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Failure To Block Adhesion of *Plasmodium falciparum*-Infected Erythrocytes to ICAM-1 with Soluble ICAM-1

ALISTER G. CRAIG,* ROBERT PINCHES, SHAHID KHAN,† DAVID J. ROBERTS, GARETH D. H. TURNER,‡ CHRISTOPHER I. NEWBOLD, AND ANTHONY R. BERENDT§

Molecular Parasitology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom

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The adhesion of *Plasmodium falciparum*-infected erythrocytes is thought to play a central role in the pathogenesis of severe malaria. ICAM-1 has been identified as one of the host receptors for parasitized erythrocytes and has been implicated as being involved in progression to cerebral malaria. Thus, intervention strategies based on the reversal of this interaction could potentially be used to reduce morbidity and mortality. We have investigated the inhibition of the interaction between ICAM-1 and infected erythrocytes by using recombinant soluble ICAM-1 as competitor and find that we are unable to reduce adhesion to ICAM-1 in vitro.

The interaction between the infected erythrocyte surface and host endothelium is thought to play an important role in the pathogenesis of severe falciparum malaria. Several endothelial proteins, including CD36 (4, 29, 31), thrombospondin (34), VCAM, E-selectin (30), chondroitin sulfate (37), and ICAM-1 (8), have been identified as receptors for parasitized cells. ICAM-1 is a 95- to 112-kDa membrane glycoprotein expressed on lymphocytes, macrophages, and vascular endothelium and is a member of the immunoglobulin (Ig) superfamily. Its primary role is in mediating cellular adhesion within the immune system via the leukocyte integrins LFA-1 (39) and Mac-1 (14). Studies on the interaction between infected erythrocytes and ICAM-1 have located the binding site to a region in the first N-terminal domain that overlaps but is distinct from the sites for LFA-1 and human rhinovirus (7, 27).

The functional significance of cytoadherence for parasite survival is unclear, but the sequestration of mature parasitized erythrocytes (PRBC) from the peripheral circulation in *Plasmodium falciparum* infections by adhesion to postcapillary venular endothelium is a key event in cerebral malaria, one of the most serious complications of malaria. The localization of sequestered PRBC has been correlated with clinical syndrome only in fatal cerebral malaria (22, 33), but sequestration is proposed to cause dysfunction in a variety of organs. Histopathological studies have demonstrated that upregulation of ICAM-1 on cerebral vessels is associated with the presence of sequestered parasites, whereas levels of CD36 on brain endothelium are low and remain unaltered in infected samples compared to uninfected controls (47).

Several reports have attempted to correlate the adhesive properties of PRBC with clinical outcome (10, 19, 28). The ability of infected erythrocytes to bind uninfected cells, rosetting, has been associated with severe malaria (9, 38, 45). However, for cytoadherence, these studies have been hampered by the difficulty in obtaining large numbers of appropriate clinical samples and the heterogeneous parasite populations within these samples. A large case-control study in Kenya has shown a correlation between malaria symptomatic disease and the ability of the parasite to adhere to ICAM-1, with a trend toward ICAM-1-binding parasites being present in more patients with severe malaria than in asymptomatic controls (26).

These and other studies show that the ability of PRBC from field isolates to bind to ICAM-1 is highly variable. Similarly, laboratory lines selected on ICAM-1 can show widely different affinities for ICAM-1 (see below). The parasite molecule present on the surface of PRBC which mediates these various adhesive properties has been identified as the variant surface antigen PfEMP1 (5, 16). Recent studies have demonstrated the existence of a large gene family encoding PfEMP1 (6, 40, 42). These genes contain conserved regions with motifs showing homology to sequences involved in adhesive interactions in erythrocyte invasion (32). The combination and content of these regions vary between genes and may explain the repertoire of ligand specificity exhibited by antigenically distinct strains. Thus, severe disease may result from the combination of induced ICAM-1 expression in the host and the ability of parasitized cells to bind to this ligand as a result of expression of the appropriate antigenic type. Disruption of this adhesive event may reduce the mortality associated with malaria. Inhibition of PRBC binding by using soluble ICAM-1 has been demonstrated for one laboratory strain of *P. falciparum* selected for high-affinity binding to ICAM-1 (41). We therefore decided to extend this study and investigate conditions under which we could block adhesion of parasites with lower affinity for ICAM-1.

