Brief Definitive Report

Soluble Intercellular Adhesion Molecule 1-Immunoglobulin G1 Immunoadhesin Mediates Phagocytosis of Malaria-infected Erythrocytes

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Summary

We describe an immunoadhesin molecule containing intercellular adhesion molecule 1 (ICAM-1) molecularly fused to hinge and C_H2 and C_H3 domains of the human immunoglobulin G1 H chain that binds Plasmodium falciparum-infected erythrocytes. This receptor-based immunoadhesin is an effective and specific inhibitor of P. falciparum-infected erythrocyte adhesion to ICAM-1-bearing surfaces, but does not inhibit leukocyte function antigen 1 (LFA-1) interaction with ICAM-1. Furthermore, the immunoadhesin promotes phagocytosis and destruction of parasitized erythrocytes by human monocytes. Each of these modes of action has potential for the therapy of malaria.

Intercellular adhesion molecule 1 (ICAM-1) belongs to the Ig superfamily of proteins and is primarily involved in cell–cell adhesive interactions of the immune system (1). ICAM-1 expression on postcapillary endothelium is regulated by the cytokines TNF and IL-1 (2). ICAM-1 functions as a natural receptor for the integrins lymphocyte function antigen 1 (LFA-1) (3) and Mac-1 (4, 5). In addition, this versatile molecule is exploited as a receptor for the major group of human rhinoviruses (6, 7) and for Plasmodium falciparum-infected erythrocytes (8, 9). Recently, we and others have demonstrated that red blood cells infected with mature intracellular forms of the malaria parasite (IRBC) bind to a region located within the NH_2-terminal Ig-like domain of ICAM-1 that is distinct from the regions recognized by LFA-1 and rhinovirus (10, 11).

The adherence of erythrocytes infected with the human malaria parasite, P. falciparum to postcapillary venular endothelium in peripheral tissues avoids clearance in the spleen and contributes to evasion of the host immune response, as well as to the pathogenesis of cerebral malaria. Disruption of intravascular erythrocyte sequestration is predicted to alleviate much of the pathology of P. falciparum malaria, and especially mortality, as a result of severe cerebral malaria or placental insufficiency. Sequestration involves interactions between multiple endothelial receptors and counter-receptors on the parasitized erythrocyte surface. ICAM-1 and CD36 bind infected erythrocytes from a majority of patients with uncomplicated and severe forms of the disease (8, 9). Disruption of these adhesion pathways may be an effective alternative to chemotherapy for treating the increasing number of drug-resistant strains of P. falciparum. Previously, we have demonstrated that soluble ICAM-1 and synthetic ICAM-1 peptides may compete IRBC binding to ICAM-1 (10). Here we characterize an ICAM-1 immunoadhesin that both competes adherence and triggers immunologic killing of IRBC.

Materials and Methods

Construction of an ICAM-1 Immunoadhesin. A 1.3 kb fragment containing the γ1 hinge, C_H2 and C_H3 sequence was generated by PCR from a plasmid containing the human gene (12) using oligonucleotide primers 5'TTTCTCGAGGGTGTCTGCTGGAAGCAG-GCTCAG and 5'TTTGCGGCCGCGGGAGCGGGGCTTGCC-GGCCGTCG. The 5' XhoI and 3' NotI sites introduced by the primers were used to subclone the IgG1 sequence into pCDM8 to produce pCDG1. To construct an ICAM-1-IgG1 chimera, a PCR fragment was generated that contains the ICAM-1 cDNA sequence for signal peptide and domains 1 and 2 terminating with the codon F185 (13) using primers 5'ACCGGAAGCTTCTAGAGATCCCTC-GACCACGAGATCCATTGTGC and 5'TTTCTCGAGTCTCAC-CAAAGGTCTGGAGCTGGTAGGGGGC. The fragment contains a 5' HindIII site, a translational stop codon following the codon for F185, the 5' donor splice that follows the γ1 C_H1 exon, and a 3' XhoI site. This fragment was subcloned into HindIII and XhoI sites of pCDG1 to produce pCDF185G1. Culture supernatants of COS cells transfected with pCDF185G1 contained ∼0.5 μg/ml ICAM-1-IgG1 chimera (IC1-2D/IgG) as determined by ELISA on day 3 after transfection. IC1-2D/IgG was purified from culture media of transfected COS cells by ICAM-1 mAb (R6.5)-Sepharose and protein A-Sepharose chromatography.

