Normal Human Erythrocytes Express CD36, an Adhesion Molecule of Monocytes, Platelets, and Endothelial Cells

By Marie Rose van Schravendijk, Shiroma M. Handunnetti, John W. Barnwell, and Russell J. Howard

We have recently shown that rosetting of Plasmodium falciparum (MC R+ line)-infected erythrocytes (parasitized red blood cells [PRBCs]) with uninfected erythrocytes (RBCs) is blocked by coating of the RBCs with anti-CD36 monoclonal antibodies (MoAbs; Handunnetti et al, Blood 80:2097, 1992). Adult RBCs have previously been considered negative for CD36. However, using fluorescence-activated cell sorter analysis with the anti-CD36 MoAbs 8A6, OKM5, and OKM8, which reverse rosetting, we consistently detect CD36 on the majority of normal adult RBCs. Absorption of the MoAb solutions with CD36-transfected Chinese hamster ovary (CHO-CD36) cells removed the reactivity against both CHO-CD36 cells and RBCs, whereas absorption with CHO cells had no effect. By comparison with staining for glycoporphin A, LFA-3, and CR1, the level of expression of CD36 appeared to be low. Nevertheless, normal RBCs were capable of adhering to plastic coated with anti-CD36 MoAbs. RBCs from one African malaria patient were identified as deficient in CD36 and these RBCs did not rosette with the patient's own P. falciparum PRBCs, even though these PRBCs were capable of rosetting with RBCs from a normal donor in a CD36-dependent manner. Therefore, the level of expression of CD36 on normal RBCs is sufficient to be important in cell adherence, and may have a biologic role in normal individuals as well as in the pathology of P. falciparum malaria.

© 1992 by The American Society of Hematology.

MATERIALS AND METHODS

Anti-CD36 antibodies. The mouse IgG1 monoclonal antibodies (MoAbs) OKM5 and OKM8 were generous gifts of Dr F. Rao (Ortho Pharmaceuticals, Raritan, NJ). The properties of these anti-CD36 MoAbs have been described.1 Other mouse IgG1 MoAbs specific for human CD36, 8A6, 1D3, and 1B1 were produced and characterized as described elsewhere and were used as protein A-Sepharose–purified antibodies. Hybridoma supernatant of 8A6 (0.2 μg/mL MoAb as determined by enzyme-linked immunosorbent assay [ELISA]) was also prepared and tested for reactivity with C32 cells, U937 cells, and RBCs. Other MoAbs. The following MoAbs were used as antibody purified from ascites by protein A-Sepharose chromatography. IgG1 MoAb 7F12 against CD61 was a gift from Dr. Anne Jackson (Becton Dickinson, San Jose, CA). IgG1 MoAb TS2/19 against LFA-3 and IgG2a MoAb W6/32 against HLA-A,B,C were gifts from Dr Lewis Lanier (DNAX Research Institute, Palo Alto, CA). IgG2a MoAb 4F9 against CD7, IgG1 MoAb 4D4 against CR1, and IgG2a MoAb 1D1.1 against human transferrin receptor were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). Isotype controls MOPC21 (IgG1) and RPCS (IgG2a) were obtained from Cappel/Organon Teknika Corp (Durham, NC). The following MoAbs were used as hybridoma supernatants:

From the Laboratory of Infectious Diseases, Molecular Biology Department, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA; and the New York University Medical Center, Department of Medical and Molecular Parasitology, New York, NY. Submitted December 23, 1991; accepted June 22, 1992. Supported by a World Health Organization Training grant and a Rockefeller Foundation Fellowship to S. Handunnetti, and by USAID Grant No. DPE-0453-G-SS-8049-00 to R.J.H. DNAX Research Institute is supported by Schering-Plough Corporation. Address reprint requests to Marie Rose van Schravendijk, PhD, DNAX Research Institute of Molecular and Cellular Biology, 901 California Ave, Palo Alto, CA 94304-1104. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/8008-0015$3.00/0
IgG1 MoAb 10F7MN (ATCC no. HB8162) against glycophospholipin A \(^{24}\) (supernatant containing 70 \(\mu\)g/mL MoAb); IgG2a MoAb W6/32 (ATCC no. HB959) against HLA-A, B, C (90 \(\mu\)g/mL MoAb); IgG1 MoAb 543 (ATCC no. HB8592) against CR1 (30 \(\mu\)g/mL MoAb); IgG1 MoAb TS2/9 (ATCC no. HB205) against LFA-3 (20 \(\mu\)g/mL MoAb). Anti-intercellular adhesion molecule-1 (anti-ICAM-1) MoAb CL203 was a gift from Dr Soldano Ferrone (New York Medical College, Valhalla, NY). Hybridoma supernatants were tested for reactivity with RBCs, C32 cells, and U937 cells, and the concentrations of antibody were determined by a sandwich ELISA with 10 \(\mu\)g/mL affinity-purified Go anti-Mu IgG (Fc specific) (Pierce Chemical Co, Rockford, IL; cat. no. 31710) as the coating antibody and 1/500 dilution of alkaline-phosphatase–labeled affinity-purified Go anti-MuIgG (whole molecule) (Cappel/Organon Teknika Corp; cat. no. 59296) as the detecting antibody. IgG1 (\(\kappa\)) MoAb L133 against CD3, purified from hybridoma supernatant, was a gift from Dr Lewis Lanier.

