (10 μg/mL)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>&gt;2</th>
<th>SD</th>
<th>% Reversal</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19</td>
<td>6</td>
<td>6</td>
<td>69</td>
<td>5.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OKM5</td>
<td>90</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>0.6</td>
<td>98.5</td>
<td></td>
</tr>
<tr>
<td>8A6</td>
<td>97</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>98.5</td>
<td></td>
</tr>
<tr>
<td>1B1</td>
<td>12</td>
<td>3</td>
<td>1</td>
<td>84</td>
<td>2.1</td>
<td>-21.7</td>
<td></td>
</tr>
<tr>
<td>1D3</td>
<td>18</td>
<td>9</td>
<td>6</td>
<td>66</td>
<td>2.0</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

Percent reversal is calculated by comparison to the control in which no antibody was added. SD is the sample standard deviation of triplicate counts of the percentage of PRBCs with >2 adherent RBCs.

Fig 2. The effect of anti-CD36 MoAbs on the reformation of rosettes when PRBCs and RBCs are incubated separately with MoAbs, then recombined in different combinations. In (A), purified PRBCs of the culture-adapted K+R+ MC strain and fresh O+ RBCs were incubated separately at 4°C for 30 minutes with 10 μg/mL of either of the anti-CD36 MoAbs 8A6 or 1B1, or no antibody, then washed before mixing PRBC and RBC samples in the 9 potential combinations shown here. Mean values ± SD are shown of triplicate counts. Eighty percent of the PRBCs reformed rosettes in the no antibody control. The results in [B] are for an isolate from a West African malaria patient (GAM425) rosetting with fresh O+ RBCs from a normal donor. Forty-seven percent of the PRBCs formed rosettes in the no antibody control. The original culture and the fresh O+ RBCs were treated separately with antibodies, washed, mixed, incubated, and the percent of rosetting determined. RBC treatments: (■) 8A6; (□) 1B1; (●) No Ab.

pretreatment of RBCs with 8A6 anti-CD36 MoAb inhibited rosette reformation regardless of the treatment of PRBCs. This inhibition ranged from 31% to 59%. These results indicate that the CD36 antigen recognized by anti-CD36 antibodies that block rosette reformation is located on the RBCs, not on the PRBCs.

In the case of the Gambian isolate 425, it was not necessary to purify PRBCs, because the patients' own
RBCs were unable to support rosetting,\textsuperscript{18} so that rosetting experiments could be performed by adding fresh RBCs from a normal donor (see Materials and Methods). Results with the Gambian isolate 425 rosetting with normal O\textsuperscript{+} RBCs are shown in Fig 2B and are similar to those obtained with MC K\textsuperscript{+}R\textsuperscript{+}.

Antibody-mediated inhibition of rosette reformation was then examined in detail with the MC strain K\textsuperscript{+} R\textsuperscript{+} parasites, using antibodies for pretreatment over the concentration range of 3 to 100 \(\mu\)g/mL and two different samples of normal human RBCs for rosette reformation with the purified PRBCs (Fig 3). Pretreatment of RBCs with MoAb 1B1 resulted in less than 18\% inhibition of rosette reformation with untreated PRBCs. Pretreatment of RBCs with MoAb 8A6 inhibited rosette reformation by 40\% to 70\% depending on the concentration used. Results with the two samples of RBCs were very similar (Fig 3).

\textit{Liquid-phase CD36 reverses rosettes specifically.} Human platelet-derived CD36 was partially purified, detergent depleted, and analyzed as described in Materials and Methods. The three fractions that spanned the CD36 gel filtration peak (7.43-44, 7.45-47, and 7.49) had total protein concentrations of 150, 230, and 200 \(\mu\)g/mL, respectively. The estimated CD36 concentrations were 50, 200, and 10 \(\mu\)g/mL. When spotted on plastic at dilutions in PBS of 1/20 to 1/640, fraction 7.45-47 strongly bound PRBCs in a standard cytoadherence assay even at 1/640 dilution, whereas fraction 7.49 bound no PRBCs at any dilution and fraction 7.43-44 bound strongly at 1/20 dilution, titrating out to weak binding at 1/320 dilution. Bovine milk CD36 was pure as judged by SDS-PAGE, had a protein concentra-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{The concentration-dependent effects of two anti-CD36 MoAbs on reformation of rosettes when RBCs are pretreated with the antibodies. PRBCs were purified from rosettes of the culture-adapted MC strain and mixed with two different samples of normal RBCs (1 and 2, both O\textsuperscript{+}) that had been pretreated with MoAb 8A6 or MoAb 1B1. In parallel control samples, untreated RBCs from both donors were added. When untreated RBCs were added, 65\% and 73\% of PRBCs reformed rosettes with RBC samples from donors 1 and 2, respectively. The effect of MoAb pretreatments on rosette reformation was calculated by normalization with the percent rosetting in the sample of untreated RBCs from the same donor. Values are shown as the percent inhibition relative to these control samples \pm SD. \textbullet \textbullet \textbullet 8A6/RBC no. 1; \textbullet \textbullet \textbullet 1B1/RBC no. 1; \textbullet \textbullet 8A6/RBC no. 2; \textbullet \textbullet \textbullet 1B1/RBC no. 2.}
\end{figure}