P. falciparum-infected Erythrocyte and Lymphoblastoid Cell Binding to ICAM-1. Soluble ICAM-1 truncated before the hydrophobic

transmembrane region was purified from the supernatants of transfected CHO cells (sICAM-1/CHO) (14) or baculovirus vector-infected insect cells (15). For IRBC binding, ICAM-1 (10 μg/ml) was adsorbed (20 μl aliquots) to plastic bacteriological plates (model 1007; Falcon Labware, Oxnard, CA) overnight at 4°C. IC1-2D/IgG (ICAM-1–IgG1 chimera) was similarly adsorbed to plastic plates that had previously been coated with protein A (50 μg/ml). Unbound sites were blocked for 30 min at room temperature with 1% BSA-PBS to reduce nonspecific binding. Laboratory-adapted intraerythrocytic *P. falciparum* parasites selected in vitro to bind to purified ICAM-1 (ItG-ICAM) (9) were maintained in synchronous continuous culture and used in adhesion assays at the trophozoite/schizont stage of development. The IRBC were added to ICAM-1–coated plates (40–50% parasitemia, 1% hematocrit) for 1 h at room temperature. In inhibition assays, ItG-ICAM IRBC were incubated in solution with increasing concentrations of IC1-2D/IgG chimera, sICAM-1/CHO, or normal human IgG for 30 min before addition to plates coated with sICAM-1/CHO (10 μg/ml). Erythrocytes not attached to the sICAM-1–coated surface were removed by gentle rinsing of the plates. Cells were fixed with 2% glutaraldehyde and stained with Giemsa. The number of malaria-infected erythrocytes bound per mm² surface area represents the mean of three separate determinations. The concentration of sICAM-1 and IC1-2D/IgG was determined with a capture ELISA assay (14), using sICAM-1/CHO as a standard.

For SKW-3 cell binding, IC1-2D/IgG at the concentration indicated was absorbed to 96-well microtiter plates that had previously been coated with protein A (20 μg/ml) and blocked with 1% BSA-PBS. SKW-3 cells in binding buffer (RPMI/10% FBS/20 mM Hepes) were treated with or without 100 ng/ml PMA for 15 min at 37°C and then labeled with 2′,7′-bis(2-carboxyethyl)-(5 and 6)-carboxyfluorescein acetomethyl ester (Molecular Probes, Inc., Eugene, OR). Binding (10⁵ cells/well) was for 1 h at 25°C.

For IC1-2D/IgG inhibition of SKW-3 binding, 96-well microtiter plates were coated with 50 μl sICAM-1 (10 μg/ml, 2 h, 37°C) and blocked with 1% BSA-PBS. PMA-treated SKW-3 (10⁵ cells) were incubated for 30 min in 50 μl of binding buffer, with or without IC1-2D/IgG or mAb TS1/18 to the LFA-1 β subunit (1:100 ascites) and then added directly to sICAM-1–coated wells. Binding was for 1 h at 37°C. Unbound cells were removed by inverting microtiter plates in a tank of PBS/1 mM Mg²⁺/0.05 mM Ca²⁺/0.1% BSA for 45 min. Bound cells were quantitated on a fluorescence concentration analyzer (Pandex Laboratories Inc., Mundelein, IL). Percent bound (±SD) was calculated by subtracting background binding to wells that were not coated with ICAM-1 from binding to ICAM-1–coated wells, divided by input fluorescence × 100.

Monocyte Phagocytosis of P. falciparum-infected Erythrocytes. Human mononuclear cells isolated from whole blood by centrifugation on a Ficoll-Hypaque density gradient were washed three times in RPMI 1640 and resuspended in medium supplemented with 10% normal human serum. Cells (10⁵ in 100 μl) were added to glass coverslips for 90 min at 37°C in 7.5% CO₂. Nonadherent cells were removed by washing coverslips three times. Attached cells were >95% monocytes by Wright-Giemsa and esterase stains. IRBC (5 × 10⁶ per 100 μl) selected in vitro for binding to ICAM-1 (ItG-ICAM) or CD36 (ItG-CD36) were incubated with IC1-2D/IgG chimera or normal human IgG (20 μg/ml final concentration) for 30 min before addition to monolayers of adherent freshly isolated human monocytes. After 2 h incubation at 37°C, unattached red blood cells were removed by washing coverslips three times with RPMI 1640. To avoid quantitating IRBC attached to the phagocyte surface but not internalized, coverslips were rinsed in hypotonic 0.85%