Secondary antibodies. For fluorescence-activated cell sorter (FACS) analysis, eight different secondary and tertiary stains were tested, all affinity-purified antibodies. Stain 1 was a fluorescein isothiocyanate (FITC)-labeled Go anti-Mu IgG (whole molecule) (Sigma Chemical Co, St Louis, MO; no. F0257) Stain 2 was a Go anti-Mu IgFc (Pierce; no. 31170) followed by FITC-Rb anti-Go IgG(H + L) (Pierce; no. 31508). Stain 3 was a Go anti-Mu IgG (H + L) (Jackson ImmunoResearch Labs, West Grove, PA; 115-005-100) followed by FITC-Rb anti-Go IgG(H + L) (Pierce; no. 31508). Stain 4 was a Rb anti-Mu IgG Fc (Jackson; 315-005-046) followed by FITC-Go anti-Rb IgG (whole molecule) (Sigma; no. F0382). Stain 5 was a R-phycocerythrin (R-PE)-labeled Go anti-Mu IgG (whole molecule) (Sigma; no. P9287). Stain 6 was a R-PE F(ab')\(\odot\) of Go anti-Mu IgG Fe (Jackson; 115-116-071). Stain 7 was an R-PE Go anti-Mu IgG1 (Catlg Labs, South San Francisco, CA; no. M32004). Stain 8 was an Rb anti-MuIgGFc (Jackson; 315-005-046) followed by R-PE F(ab')\(\odot\) of Go anti-RbIgG (H + L) (Catlg Labs; no. L43004).

Cells. O+ RBCs obtained from healthy adult donors who had tested negative for hepatitis B and human immunodeficiency virus (HIV) antibody were passed through an RC50 transfection grade leukocyte removal filter (Pall Biomedical Products Co, East Hills, NY). The absence of leukocytes was established by light microscopy of Giemsa-stained blood films and by forward and side scatter in FACS analysis. RBCs from six different O+ donors were tested. The Plasmidispora isolate from Gambian patient 425 (CA043) was collected in 1985 from a patient reporting to the clinic of the Medical Research Laboratories (Fajara, The Gambia) and the rosetting properties of this sample of parasites have been described elsewhere.\(^{22,26}\) Cryopreserved GAM425 RBCs (blood group O) containing 15% ring-stage P. falciparum-infected RBCs were thawed in parallel with a cryopreserved sample of normal O+ RBCs, washed, and allowed to equilibrate in RPMI at 4°C for 24 hours before staining and analysis. Freshly outdated platelets were obtained from Peninsula Blood Bank (San Mateo, CA). Stable transfecants of Chinese hamster ovary (CHO) cells bearing CD63 were derived in this laboratory\(^{34}\) and are denoted CHO-CD63. CHO cells, CHO-CD63 cells, C32 human malignant melanoma cells (ATCC no. CRL1585), and U937 myelomonocytic cells (ATCC no. CRL1593) were grown in RPMI 1640 containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin (all media from JRH Biosciences, Lenexa, KS).

Cell staining for FACS analysis. Fifty-microliter amounts of leukocyte-depleted, washed O+ RBCs at 0.2% hematocrit (Hct) in phosphate-buffered saline (PBS) containing 2 mmol/L EDTA and 1 mmol/L bovine serum albumin (BSA; Sigma globulin-free grade, no. A7638) (PBS/BSA1) were aliquotted into V-shaped 96-well plates (Costar SeroClone plate no. 3898; Costar, Cambridge, MA). Platelets were washed in PBS/BSA1 and aliquotted into Eppendorf tubes at 10^\(\mu\)L per tube. Fifty-microliter aliquots of 10 \(\mu\)g/mL solutions of MoAbs in PBS/BSA1 were added and incubated on ice for 1 hour. Anti-glycophospholipin MoAbs were used as a 1/1,000 dilution of hybridoma supernatant to minimize agglutination. Other hybridoma supernatants were used undiluted at 50–50 \(\mu\)L aliquots, after the addition of 2 mmol/L EDTA and 25 mmol/L HEPES, pH 7.4. RBCs were washed by centrifuging plates for 5 minutes at 1,000g, flicking the plates, and resuspending cells in PBS/BSA1 for each wash. Platelets were washed by microfuging at 7,000g for 1 minute and aspirating the supernatant. After two washes with 200 \(\mu\)L PBS/BSA1, cells were resuspended in 50 \(\mu\)L 1/50 dilution of secondary antibody and incubated on ice for 45 minutes. For tertiary staining, washing and incubation were repeated with the tertiary antibody. Finally, cells were washed once with PBS/BSA1, twice with PBS, and resuspended in 150 \(\mu\)L PBS. Fifty microliters of 2% paraformaldehyde in PBS, pH 7.4, was added and cells left for at least 1 minute at room temperature (RT) to inactivate any possible HIV present. Six hundred microliters of 0.5% paraformaldehyde in PBS, pH 7.4, were then added before FACS analysis.