MATERIALS AND METHODS

Construction of ICAM-1–Fc plasmids. Three chimeric constructs were made for expression in COS cells containing the N-terminal two (h12), three (h123), or all five (h12345) Ig-like domains of human ICAM-1 combined with the hinge region and C_H2 and C_H3 domains of human IgG1. These were produced by digestion of a CD8-Fc expression plasmid (18) with *Xho*I and *Bam*HI to remove the CD8-encoding portion. This was replaced with ICAM-1 fragments (derived by PCR from a five-domain expression plasmid) encoding the leader sequence and either two (5′-ACGGATCCACAAAGGTCTGGAGCTG), three (5′-ACG

* Corresponding author. Mailing address: Molecular Parasitology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom. Phone: 44 1865 222303. Fax: 44 1865 222444. E-mail: acraig@hammer.imm.ox.ac.uk.

† Present address: Department of Pathology, University of Cambridge, Cambridge, CB2 1QP, United Kingdom.

‡ Present address: Department of Cellular Science, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom.

§ Present address: Adhesion and Infection Laboratory, Centre for Tropical Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom.

GATCCTTGGGCGCCGGAAAGC), or five (5′-ACGGATCCATCTCATACC GGGGGGAG) domains. The primer sequences indicate the boundaries of each construct.

Preparation and purification of ICAM-1–Fc. COS7 cells were transfected with the ICAM-1–Fc plasmids by using DEAE-dextran (3). Transfected cells were incubated in Dulbecco modified Eagle medium (DMEM)–10% fetal bovine serum (Gibco) for 18 h before the medium was replaced with DMEM–1% fetal bovine serum–1% NuSerum (Calbiochem). The supernatants were harvested after 5 to 6 days and passed through a 0.4-μm-pore-size nylon filter (Gelman) prior to application onto protein A-Sepharose (Pharmacia). The column was washed with 5 to 10 column volumes of 20 mM sodium phosphate (pH 7.0)–1 mM EDTA, and the contaminating bovine Igs were eluted with 0.1 M sodium citrate (pH 5.0). The ICAM-1–Fc was eluted with 0.1 M sodium citrate (pH 2.7) and dialyzed immediately against phosphate-buffered saline (PBS)–0.05% sodium azide. The purity of the protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration was determined by absorption at 280 nm.

Gel chromatography of ICAM-1–Fc protein. Samples of the three different ICAM-1–Fc chimeric proteins were labelled with ^{125}I by using iodogen (15). Unincorporated label was removed, and the samples were applied separately to a 1- by 79-cm Sephacryl S400 column. The samples were eluted with 150 mM sodium chloride–50 mM Tris-HCl (pH 7.5)–0.02% sodium azide at a flow rate of 16.5 ml/min, and fractions were collected for counting. Standards were included in the elution to calibrate the column.

Parasite culture. *P. falciparum* parasites were cultured in group O human erythrocytes as previously described (44). PRBC were purified from uninfected cells by flotation on plasmagel (Rhone-Poulenc) and cultured for 2 to 3 h prior to the adhesion assays.

Cytoadhesion assays. Plastic dishes (Falcon code 1007) were spotted (2-μl volume) with ICAM-1 (25 μg/ml) and CD36 (12.5 ng/ml) in a radial pattern unless otherwise stated. Triplicate spots were made on each dish and incubated in a wet box for 2 to 3 h at 37°C. The protein droplets were aspirated by using a fine tip, and the dishes were blocked with PBS–1% bovine serum albumin (Sigma) overnight at 4°C. The dishes were washed twice in binding medium (RPMI–20 mM HEPES–5 mM glutamine–20 mM glucose–1% bovine serum albumin prior to the addition of PRBC. The infected erythrocytes were washed in binding medium, and the hematocrit was adjusted to 2%. The binding assays were carried out in a volume of 1.25 ml at 37°C with five resuspensions (evey 10 min). For inhibition assays, proteins were added as indicated (for ICAM-1–Fc) or at 5 μg/ml for monoclonal antibodies (MAbs). The dishes were washed carefully with binding medium (five to six times), and the adherent cells were fixed in PBS–1% glutaraldehyde. The PRBC were stained with Giesma and counted under ×400 magnification.