NH₄Cl to lyse attached IRBC. Preincubation of monocyte monolayers with anti-CD36 mAb OKM5 completely blocked adhesion of ItG-CD36-infected erythrocytes to monocytes without any effect on subsequent phagocytosis of ItG-ICAM malaria-infected erythrocytes (not shown). Coverslips were fixed with 2% glutaraldehyde followed by staining with Giemsa. The percentage of monocytes that contained intracellular intact infected red cells or degraded parasite pigment was quantitated by light microscopy.

Results

Since adhesion of IRBC to microvascular endothelium is an absolute requirement for survival of *P. falciparum* parasites in vivo (16), a strategy was fashioned to both inhibit infected
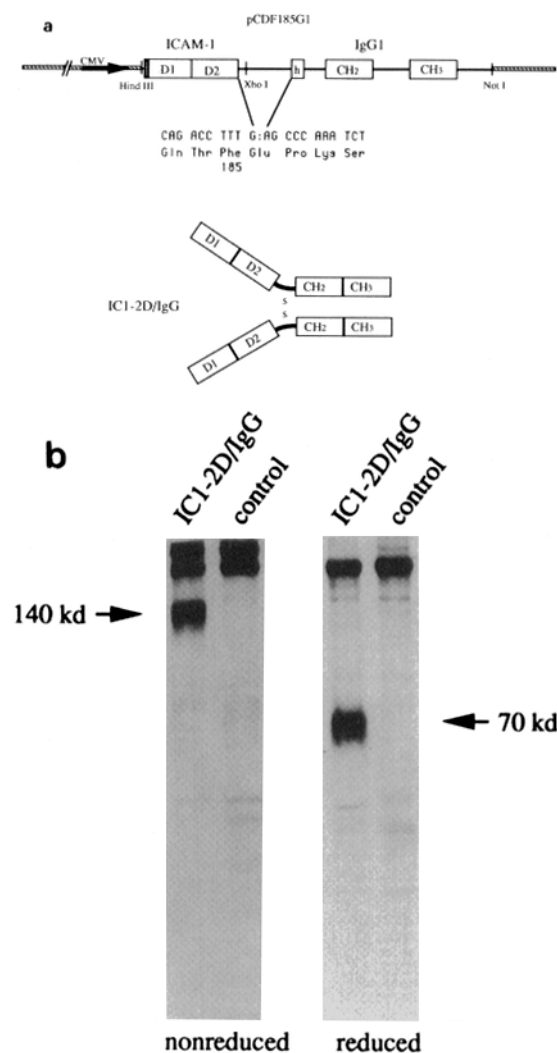