FACS analysis. Cells were analyzed on a Becton Dickinson FACScan instrument. Five thousand or 10,000 cells were analyzed for each determination. Data were analyzed by overlays and subtraction of histograms after 3-point smoothing using the Becton Dickinson LYSYS-2 program. Staining by the test antibodies was compared with that by secondary reagents alone, and with completely unstained cells. Two parameters were noted, the mean fluorescence of the cell population and the number of cells positive. Mean fluorescence was standardized between different experiments by taking the ratio of mean relative fluorescence of the histogram obtained in the presence of primary antibody to that obtained in its absence in the same experiment. The number of RBCs positive was obtained by subtracting the histogram obtained in the absence of primary antibody from that obtained in its presence, and counting only cells whose fluorescence was greater or equal than the mean fluorescence of the cells with no primary antibody. Data are shown as a percentage of the total cells analyzed. In the case of weak staining in which the entire histogram is shifted, but not sufficiently to avoid overlap with the control histogram, the percentage of RBCs positive measured in this way represents only a minimum estimate of the percentage of positive cells. To ascertain that the parameters measured represented a saturating level of binding of antibody, titrations over 10 doubling dilutions were analyzed for MoAbs 8A6, OKM5, TS2/9, and 543. By contrast, data with 10F7MN were purposely obtained with concentrations of hybridoma supernatant far below saturating, because at higher levels of antibody the fluorescence of the cells was offscale on the instrument. Under the conditions used with 10F7MN, the presence of agglutinates was not significant during FACS analysis, as seen from forward and side scatter.

Absorption of 8A6 and OKM5 MoAbs with cells bearing CD36. CHO-CD36 cells and untransfected CHO cells were grown to near-confluency. Cells were detached with PBS containing 2 mmol/L EDTA, washed with PBS containing 2% FCS and 2 mmol/L EDTA (PBS/FCS), and kept on ice during all subsequent operations. To 10^\(\text{7}\) CHO-CD36 or CHO cells, or to 10^\(\text{6}\) RBCs, 0.4 mL of a 5 \(\mu\)g/mL MoAb dilution in PBS/FCS was added and the suspensions left on ice for 45 minutes with occasional mixing. Cells were centrifuged at 2,000g for 5 minutes and the supernatants were removed (supernatant no. 1). The absorption was repeated twice by adding part of the supernatant successively to two fresh aliquots of cells, yielding supernatants no. 2 and 3. All supernatants were microfuged at 15,000g for 5 minutes to remove any particulate material before FACS staining. FACS staining was performed as
CD36 ON ERYTHROCYTES

described for RBCs above, except that PBS/FCS was used as the diluent buffer, 100 µL MoAb dilution was used per well, and cells stained included also CHO or CHO-CD36 at 2 × 10³ cells per well. Stain 6 (see Antibodies) was used as the secondary reagent. In parallel, CHO-CD36 cells and RBCs were stained with a series of doubling dilutions of the original 5 µg/mL solution of MoAb, so that the extent of absorption at each step could be determined by comparison with this standard titration. Mean fluorescence was used as the titration parameter with different concentrations of MoAb. With RBCs the percentage of positive cells (as defined by subtraction of the control with secondary reagent alone) could also be used to monitor the titration.

Adherence of RBCs to antibody immobilized on plastic. Ten-microliter aliquots of 10 µg/mL dilutions of MoAbs, 10 µg/mL dilutions of affinity-purified Rb anti-MuIgG (Fc specific, Hu serum absorbed; Jackson Immunoresearch Labs; cat. no. 315-005-046), or 50 µg/mL dilutions of protein A (Pharmacia-LKB, Piscataway, NJ) in PBS were spotted onto bacteriologic petri dishes (Falcon Optiplex no. 1005) inside larger wells demarcated with adherent paraffin. In the case of the protein A spots, all PBS solutions used in the assay were adjusted to pH 8 to enhance binding of IgG1 MoAbs. After standing at RT for 1 hour, the spots were aspirated and immediately overlaid with 50 µL PBS containing 10 mg/mL BSA (Sigma globulin-free grade, no. A7638) (PBS/BSA10). The wells were aspirated again and overlaid with PBS/BSA10, then left at RT for 1 hour to block protein binding sites on the plastic. The wells were then washed three times with 50 µL PBS/BSA10. In the case of protein A or Rb anti-MuIgG spots, 30 µL of 10 µg/mL dilutions of MoAbs or twofold dilutions of hybridoma supernatants were added to the wells, incubated for 1 hour at RT, and washed five times with PBS/BSA10. The wells were then overlaid with 50 µL of a 1% Hct suspension of leukocyte-depleted normal O+ RBCs freshly washed with PBS/BSA10, and incubated at 37°C for 45 minutes. The paraffin was carefully removed and 10 mL PBS/BSA10 carefully dripped over the entire plate without disturbing the spots. After blocking for 30 minutes at RT, nonadherent cells were resuspended by careful swirling and aspiration and the plates washed in this manner one further time with PBS/BSA10 and twice with PBS. Ten milliliters of 2% glutaraldehyde in PBS was gently added and the plates left to fix at RT for 18 hours. The fixed plates were washed twice with H₂O₂ and rapidly dried with a hair dryer. The plates could then be inspected under the microscope. For photography, the plates were subsequently stained with 1% wt/vol Naphthol BlueBlack (Sigma N3005) in 7.5% HAc for 2 hours, washed three times with 7.5% HAC, and once with H₂O₂, and dried rapidly with a hair dryer.