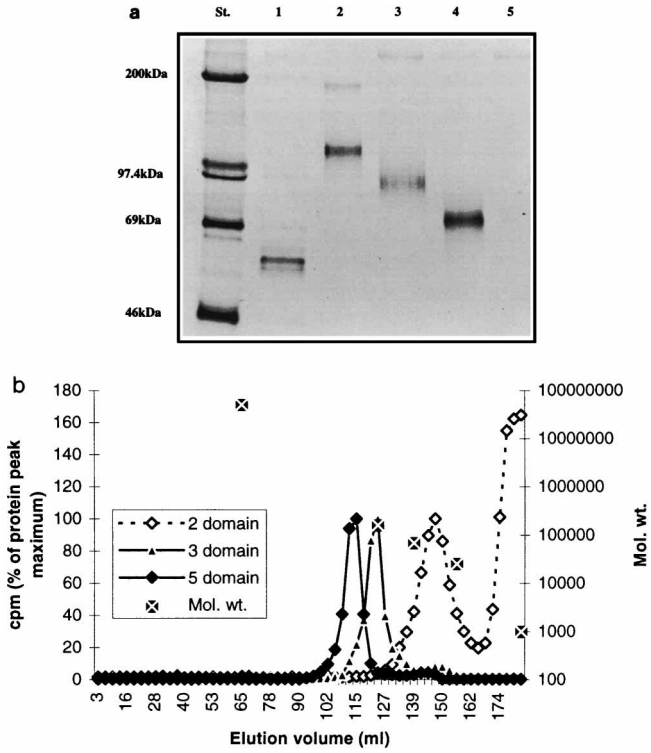


FIG. 1. (a) SDS-PAGE of [^{35}S]methionine-labelled proteins derived from transfection of COS cells. Track 1, CD8Fc (positive control); track 2, ICAM-1–Fc(h12345); track 3, ICAM-1–Fc(h123); track 4, ICAM-1–Fc(h12); track 5, mock transfection (negative control). St., standards. The sizes obtained by expression in COS cells are concordant with the molecular weights predicted from protein derived from endothelium. (b) Elution profiles of nondenatured ICAM-1–Fc protein samples from a Sephacryl S400 column indicating dimerization of the ICAM-1–Fc proteins under nondenaturing conditions.

RESULTS

Production and characterization of ICAM-1–Fc. Several reports demonstrating the inhibition of adhesive interactions with soluble ligand have been published (20, 23, 25). To investigate whether it was possible to inhibit adhesion of PRBC to ICAM-1 by using soluble ICAM-1, we made recombinant ICAM-1–Ig chimeras. ICAM-1–Fc was purified by protein A-Sepharose chromatography from culture supernatants of COS cells transfected with these constructs. The expressed proteins migrated as a single band of the predicted molecular weight in SDS-PAGE under reducing conditions (Fig. 1a). Due to the Fc portion of the expressed proteins, it was predicted that the secreted proteins would exist as a dimers. To test this, ^{125}I-labelled protein was analyzed by gel filtration, confirming that all three versions of the ICAM-1–Fc protein exist as dimers in solution (Fig. 1b). The structure in solution of the ICAM-1–Fc proteins was investigated by using a capture enzyme-linked immunosorbent assay. The two, three, and five ICAM-1 domain proteins were able to bind from solution to five anti-ICAM-1 MAbs (RR1/1, 84H10, 15.2, DH8.4, and LB2) bound to plastic via an anti-mouse Fc antibody (data not shown), indicating the correct conformation of the protein in solution.

Inhibition of adhesion of PRBC to ICAM-1-Fc by using a panel of anti-ICAM-1 MAbs. We had shown previously that a subset of MAbs to human ICAM-1 were able to inhibit the adhesion of PRBC to COS cells transfected with ICAM-1 (7). To validate both the structure of the ICAM-1–Fc on plastic and specificity of the adhesion assay, we performed binding studies using a panel of MAbs derived from the 5th Leukocyte Typing Workshop (13) (subpanel 4 [Fig. 2]). We used this panel of inhibitory and noninhibitory anti-ICAM-1 MAbs to confirm the specificity of binding to protein on plastic. Antibodies which had been shown to block binding to human ICAM-1 expressed on COS cells were also able to inhibit adhesion to protein on plastic and vice versa. CD36 (prepared from platelets as previously described [43]) was included as a positive control for binding on all dishes as an indicator of parasite integrity.