Figure 1. (*a*) Schematic diagram of the IC1-2D/IgG expression construct (pCDF185G1) and the IC1-2D/IgG immunoadhesin. (*b*) SDS-PAGE of the immunoadhesin. COS cells were transfected with the plasmid pCDF185G1 or as a control CDM8 and labeled with [³⁵S]methionine and cysteine. Secreted material was precipitated with protein A-Sepharose and subjected to SDS-PAGE and fluorography. Identical results were obtained with immunoprecipitations using anti-ICAM-1 mAb R6.5 (data not shown).
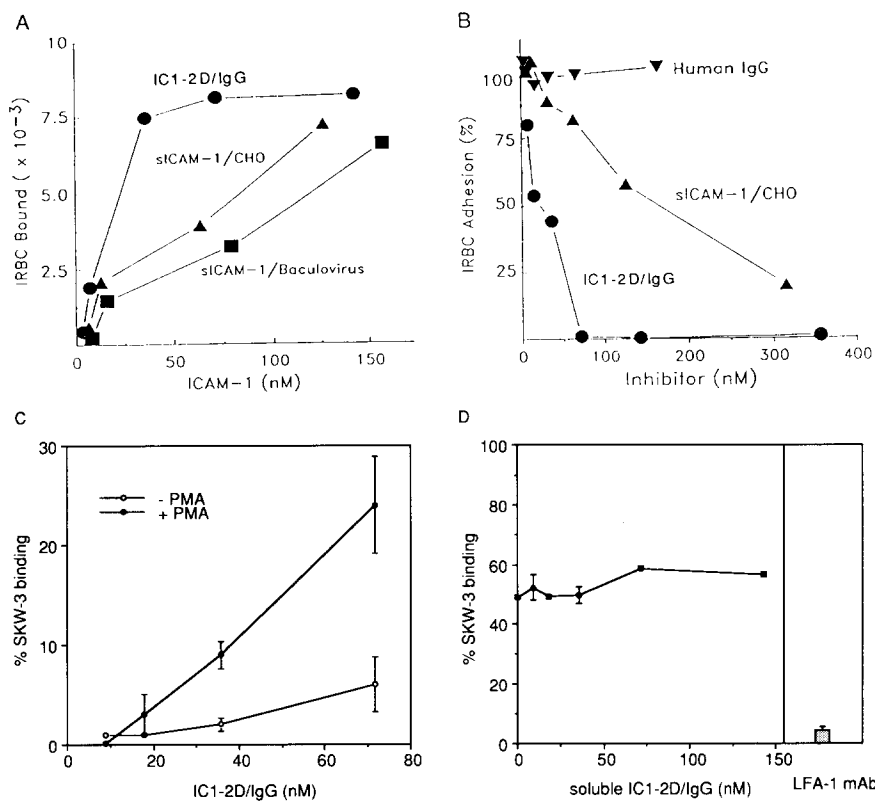
Figure 2. *P. falciparum*-infected erythrocyte and T-lymphoblastoid cell binding to recombinant ICAM-1. (*A*) Adhesion of ItG-ICAM-1-IRBC to surfaces coated with the indicated concentrations of ICAM-1-IgG chimera (*IC1-2D/IgG*), CHO cell-derived soluble ICAM-1 and baculovirus-derived soluble ICAM-1. (*B*) Inhibition of IRBC adhesion to ICAM-1–coated surfaces by IC1-2D/IgG chimera, sICAM-1, or human IgG. (*C*) Binding of T lymphoblastoid cells ± PMA to IC1-2D/IgG coated surfaces. (*D*) Inhibition of PMA-stimulated SKW-3 adhesion to sICAM-1–coated surface by IC1-2D/IgG.

erythrocyte adhesion and kill the intracellular parasite. We designed an immunoadhesin consisting of the first two NH₂-terminal Ig-like domains of ICAM-1 fused to the hinge region and C_H2 and C_H3 domains of human IgG1 H chain and expressed it in COS cells (Fig. 1 *a*). The secreted mature immunoadhesin, IC1-2D/IgG, exists as a dimer migrating at 140,000 M_r when not reduced, and 70,000 M_r when reduced (Fig. 1 *b*). These sizes agree with that predicted for IC1-2D/IgG.

The adhesion of IRBC to IC1-2D/IgG immunoadhesin was compared with that of a soluble form of ICAM-1 (sICAM-1) possessing all five Ig-like domains that was produced in CHO cells (14) or insect cells (15). Malaria-infected erythrocytes bind in a dose-dependent manner to sICAM-1 and IC1-2D/IgG coated on surfaces (Fig. 2 *A*). The immunoadhesin did not bind uninfected erythrocytes or erythrocytes infected with malaria parasites which bind to an alternative endothelial receptor, CD36 (data not shown). The ICAM-1 immunoadhesin is a more effective inhibitor of IRBC adhesion to an ICAM-1–coated surface than sICAM-1 (Fig. 2 *B*). The K_i for IC1-2D/IgG is ∼20 nM, whereas for sICAM-1 it is 150 nM (10). Enhanced binding may reflect the divalent nature of IC1-2D/IgG.