Analysis of RBCs adherent to plastic coated with anti-CD36 MoAbs. Using the above protocol, entire 3 cm bacteriologic petri dishes (Falcon no. 1007) were coated with 3 mL 10 µg/mL Rb anti-MuIgG (Fc specific), followed by 3 mL 8A6 hybridoma supernatant, TS2/2 hybridoma supernatant, or 10 µg/mL dilutions of purified 8A6 or purified OKM5. The washed plates were incubated for 45 minutes at 37°C with 3 mL RBC suspension in RPMI 1640 containing 10% FCS and 25 mmol/L HEPES, then washed with the same medium until only adherent cells remained, as seen microscopically. The adherent cells were resuspended by vigorous pipetting close to the plate surface and collected in two washes with medium. The washed plates were inspected microscopically for removal of cells. Eluted cells were then stained for reticulocytes using Brilliant Cresyl Blue followed by Wright’s stain (both from Sigma). Ten thousand cells were counted to determine the percentage of reticulocytes in each sample.

RESULTS

FACS analysis of normal human RBCs with anti-CD36 and control MoAbs. We could consistently detect a CD36-like antigen on RBCs by FACS analysis. Normal RBCs were stained with eight different FITC- and R-PE–labeled reagents in secondary and tertiary combinations and analyzed by FACScan. All reagents gave weak positive staining with purified 8A6, OKM5, and OKM8 as primary antibody, but were negative with IB1, 1D3, and MOPC21 (results for stain 6 shown in Fig 1). Two reagents (stain 1 and stain 5) were relatively insensitive compared with the other six, which gave comparable levels of staining. The six sensitive reagents shifted the mean fluorescence of the RBCs by a factor of 1.5 to 3 depending on the anti-CD36 MoAb and the secondary reagent, whereas the control MoAbs remained within 10% of the control without primary MoAb (Table 1). The 8A6 hybridoma supernatant was also clearly positive (Table 1), staining RBCs, C32 cells, and U937 cells at a level shown in titrations to be consistent with the low MoAb concentration (0.2 µg/mL) in this supernatant (data not shown). Upon staining of RBCs with anti-CD36 MoAbs, the overall shape of the fluorescence histograms remained similar to the unstained cells, suggesting that a high percentage of cells is positive (Fig 1). This percentage could not be determined exactly because the histograms of the stained cells still overlapped with the unstained cells at this level of fluorescence. A minimum estimate of the proportion of cells that are positive was obtained by subtracting the histograms of cells stained only with secondary and tertiary reagents from the histograms obtained when primary antibody was included (see Materials and Methods). Thirty percent to 60% of the cells scored positive by this criterion (Table 1). Control MoAbs showed at most 10% positivity, and usually had cells in the subtracted plot ranging over the entire fluorescence range of the histogram rather than being concentrated at the higher fluorescence levels, as with the anti-CD36 MoAbs. With the antiglycophorin MoAb, the dilution of hybridoma supernatant used in Fig 1 was chosen to be high enough (1,000-fold) so as to obtain histograms within the measurable range of fluorescence; at higher concentrations of MoAb, both antiglycophorin MoAbs gave relative mean fluorescence greater than 2,000 times the levels in the absence of primary MoAb (data not shown). RBCs from six different normal donors gave similar results (data not shown). Cell samples sorted with several different forward and side scatter gate settings gave similar results. Similar results were obtained whether the buffer contained 1 mg/mL BSA or 2% FCS, and 0 or 2 mmol/L EDTA.

Comparative FACS analysis of RBCs and platelets. To evaluate whether fragments of leukocytes and especially platelets might adhere to the RBCs and be responsible for the apparent expression of CD36 on RBCs, we tested RBCs with several other antileukocyte MoAbs: MoAb CL203, which reacts with ICAM-1, which is expressed on activated B cells and on peripheral blood monocytes; MoAb 4H9, which reacts with CD7 expressed on peripheral T cells; MoAb 7T12, which reacts with CD61, which is expressed on platelets and peripheral blood monocytes; and MoAb L133.
reactive with CD31, which is expressed on platelets, peripheral blood granulocytes, monocytes, and T cells. All four MoAbs were negative with RBCs (Fig 1 and Table 1). With platelets, MoAb 7F12 and MoAb L133 were strongly positive (Fig 2), as were anti-CD36 MoAbs 8A6, OKM5, and OKM8. 1B1 and 1D3 gave 10- to 20-fold lower staining relative to 8A6, OKM5, and OKM8. MoAb 7F12 stained platelets more strongly than the anti-CD36 MoAbs (Fig 2), but was negative with RBCs (Fig 1).

**Comparative FACS analysis of RBCs with MoAbs against other RBCs and erythroid-lineage antigens.** RBCs were tested with MoAbs 44D and 543 against CR1 and MoAb TS2/9 against LFA-3. All three MoAbs reacted more strongly with RBCs than the anti-CD36 MoAbs (Table 1). FACS analysis of samples stained with a range of dilutions of these MoAbs showed that the levels of staining obtained with the anti-CR1 and anti-LFA-3 MoAbs when used at 10 μg/mL purified MoAb or 50 μL hybridoma supernatant, were saturating (data not shown). Binding of purified 8A6 and OKM5 was also shown to be saturating (data not shown). RBCs were also tested with MoAbs L01.1 and W6/32, neither of which gave appreciable staining with stain 6 (Table 1).