Properties of adhesion of PRBC to ICAM-1–Fc. The levels of adhesion of ITO4-A4 (35) parasites to two-, three-, and five-domain versions was equivalent (Fig. 3a), with minor differences occurring at lower coating concentrations. The binding behavior appears to have a threshold at a coating concentration of approximately 12 μg/ml. The presence of EDTA had no effect on binding (Fig. 3b), nor did pH within the range of 6.8 to 7.5 (Fig. 3c).

From field studies, it is clear that the ability of PRBC to bind to ICAM-1 is variable (26). This may be due to differences in affinity between parasite lines or variation in the number of ICAM-1-binding parasites within the field sample. An independently derived ICAM-1-binding clone ItG-ICAM (28) was tested in parallel with the A4 clone for binding to ICAM-1. The ItG-ICAM clone is antigenically distinct from A4, as shown by mixed agglutination (data not shown). There is a dramatic difference in the relative binding abilities of these clones (Fig. 4), suggesting that the affinities and avidities for ICAM-1 binding are different. The threshold value for the

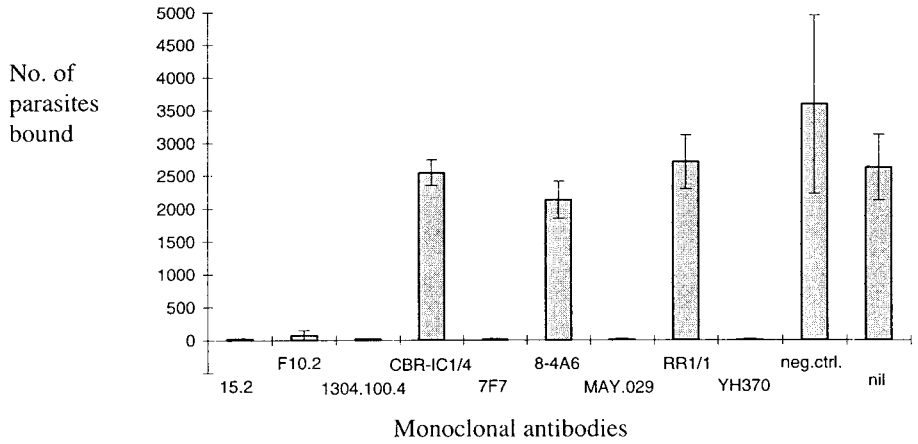


FIG. 2. Inhibition of ITO4-A4 binding to ICAM-1–Fc(h12345) on plastic, using a panel of anti-ICAM-1 MAbs (13). neg.ctrl., negative control.

spotting concentration below which binding is not seen does not differ significantly between these two isolates, suggesting that the minimum site density required for binding is the same for both parasites. No differences in the panel of inhibitory MAbs have been found to differentiate between the ICAM-1 adhesion for ITO4-A4 and ItG-ICAM.

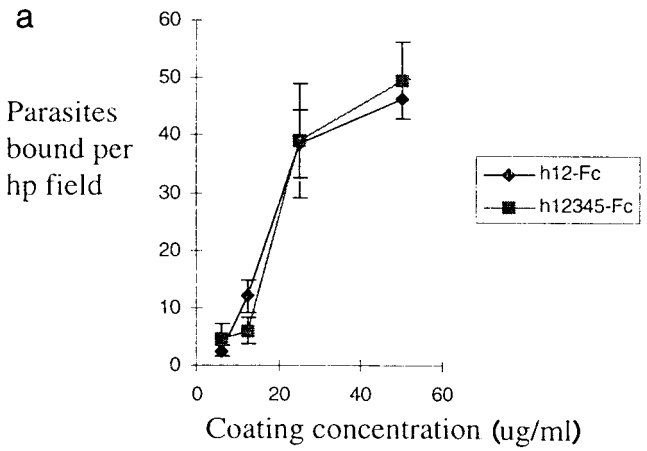
Soluble ICAM-1 does not inhibit adhesion of ITO4-A4 and ItG-ICAM to ICAM-1 on plastic. It may be possible to capitalize on the differences observed between LFA-1 and PRBC adhesion to ICAM-1 by developing specific therapeutic inhibitors of cytoadherence which do not interfere with normal host mechanisms. To explore this possibility, we have, in the first instance, attempted to inhibit the interaction between *P. falciparum*-infected erythrocytes and ICAM-1 with soluble ICAM-1. Using both the ITO4-A4 and ItG-ICAM lines of *P. falciparum*, we were unable to demonstrate inhibition of binding with levels of soluble ICAM-1 up to 100 μg/ml (Fig. 5). Although the recombinant ICAM-1–Fc generated from this study has been shown to be conformationally intact, since it binds to a range of MAbs, there was some concern that the lack of inhibition might be due to other undetected deficiencies in the ICAM-1–Fc. Therefore, the experiments were repeated by using soluble ICAM-1 obtained from Boehringer Ingelheim, which had been used in the previous study, with no differences (data not shown). It has been reported that the blockade of the adhesive interaction between ICAM-1 and rhinovirus can be increased dramatically by the use of alternative classes of Fc domains (24). To test whether this would have an effect on our results, we obtained ICAM-1–IgA-Fc, which had been shown to be 200 times more effective than soluble ICAM-1 in preventing rhinovirus plaque formation (24). This also had no effect on the binding to ICAM-1 on plastic (Fig. 5).