Binding of IC1-2D/IgG to IRBC was compared with binding to LFA-1 on the T lymphoblastoid line SKW-3. SKW-3 cell adherence to immobilized IC1-2D/IgG is enhanced by PMA (Fig. 2 *C*), in similarity to results with immobilized ICAM-1 (17). Concentrations of soluble IC1-2D/IgG as high as 150 nM do not inhibit LFA-1–dependent SKW-3

binding to sICAM-1–coated surfaces (Fig. 2 *D*). In addition, binding of soluble IC1-2D/IgG to lymphoblastoid cells with or without PMA treatment cannot be detected by indirect immunofluorescence (data not presented). Adhesion receptors have been found to be of low affinity, on the order of 1 μM (18, 19). These findings show that the affinity of IC1-2D/IgG is higher for the receptor on IRBC than for LFA-1. This difference in affinity should make it possible to use IC1-2D/IgG to disrupt sequestration of IRBC through ICAM-1 without
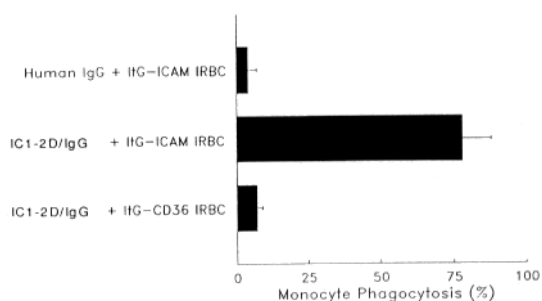