### Table 3. Reversal of Rosettes With CD36 Column Fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction*</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>&gt;2</th>
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<tr>
<td>CD36f</td>
<td>7.43-44</td>
<td>29</td>
<td>24</td>
<td>17</td>
<td>30</td>
<td>3.6</td>
</tr>
<tr>
<td>CD36f</td>
<td>7.45-47</td>
<td>93</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>CD36f</td>
<td>7.49</td>
<td>33</td>
<td>21</td>
<td>16</td>
<td>30</td>
<td>8.3</td>
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<td>Binding medium</td>
<td>—</td>
<td>17</td>
<td>7</td>
<td>6</td>
<td>70</td>
<td>11.5</td>
</tr>
</tbody>
</table>

*Column fractions numbered 7.43-44, 7.45-47, and 7.49 contained total protein concentrations of 150, 230, and 200 \(\mu\)g/mL, of which approximately 50, 200, and 10 \(\mu\)g/mL was CD36 as measured by SDS-PAGE and silver-staining, respectively.

\textsuperscript{†Standard deviation for percentage of PRBCs with >2 adherent RBCs.}

\textsuperscript{‡Human platelet CD36 was partially purified (see Materials and Methods) and fractions containing CD36 eluted from the final gel filtration column were detergent depleted on an Extractigel column, diluted 10-fold in binding medium, and then added to R\textsuperscript{+} PRBCs.

\textsuperscript{§Binding medium (RPMI without sodium bicarbonate, containing 2 g/L additional glucose, 30 mmol/L HEPES, pH 6.8, 100 \(\mu\)g/mL gentamicin, and 10\% fetal bovine serum) used for suspension of the cells under test.}

This extent of reversal was shown to be dependent on the concentration of exogenous CD36 (Fig 4). The 3 CD36-containing fractions (7.43-44, 7.45-47, and 7.49) were added to blood containing rosettes over a range of 0.02 to 20 \(\mu\)g total protein/mL total. There was no effect on rosetting at 0.2 \(\mu\)g protein/mL for the two fractions of lowest CD36 concentration. Fraction 7.45-47, containing the highest CD36 concentration, did reverse rosetting by approximately 98.5\%, while the fractions eluted on either side reversed rosetting by approximately 53\%. Fraction 7.45-47 converted almost all rosettes with greater than 2 RBCs per PRBC to free PRBCs, while the fractions containing less CD36 converted only half of the rosettes with greater than 2 RBCs per PRBC to PRBCs (Table 3). Bovine milk CD36 at 20 \(\mu\)g/mL, a protein concentration similar to the human CD36 in fraction 7.45-47, did not reverse rosettes (data not shown).

DISCUSSION

The results in Tables 1 and 2 and Fig 1 indicate that 3 different MoAbs (8A6, OKM5, and OKM8) specific for human CD36 are able to reverse rosetting of human RBCs around \textit{P falciparum} PRBCs. These antibodies reverse rosetting very effectively at sub-microgram per milliliter
concentrations. These MoAbs also react with native CD36 expressed on several types of cells and block cytoadherence. The MoAbs probably react with related epitopes on CD36 because OKM5 and OKM8 compete with each other for binding to mononuclear cells, and 8A6 and OKM5 compete with each other in differential radioimmunoassay (RIA) on purified CD36. Two of the anti-CD36 MoAbs (1B1 and 1D3) had no effect on rosettes, and these MoAbs have a reactivity pattern (Table 1) suggesting that they recognize an epitope of CD36 that is not available on the cell surface, for reasons of either conformation or location. The correlation between the capacity of an anti-CD36 MoAb to block adherence of PRBCs to C32 cells and the capacity to reverse rosetting may suggest that the disposition of CD36 epitopes accessible on the surface of C32 cells is similar to those important in the rosetting phenomenon.

The results of Figs 2 and 3 indicate that 8A6, OKM5, and OKM8 reverse rosetting through binding to the uninfected RBCs rather than the PRBCs. The simplest interpretation of these data is that a CD36-like antigen is expressed on human RBCs and can act as host receptor for rosetting of the MC R+ PRBCs. The CD36-like antigen appears to be like CD36 not only in its antigenic properties, but also in its receptor properties, because rosettes can be reversed by addition of liquid phase CD36 protein (Fig 4). For convenience we will refer to the CD36-like RBC surface antigen as CD36 for the rest of this discussion.