**Absorption of 8A6 and OKM5 MoAbs by cells bearing CD36.** Titration of CHO-CD36 and RBCs using mean fluorescence or percent positive RBCs as the titration parameter measured showed that staining only becomes sensitive to MoAb concentration when the MoAb concentration falls below 1 μg/mL, ie, when more than 80% of added MoAb has been absorbed (data not shown). With 8A6 purified from ascites, a single absorption with CHO-CD36 cells was sufficient to remove greater than 99.5% of reactivity with either CHO-CD36 cells or RBCs, and two absorptions removed almost all activity (Fig 3). All detectable OKM5 reactivity was absorbed from solution in a single absorption with CHO-CD36 (data not shown). CHO cells were negative with both MoAbs (data not shown), and MoAb solutions absorbed with three successive aliquots of CHO-CD36 cells and RBCs as the original 5 μg/mL MoAb dilution (Fig 3 and data not shown). Absorption with three successive aliquots of RBCs also did not remove a detectable fraction of the anti-CD36 reactivity (Fig 3). For comparison, we also tested a sample of a commercially available mouse MoAb purified from ascites on protein A-Sepharose, which was found to be contaminated with antiblood group reactivity such that it agglutinated RBCs and stained them at a level of 270-fold autofluorescence (data not shown). In this instance, absorption of this antibody with a single aliquot of RBCs was sufficient to remove almost all antiblood group reactivity (data not shown).

**Analysis of RBCs from Gambian patient 425.** RBCs from patient 425 failed to stain with MoAb 8A6 (purified or hybridoma supernatant) or OKM5 (Fig 4). In t-tests, the difference between the means of percent RBCs positive
those obtained with stains, 2, 6, and 8, as only one sample was run with stain 6. Data shown for 44D show a high degree of variation presumably due to anti-CD36 MoAbs. Mean fluorescence ratio is shown as the ratio of mean relative fluorescence of the histogram obtained in the presence of primary antibody to that obtained in its absence in the same experiment. The specificity of MoAbs 1B1 and 1D3 is shown as CD36\textsuperscript{+} because these MoAbs react with CD36 on Western blot, but react only very weakly with native CD36 as expressed on cells. Data shown for MoAb 4H9 are an average of two experiments. At higher concentrations of culture supernatant, the fluorescence of these cells was offscale on the instrument. By contrast, fluorescence obtained with TS2/9 and anti-CR1 antibodies was at saturating levels for these antibodies.

with normal RBCs versus GAM425 RBCs when staining with 8A6 hybridoma supernatant, purified 8A6, or OKM5 were significant at the 1% level for each antibody. Although staining of GAM425 RBCs with these MoAbs is close to that of the negative controls, our data cannot distinguish between lack of CD36 on GAM425 RBC or a fourfold decrease in the level of expression, given that the level of expression on normal RBCs is already low.

Adhesion of RBCs to anti-CD36 MoAb-coated plastic. Expression of CD36 epitopes on RBCs was also detected using anti-CD36 MoAbs immobilized on plastic. Similar results were obtained whether purified MoAbs were spotted directly on plastic (data not shown), or using a coating of protein A (Fig 5) or Rb anti-Mu IgG (data not shown) to bind MoAbs to the plate. All three MoAbs capable of reversing rosettes (OKM5, OKM8, and 8A6, all three of the IgG1 subclass) mediated adherence of RBCs to plastic. RBCs did not adhere to plastic coated with either anti-CD36 MoAbs that do not bind to cell surface CD36 (1B1 and 1D3) or with IgG1 myeloma protein MOPC21 (Fig 5). Spots with protein A alone were negative and spots overlaid with antiglycophorin MoAbs were strongly positive (Fig 5). By Brilliant Cresyl Blue and Wright's staining, the cell populations adherent to plates with anti-CD36 MoAbs were indistinguishable from the original RBCs added to the plate. The original RBCs, on counting 10 samples of 1,000 cells, contained no detectable white blood cells (WBCs).

Table 1. FACS Analysis of Normal Human RBCs Stained With Anti-CD36 and Control MoAbs