Figure 3. Phagocytosis of *P. falciparum*-infected erythrocytes by human monocytes. *P. falciparum*-infected erythrocytes selected for binding to ICAM-1 (*ItG-ICAM*) or CD36 (*ItG-CD36*) were incubated with IC1-2D/IgG chimera or normal human IgG before addition to monolayers of freshly isolated human monocytes. After 2 h, noninternalized IRBC were lysed and monocytes with intracellular infected red cells or degraded parasite pigment were quantitated by staining with Giemsa and light microscopy. Results indicate the mean ± SD of three determinations.
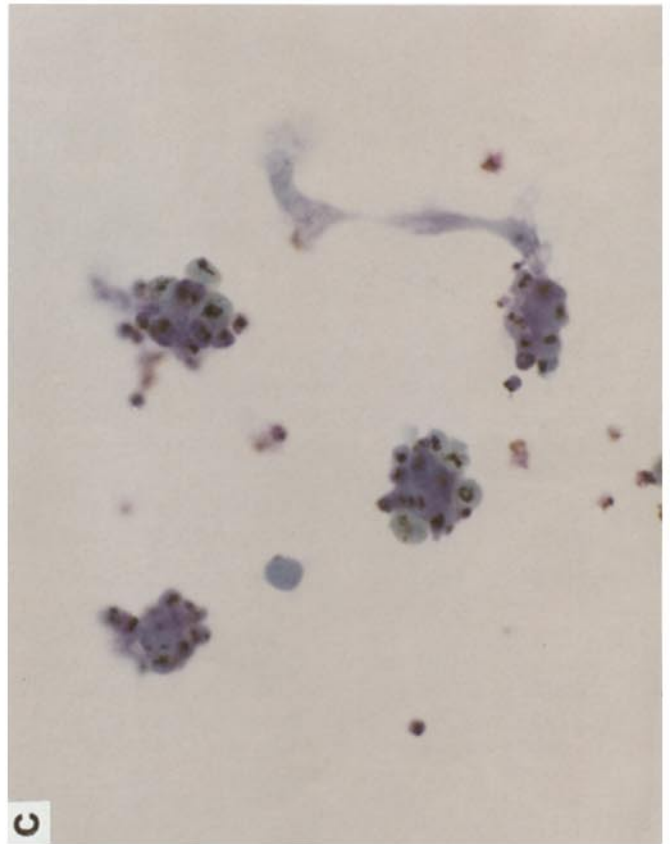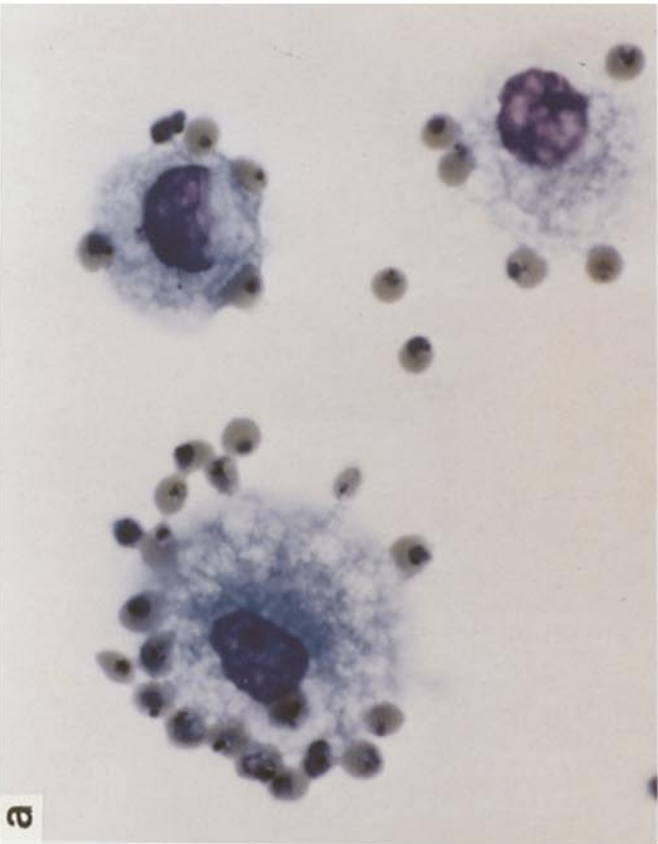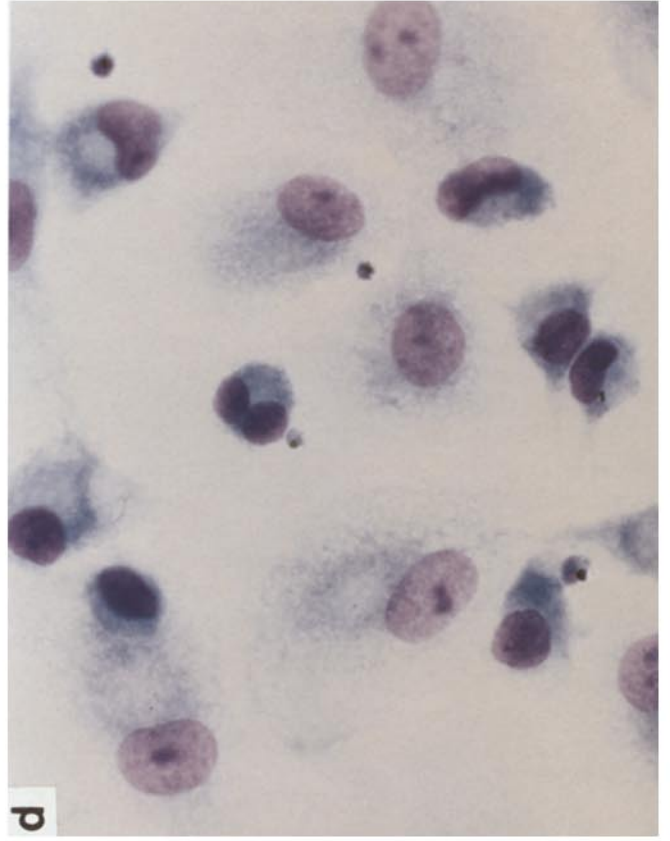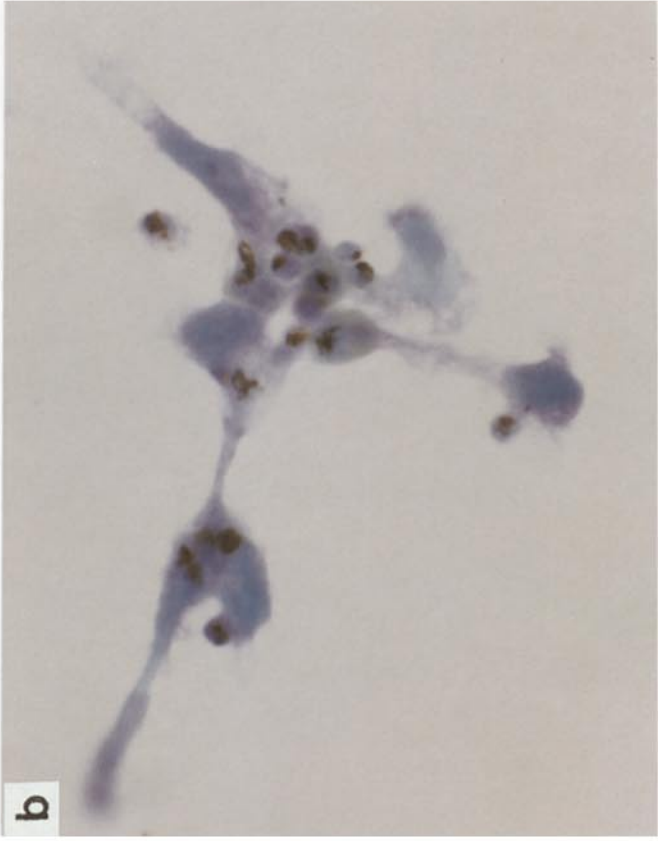
disrupting ICAM-1/LFA-1 interactions that are important in inflammation and the immune response.