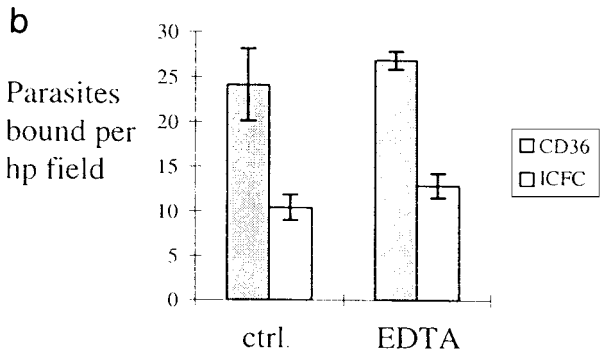
DISCUSSION

ICAM-1 is a receptor for *P. falciparum*-infected erythrocytes. Recent evidence supports its role in the pathogenesis of cerebral malaria (26, 47). We have therefore carried out preliminary studies on the use of soluble ligand as a prelude to developing potential chemotherapeutic agents.

Previous studies have demonstrated the efficacy of expressing soluble ICAM-1 as a human Fc chimera (which replaces the transmembrane region) and that human ICAM-1 expressed in COS cells retains a diverse range of immunological epitopes. Therefore, we based our constructs on a transient COS cell expression system which incorporated the signal sequence from human ICAM-1 and the human IgG1 region. The protein expressed using this system existed as dimers with no detectable higher-order aggregation during this study and contained the epitopes for a wide range of MAbs raised against native human ICAM-1. The role of ICAM-1 in the adhesion of lymphocytes to activated endothelium is well characterized and is dependent on Ca^{2+}. The lack of effect of EDTA treatment (Fig. 3b) suggests a mechanism for PRBC binding different from that seen with the integrin LFA-1 and implies that other *P. falciparum* proteins which have been shown recently to contain Ig-domain-like sequences (46) are also not involved. This suggestion is supported by our previous data on the binding sites on ICAM-1 for these two interactions. Whereas LFA-1 binding appears to involve one face of the N-terminal domain of ICAM-1 (plus some contribution from the second domain), PRBC adhesion is more circumscribed, involving loops on the side of the Ig domain opposite the LFA-1-binding face. In this earlier study, there was some question as to whether there was any contribution to the PRBC binding site from the third Ig-like domain in ICAM-1, as COS cells expressing the two N-terminal domains mediated adhesion of PRBC at a significantly lower level than cells expressing the three N-terminal domains. This effect was ascribed to steric inhibition by the cellular glycocalyx, a conclusion supported by the binding titration curves for protein adsorbed onto plastic presented here, which show no differences between binding between chimeric proteins containing the two N-terminal domains and those with three or five N-terminal domains (Fig. 3a).