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Isotype</th>
<th>Antigen</th>
<th>Mean % of RBCs Positive</th>
<th>SD</th>
<th>Mean Fluorescence Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>8A6p</td>
<td>IgG1</td>
<td>CD36</td>
<td>40.2</td>
<td>10.0</td>
<td>1.98</td>
</tr>
<tr>
<td>8A6s</td>
<td>IgG1</td>
<td>CD36</td>
<td>26.2</td>
<td>7.10</td>
<td>1.54</td>
</tr>
<tr>
<td>OKM5p</td>
<td>IgG1</td>
<td>CD36</td>
<td>37.1</td>
<td>6.64</td>
<td>1.83</td>
</tr>
<tr>
<td>OKM8p</td>
<td>IgG1</td>
<td>CD36</td>
<td>57.0</td>
<td>9.03</td>
<td>2.63</td>
</tr>
<tr>
<td>1B1p</td>
<td>IgG1</td>
<td>[CD36]</td>
<td>7.2</td>
<td>4.42</td>
<td>1.08</td>
</tr>
<tr>
<td>1D3p</td>
<td>IgG1</td>
<td>[CD36]</td>
<td>5.6</td>
<td>2.90</td>
<td>1.05</td>
</tr>
<tr>
<td>MOPC21p</td>
<td>IgG1</td>
<td>None</td>
<td>4.2</td>
<td>2.10</td>
<td>1.04</td>
</tr>
<tr>
<td>RPC5p</td>
<td>IgG2a</td>
<td>CD61</td>
<td>3.8</td>
<td>1.87</td>
<td>1.01</td>
</tr>
<tr>
<td>7F12p</td>
<td>IgG1</td>
<td>CD61</td>
<td>10.4</td>
<td>4.31</td>
<td>1.13</td>
</tr>
<tr>
<td>L133p</td>
<td>IgG1</td>
<td>CD31</td>
<td>7.5</td>
<td>3.81</td>
<td>1.08</td>
</tr>
<tr>
<td>CL203s</td>
<td>nd</td>
<td>ICAM-1</td>
<td>6.6</td>
<td>3.43</td>
<td>1.07</td>
</tr>
<tr>
<td>4H9p</td>
<td>IgG2a</td>
<td>CD7</td>
<td>2.2</td>
<td>1.57</td>
<td>1.04</td>
</tr>
<tr>
<td>2.06s</td>
<td>IgG1</td>
<td>HLA-D</td>
<td>2.7</td>
<td>0.56</td>
<td>1.02</td>
</tr>
<tr>
<td>LO1.1p</td>
<td>IgG2a</td>
<td>TR</td>
<td>6.2</td>
<td>4.21</td>
<td>1.07</td>
</tr>
<tr>
<td>W6/32p</td>
<td>IgG2a</td>
<td>HLA-ABC</td>
<td>12.3</td>
<td>5.56</td>
<td>1.27</td>
</tr>
<tr>
<td>W6/32s</td>
<td>IgG2a</td>
<td>HLA-ABC</td>
<td>8.8</td>
<td>2.03</td>
<td>1.16</td>
</tr>
<tr>
<td>44Dp</td>
<td>IgG1</td>
<td>CR1</td>
<td>98.7</td>
<td>15.4</td>
<td>10.1</td>
</tr>
<tr>
<td>5A3s</td>
<td>IgG1</td>
<td>CR1</td>
<td>97.3</td>
<td>0.56</td>
<td>18.6</td>
</tr>
<tr>
<td>TS2/9p</td>
<td>IgG1</td>
<td>LFA-3</td>
<td>99.8</td>
<td>0.11</td>
<td>81.8</td>
</tr>
<tr>
<td>TS2/9s</td>
<td>IgG1</td>
<td>LFA-3</td>
<td>99.9</td>
<td>0.15</td>
<td>87.5</td>
</tr>
<tr>
<td>10F7MNs</td>
<td>IgG1</td>
<td>GpA</td>
<td>99.8</td>
<td>0.13</td>
<td>308.0</td>
</tr>
</tbody>
</table>

Leukocyte-depleted normal O+ RBCs from four different donors were stained with the above primary antibodies and R-PE-labeled F(ab')\textsubscript{2} of Go anti-Mu IgG Fc (stain 6 in Materials and Methods). Antibodies used as purified MoAb originating from ascites are suffixed p. These were used at 10 µg/mL. Antibodies used as hybridoma supernatant are suffixed s. Figures shown are the means and SD of n samples analyzed by FACScan. The number of RBCs positive was obtained by subtracting the histogram obtained in the absence of primary antibody from that obtained in their presence, and counting only cells whose fluorescence was greater than equal to the mean fluorescence of the histogram with no primary antibody. Data are shown as a percentage of the total cells analyzed and represent only a minimum estimate of the percentage of positive cells in the case of the anti-CD36 MoAbs. Mean fluorescence ratio is shown as the ratio of mean relative fluorescence of the histogram obtained in the presence of primary antibody to that obtained in its absence in the same experiment. The specificity of MoAbs 1B1 and 1D3 is shown as CD36\textsuperscript{+} because these MoAbs react with CD36 on Western blot, but react only very weakly with native CD36 as expressed on cells. Data shown for MoAb 4H9 are an average of those obtained with stains, 2, 6, and 8, as only one sample was run with stain 6. Data shown for 44D show a high degree of variation presumably because of the allelic variation in the level of CR1 expression. Cells from only two different donors were tested with 5A3 supernatant and these had similar levels of expression. Data obtained with 10F7MN were at 1/1,000 dilution of culture supernatant and were far below saturating. At higher concentrations of culture supernatant, the fluorescence of these cells was offscale on the instrument. By contrast, fluorescence obtained with TS2/9 and anti-CR1 antibodies was at saturating levels for these antibodies.

DISCUSSION

The presence of CD36 on adult erythrocytes has previously been discounted. Earlier studies with an anti-CD36 murine IgG1 MoAb (FA6-152) did not detect any CD36 on adult RBCs, although positive reactivity was noted with a minority of adult reticulocytes. In these studies, it was...
concluded that CD36 is a marker of early erythroid differentiation because FA6-152 reacted with erythroblasts generated from adult or fetal progenitors and with fetal erythrocytes. MoAb FA6-152 failed to identify CD36 on adult RBCs by antibody-mediated cell agglutination, immunofluorescence microscopy, or immunoprecipitation of radiolabeled RBC glycoproteins. In addition, RBCs were reported as essentially negative with 5 anti-CD36 MoAbs in the Leukocyte Typing Workshop IV. We were similarly unable to detect CD36 on RBCs by agglutination and immunofluorescence microscopy (S.M. Handunnetti, unpublished results). However, using FACS analysis, we were consistently able to identify CD36 antigenic activity on RBCs from all six normal donors tested. For the rest of the discussion we will refer to this antigen as CD36 for brevity, while remaining aware that the antigen may be a different form of CD36 than that present on other cell types. For instance, the form of CD36 on fetal erythroblasts and fetal mature erythrocytes is 78 Kd, whereas adult platelet CD36 is 88 Kd.