We tested the ability of ICAM-1 immunoadhesin to support phagocytosis of IRBC. The Fc region of IgG1 was chosen for the immunoadhesin because this subclass is the most effective in triggering antibody-dependent cellular cytotoxicity (20) and binds avidly to all three classes of Fcγ receptor (21). Incubation of parasitized erythrocytes that bind to ICAM-1 (ItG-ICAM IRBC) with the IC1-2D/IgG chimera resulted in their phagocytosis suggesting that the FcR binding function of IC1-2D/IgG is intact (Fig. 3). Infected erythrocytes incubated with or without normal human IgG were not phagocytosed. CD36-binding IRBC incubated in the presence or absence of IC1-2D/IgG chimera were not phagocytosed. The IC1-2D/IgG–treated internalized IRBC are quickly degraded and residual parasite-derived hemozoin pigment observed intracellularly (Fig. 4, b and c). CD36-binding IRBC attach to CD36 on the surface of monocytes but are not phagocytosed through this receptor (Fig. 4 a). The rosetting of ItG-CD36 IRBC with monocytes was blocked completely by the anti-CD36 mAb OKM5 (data not shown). The ICAM-1–binding IRBC are not rosetted or phagocytosed in the absence of IC1-2D/IgG (Fig. 4 d).

Discussion

We have designed an ICAM-1 immunoadhesin that is effective against *P. falciparum* parasitized erythrocytes at concentrations that do not block lymphocyte binding to ICAM-1. Sequestration of *P. falciparum* IRBC plays a pivotal role in the pathology of malaria, probably by triggering a cascade of deleterious events including local anoxia, induction of toxic inflammatory mediators, edema, and tissue damage. Sequestration in the brain leads to the most fatal form of the disease, cerebral malaria (22). Immunoadhesins mimicking *P. falciparum* sequestration receptors can be therapeutically effective through two distinct mechanisms. First, they should reverse sequestration. A combination of adhesins, including ICAM-1 and CD36 immunoadhesin, may be required for maximal effectiveness. Reversal of sequestration is predicted to alleviate much of the associated pathology and especially mortality resulting from cerebral malaria or placental insufficiency. Second, immunoadhesins can sensitize parasitized erythrocytes for recognition and elimination by the immune system, as exemplified here by monocyte phagocytosis and destruction mediated by an ICAM-1 immunoadhesin. Release from sequestration is not necessarily required for this effector mechanism, as it could presumably be mediated by monocytes and granulocytes at sites of sequestration in postcapillary venules. A side benefit of clearance of parasites by phagocytes is that it may boost host humoral and cellular immunity to *P. falciparum*. Cytoadherence receptor binding must be conserved and thus pathogen strain variation, which is extensive for *P. falciparum*, should not be an effective mechanism for evasion of this therapy.

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Figure 4. Monocyte phagocytosis of *P. falciparum*-infected IRBC. (*a*) CD36-binding IRBC preincubated with IC1-2D/IgG chimera bind to the monocyte surface but are not phagocytosed. (*b* and *c*) ICAM-1-binding IRBC preincubated with IC1-2D/IgG chimera are phagocytosed and internally degraded by monocytes. (*d*) ICAM-1-binding IRBC in the absence of ICAM-1 immunoadhesin are not phagocytosed by monocytes. Conditions for IC1-2D/IgG mediated IRBC phagocytosis were as described in the Fig. 3 legend.

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