In principle, rosette reversal by antibodies and soluble CD36 could reflect a secondary effect on receptor interactions that does not involve CD36 directly, such as steric blocking or changes in deformability of the RBC. It is not known whether the deformability of RBCs affects their capacity to rosette with R+ PRBCs, nor is it known whether binding of anti-CD36 MoAbs affects the deformability of the RBCs, and these issues should be investigated in the future. In the case of inhibition of invasion of RBCs by malaria merozoites, it has been shown that invasion is affected by changes in RBC deformability induced by antiglycophorin MoAbs. These experiments were performed with 1 to 6 x 10^5 sites bound per cell. Given the very low number of CD36 molecules per RBC, it seems unlikely that a change in deformability could explain reversal of rosetting by anti-CD36 MoAbs. Furthermore, the presence of receptors for CD36 on the R+ PRBCs suggests a direct involvement of CD36 as receptor for rosetting.

Studies on other rosetting lines of P. falciparum found that anti-HRP1 MoAb 89 and anti-HRP1 rabbit serum MC1 reversed rosettes of the R+ PA1 and TM180 strains, whereas anti-CD36 MoAb OKM5 did not. With the MC R+ line we could not reverse rosettes with either of the same anti-HRP1 reagents (Fig 1 and Handunnetti, unpublished results), whereas anti-CD36 MoAbs strongly reversed rosettes. Because most patient isolates tested have CD36 receptors, one might expect that CD36 commonly acts as host receptor for rosetting. Indeed, we found that the Gambian isolate 425 behaved similarly to MC R+ in its dependence on RBC CD36 for rosetting. However, given the diversity of putative endothelial cell host receptors identified in vitro, there may be a similar diversity in host receptors for rosetting. Wahlgren et al have tested 22 rosetting parasite lines for reversal of rosetting by MoAb OKM5, and only two, including MC R+, were susceptible to MoAb OKM5, whereas R+ PA1 and TM180 were not (M. Wahlgren, personal communication).

We have been able to select both rosetting-positive and rosetting-negative lines of MC through appropriate selections. PRBCs from both lines adhere to CD36, provided that, in the case of the MC R+ line, rosettes are first disrupted before performing the cytoadherence assay to avoid steric interference of rosettes with binding of PRBCs to the plate. Given that both lines have receptors for CD36 but only the MC R+ line rosettes with RBCs, the question arises why the CD36 receptors on the R- line do not support rosetting. There are several possible explanations. First, as discussed above, anti-CD36 MoAbs may indirectly affect rosetting receptor interactions, without CD36 itself being the receptor on RBCs. Second, rosetting may involve several receptor molecules simultaneously, with CD36 being only one of these receptors. The R+ line would then be lacking receptors for one of the other host molecules involved. Third, the form of CD36 on RBCs may be somewhat different from that on other cells. For instance, there may be structural differences such as glycosylation or proteolytic degradation, or there may be differences in clustering or accessibility of CD36 in the membrane of different cells. In this respect, it is interesting to note that the form of CD36 on fetal erythrocytes and fetal erythro-
blasts has a lower molecular weight in SDS-PAGE than adult platelet CD36.\textsuperscript{20} Thus, it is possible that the receptor on R\textsuperscript{+} PRBCs can bind to the form of CD36 on RBCs as well as other cells, whereas the receptor on R\textsuperscript{−} PRBCs is more restricted in its specificity. Fourth, there may be a difference in the density or affinity of receptors for CD36 on PRBCs, with R\textsuperscript{−} PRBCs expressing fewer or lower affinity receptors than R\textsuperscript{+} PRBCs. The number of molecules of CD36 on C32 melanoma cells, as measured by OKM5-inhibitable binding of 125I-TSP, is approximately 700,000.\textsuperscript{24} This high level of CD36 may support attachment of R\textsuperscript{−} PRBCs, while the very low level of CD36 on RBCs is not sufficient for those PRBCs. Similar factors are likely to determine the rosetting phenotype of CD36-binding PRBCs from natural isolates, because most natural isolates have CD36 receptors,\textsuperscript{18} but only a proportion rosette.\textsuperscript{7,18,33}

The results presented here may have important implications for disease pathology. Rosetting has been shown to correlate with cerebral malaria\textsuperscript{7} and to promote obstruction of blood vessels in an ex vivo model.\textsuperscript{5} Previous studies have shown diversity in \textit{P. falciparum} in that some parasites are able to rosette, whereas others cannot.\textsuperscript{7,18,33} Some patients have antibodies that reverse rosetting, whereas others do not.\textsuperscript{7,33} In addition, we show here that RBCs from Gambian patient 425 did not support rosetting. Thus, there are multiple factors determining whether rosetting takes place in an infected host. Because CD36 is thought to be involved in binding to endothelial cells in sequestration as well as rosetting, variations in parasites that affect rosetting may reflect the specificity of their receptors for CD36 and therefore also affect patterns of sequestration between different tissues. Finally, as discussed above, it seems likely some parasite isolates may use RBC molecules other than CD36 as rosetting receptors. These complications will need to be taken into account in the design of clinical studies on the importance of rosetting and sequestration in the disease process.

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Involvement of CD36 on erythrocytes as a rosetting receptor for Plasmodium falciparum-infected erythrocytes

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