The level of CD36 we were able to detect by FACS analysis is clearly very low and close to the limits of detection. To confirm that staining is due to expression of CD36 on RBCs, we tested two possible artifactual causes of staining with anti-CD36 MoAbs. First, the possibility that fragments of platelets became adherent to RBCs during processing of blood was tested by FACS analysis with antiplatelet MoAbs 7F12 and L133. Both MoAbs were negative with RBCs, even though with platelets these MoAbs stain similarly or more strongly than the anti-CD36 MoAbs (Fig 2). Thus, the staining of RBCs with anti-CD36 MoAbs cannot be accounted for by any adherence of platelet fragments. Second, ascites of some individual mice may contain small amounts of normal mouse Ig with antihuman RBC reactivity, some of which will copurify on protein A-Sepharose. We therefore tested by an absorption experiment (Fig 3) whether the reactivity of the anti-CD36 MoAbs was specific to CD36 or might represent low levels of antihuman RBC normal mouse Ig. We found that absorption of 8A6 and OKM5 MoAb solutions with CHO-CD36 completely removed the reactivity with RBCs, whereas absorption with CHO cells or RBCs had no effect. In addition, we found that a hybridoma supernatant of 8A6 was positive with RBCs (Table 1). We conclude that staining of RBCs with anti-CD36 MoAbs is specific for CD36.

Transferrin receptor and HLA-A,B,C, like CD36, are present on erythroblasts and at low levels on some reticulocytes. In addition, low levels of HLA-A,B,C have been reported to be present on mature RBCs of some normal donors, using the W6/32 MoAb as the detecting antibody. With MoAbs W6/32 and L01.1 and stain 6, we could detect little or no expression of either HLA-A,B,C or transferrin receptor on the RBCs of the donors we tested.
The fluorescence with anti-CR1 MoAbs was 9- to 18-fold autofluorescence (Table 1). Staining with antiglycophorin A antibodies was capable of giving enhancements of more than 2,000-fold, the maximum level of staining being off-scale on the instrument (data not shown). Although accurate quantitation of the amount of CD36 on RBCs will require detailed studies with multiple 125-I-labeled MoAbs and Fab fragments,44 our data with anti-CR1, anti-LFA-3, and antiglycophorin MoAbs consistently suggest that the number of CD36 molecules per cell is below 500. The plate adherence assay (Fig 5) and its lack of selectivity for reticulocytes suggest that, although the amount of CD36 expressed on RBCs is low as detected by FACS analysis, there are enough CD36-like molecules on the surface of normal RBCs to mediate adherence to a surface presenting suitable receptors, such as anti-CD36 MoAbs in this assay. It is also possible that CD36 on RBCs may act together with other receptors in mediating adhesion phenomena, because many cell adhesion phenomena involve multiple receptors.59,60,61 With respect to the adhesion of P. falciparum-infected cells to uninfected erythrocytes, it is interesting to note that ICAM-1, which has been identified as an additional adherence receptor on endothelial cells for some isolates of P. falciparum,23,18 could not be detected on RBCs with the CL203 MoAb (Table 1), so that it seems unlikely to be a candidate rosetting receptor. However, it (Table 1). The use of a single MoAb to test the presence of these antigens is subject to the limitation that the antigens might still be present in a tissue-specific form lacking reactivity with the test MoAb. Subject to this limitation, our data would suggest that the retention of CD36 on mature erythrocytes may be relatively specific and may indicate a biologic role for this molecule on RBCs in the normal individual.

The enhancement of relative mean fluorescence we obtained with anti-CD36 antibodies was approximately a factor of 2, ie, the fluorescence due to R-PE-labeled antibody bound is approximately equal to the autofluorescence of the cells (Table 1). We also determined the mean fluorescence obtained with antibodies for which the number of sites per cell is well-characterized: LFA-3, 4,000 molecules/cell19,80; CR1, which shows allelic variation in the amount expressed, with a population average of 600 molecules per cell61,46; and glycophorin A, 1,000,000 molecules of glycophorin per cell.44 The fluorescence with anti-LFA-3 antibodies was 84-fold autofluorescence, and
cannot be ruled out that there might be alternative form of ICAM-1 on RBCs that does not react with this MoAb, or that molecules present at levels undetectable by this assay could still be involved in adhesion.

The expression of CD36 is thought to decrease as the erythroblast matures to reticulocyte and mature erythrocyte. The results we obtained suggest that, among circulating erythrocytes in a normal individual, the level of CD36 remains relatively constant as the erythrocyte ages, because the fluorescence histograms (Fig 1) suggest rather homogeneous staining of RBCs with anti-CD36 MoAbs. In addition, we found that the plate adherence assay with anti-CD36 MoAbs did not selectively retain reticulocytes. Nevertheless, the level of expression of CD36 on reticulocytes, old and young RBCs, and the relative ability of these cells to support rosetting in *P. falciparum* malaria deserve further quantitative study. In anemia, depending on the mechanism or stage of anemia, the proportion of reticulocytes and young RBCs may increase or decrease. If there is a decrease in CD36 expression with erythrocyte age, CD36-dependent rosetting of infected erythrocytes may be enhanced or reduced in an anemic individual.