Despite the use of relatively high levels of soluble ICAM-1 (100 μg/ml), we were not able to block adhesion of PRBC to ICAM-1–Fc. The lack of inhibition is consistent with a model of ICAM-1 adhesion involving rapid k_{off} rates such that ICAM-1 dimers in solution are unable to compete with the multivalent nature of this ligand on plastic. The transient nature of the interaction between the infected erythrocyte surface and ICAM-1 is supported by our repeated inability to detect significant levels of surface-bound ICAM-1-Fc on PRBC by using indirect immunofluorescence and flow cytometry (data not shown) and the finding that ITO4-A4-infected erythrocytes roll on ICAM-1-coated surfaces under flow (12). Fast k_{on} and k_{off} rates have been associated with rolling behavior enabling rapid ligand-receptor bonding under flow conditions (high k_{on}) but resulting in an unstable interaction (high k_{off}) which is observed as rolling. It is likely that the ICAM-1 in solution does not form a stable complex with the surface of the infected cell so that although there will be some ICAM-1 bound, the amount will





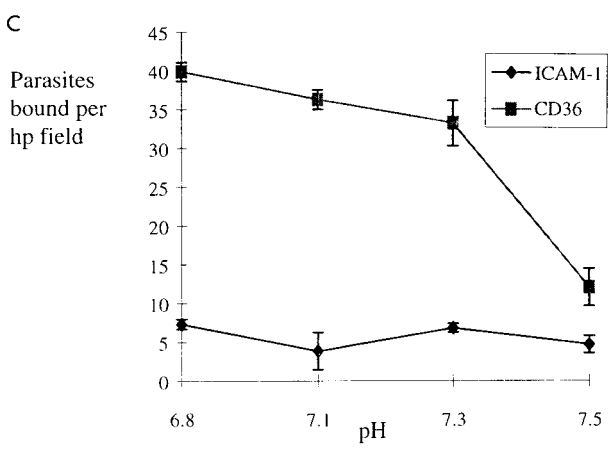


FIG. 3. (a) Titration of ITO4-A4 binding to ICAM-1-Fc(h12) and ICAM-1-Fc(h12345). ICAM-1–Fc coating concentrations were as indicated on the *x* axis. (b) Effects of EDTA on binding of ITO4-A4 to ICAM-1-Fc(h12345) and CD36. c, Effects of pH on binding of ITO4-A4 to ICAM-1-Fc(h12345) and CD36.

be dependent on the equilibrium constant for this association (and the concentration of ICAM-1 in solution). One study showed that PRBC can retain ICAM-1–Fc on their surface, where it was able to promote phagocytosis of the infected cells by monocytes specifically by their interaction with ICAM-1–Fc protein (41). It is possible that phagocytosis can be facilitated by levels of protein that are subinhibitory for adhesion. Binding of soluble ICAM-1 to the cell surface may be improved by using multimers as the competitive inhibitor either through the use of IgM-Fc expression constructs, for example, for rhinovirus adhesion (24, 36), or, in the case of LFA-1–ICAM-1 interaction, through the use of ICAM-1-coated microspheres (48). Both of these methods have been shown to inhibit ICAM-1-dependent adhesion reactions more efficiently than monomer or dimer molecules.

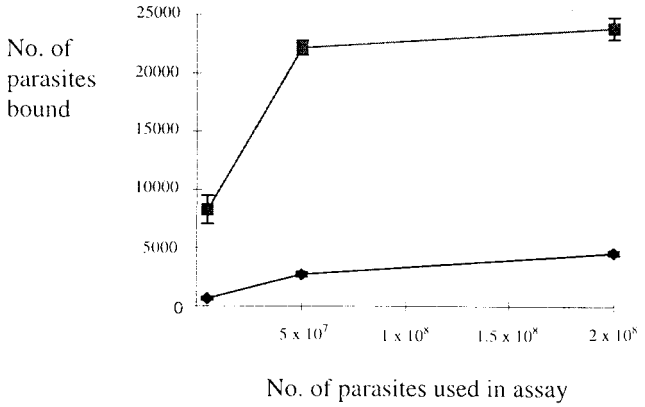


FIG. 4. Titration of PRBC binding to ICAM-1-Fc(h12345), using ITO4-A4 (◆) and ItG-ICAM (■). These two laboratory lines of *P. falciparum* show widely differing affinities for ICAM-1.

The inconsistencies between the results reported here and those found previously (41) are difficult to reconcile but may be due to differences in the binding assays. Binding is clearly related to the amount of ICAM-1 on plastic (Fig. 3a) and number of parasites used in the assay (Fig. 4). The site densi-

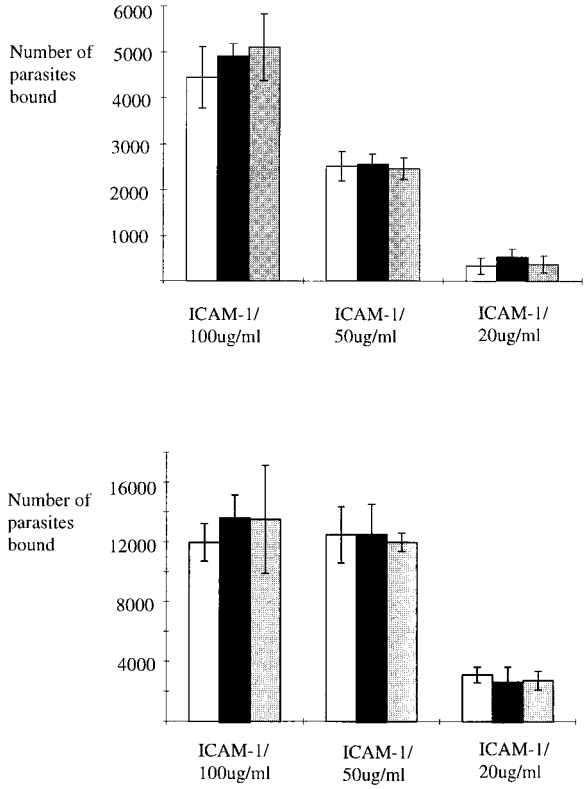


FIG. 5. Inhibition of ITO4-A4 and ItG-ICAM binding to ICAM-1-Fc (h12345), using soluble ligand. ICAM-1 coating concentrations were as indicated on the *x* axis. No inhibition was observed over a range of ICAM-1 coating concentrations. □, ITO4-A4 control; ■, ITO4-A4–IgG-Fc (100 μg/ml); ▨, ITO4-A4–IgA-Fc (100 μg/ml).

ties on plastic produced with the coating concentrations of soluble ICAM-1 used in this study (20 to 100 μg/ml) are in the same range as those seen on activated endothelium (24a) and cover a wide range of binding levels, whereas the previous study used a standard coating concentration of 10 μg/ml. Furthermore, the weak interaction with ICAM-1 makes the washing conditions critical, as it is possible to wash off adherent PRBC. Variations in any of these conditions could easily lead to large differences in results, as would differences in the degree of aggregation or multimerization of the ICAM-1 or ICAM-1–Fc. More importantly, the differing affinities with which the ItG-ICAM and ITO4-A4 lines bind to ICAM-1 (Fig. 4) could explain the observed differences in inhibition such that under marginal binding conditions, it may be possible to inhibit binding of the high-affinity isolate ItG-ICAM but not ITO4-A4. It should be noted that inhibition studies using a range of anti-ICAM-1 MAbs indicate that the contact residues on ICAM-1 utilized by each parasite line are probably the same. The binding characteristics of clinical isolates to ICAM-1 are highly variable, and a significant proportion show behavior similar to that of the ITO4-A4 line and would therefore not be expected to be affected by relatively high concentrations of ICAM-1 in solution.

Human rhinovirus replication can be blocked in vitro by concentrations of soluble ICAM-1 within the range 0.1 to 7.9 μg/ml (2). This inhibition is thought to be attributable to direct interference with binding, interruption of viral entry, and the induction of capsid uncoating by soluble ICAM-1 (17). Human rhinovirus variants that are relatively resistant to inhibition by soluble ICAM-1 (30-fold increase in 50% effective concentration [1]), a property which is also observed in poliovirus neutralization with soluble cell receptors (21), have been selected. Thus, the use of soluble receptors as inhibitors of host-pathogen interactions must be viewed with some caution. For infection with *P. falciparum*, inhibition of binding to ICAM-1 would not be expected to prevent PRBC adhesion to endothelium due to the range of receptors utilized by infected erythrocytes (11), but by specifically interfering with the interaction with ICAM-1, it may beneficial in the treatment of acute severe malaria. This study shows that soluble ICAM-1 is not an efficient competitive inhibitor of adhesion to immobilized ICAM-1 and that more sophisticated solutions need to be investigated.

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