The presence of CD36 on normal human RBCs also raises interesting questions regarding the role of this CD36 in normal individuals. Is the form of CD36 on RBCs also able to bind TSP (whether soluble, aggregated, or degraded) and, if so, what is the role of TSP binding? If CD36 on RBCs binds TSP, then, by analogy with the role of the CR1 receptor on RBC, it might be interesting to investigate its possible role in clearance and degradation of TSP. CD36 on RBCs might also be involved in adhesion phenomena involving TSP or collagen. Erythroid progenitor cells at the burst-forming unit-erythroid (BFU-E) or colony-forming unit-erythroid (CFU-E) stage have been shown to attach to TSP, but studies so far have not been able to detect RBC adhesion to TSP-coated plastic (van Schraardijk, unpublished results). The low levels of CD36 present may control adhesion of RBCs, so that adhesion to cells or matrix carrying TSP might only be seen in the presence of synergistic interactions with other receptors, or with a high density or aggregated form of TSP. A possible analogy might be the situation with von Willebrand factor (vWF), which mediates RBC adhesion to endothelial cells when multimeric forms of vWF are induced with desmopressin. Alternatively, the level of negative surface charge on RBCs and other cells may be critical in controlling adhesion. For instance, the ability of LFA-3 and CD2 to mediate rosetting of human RBCs specifically with activated T cells, rather than resting T cells, is thought to be controlled by a change in surface charge on T-cell activation. Finally, it remains to be seen whether the signal transduction properties of CD36 are relevant to CD36 on RBCs. It has been postulated that cell-cell interactions between erythrocytes and platelets enhance platelet reactivity and may activate erythrocytes to release ADP.

Three percent to 11% of phenotypically normal Japanese individuals and 0.34% of normal United States donors lack detectable platelet CD36. These NAK- individuals have no overt hemostatic problems and were identified by screening healthy blood donors. Recently, it has been shown that CD36 is also missing from monocytes of these individuals, and that PRBCs of the Ibg strain of *P. falciparum* did not adhere to NAK- platelets and monocytes. We identified here a Gambian patient with *P. falciparum* ma-
laria (patient 425), who was deficient in the expression of CD36 on erythrocytes. We have no information as to whether this deficiency was transient or permanent. We have shown\textsuperscript{29} that the \textit{P. falciparum} strain present in this patient was dependent on CD36 for rosetting and did not rosette with the patient’s own RBCs. In this way, some African patients may be protected from rosetting of CD36-deficient RBCs. The frequency and etiology of the CD36-deficient RBC phenotype in African populations remains to be determined, as does any correlation with the clinical picture of malaria in these patients. Furthermore, if such individuals are deficient in CD36 on all cell types, the course of malaria in these individuals could give important information about the importance of CD36 in sequestration in vivo in humans. The ability to bind to CD36 is the most widely distributed cytoadherence property among wild isolates of \textit{P. falciparum},\textsuperscript{22,23} and this is thought to reflect the importance of CD36 for PRBC sequestration via adherence to endothelial cells in the microvasculature. If individuals lacking CD36 were to be found to have normal susceptibility to CD36-dependent strains of malaria and normal infected cell sequestration, our understanding of the molecular basis of cytoadherence in malaria would need to be profoundly changed.

**ACKNOWLEDGMENT**

We thank Dr A. Facello and Dr P. Rao of Ortho Pharmaceuticals for the gifts of OKM5 and OKM8 MoAbs, Dr Anne Jackson of Becton Dickinson Immunocytometry Systems for the gift of the 7F12 antibody, Dr Soldano Ferrone of New York Medical College for the gift of the CL203 anti-ICAM-1 antibody, and Dr Lewis Lanier of DNAX Research Institute Inc for the gift of several MoAbs and for helpful discussions. We thank Dr James Cupp, Dixie Polakoff, and Jean Herrman for help and advice with FACS analysis; Kerstin Morehead for assistance in characterization of the anti-CD36 antibodies; and Dr K. Marsh and the staff at the Medical Research Laboratories at Fajara, The Gambia, for the GAM425 malaria isolate.

**REFERENCES**


35. Robinson J, Sieff C, Delia D, Edards P, Greams V: Expression of cell-surface HLA-DR, HLA-ABC and glycophorin during erythropoiesis differentiation.


37. de Villarta JP, Rouger P, Muller JY, Salmon C: HLA antigens on peripheral red blood cells: Analysis by flow cytofluorimetry using monoclonal antibodies. Tissue Antigens 26:12, 1985

38. Rivera R, Scornik JC: HLA antigens on red cells. Implications for achieving low HLA antigen content in blood transfusions. Transfusion 26:375, 1986


47. Bernard A, Tran HC, Boumsell L: Three different erythrocyte surface molecules are required for spontaneous T cell rosette formation. J Immunol 139:18, 1987


From www.bloodjournal.org by guest on October 3, 2017. For personal use only.
Normal human erythrocytes express CD36, an adhesion molecule of monocytes, platelets, and endothelial cells

MR van Schravendijk, SM Handunnetti, JW Barnwell and RJ Howard