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Plasmodium falciparum–Infected Erythrocytes Bind ICAM-1 at a Site Distinct from LFA-1, Mac-1, and Human Rhinovirus

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Summary

The attachment of erythrocytes infected with P. falciparum to human venular endothelium is the primary step leading to complications from severe and cerebral malaria. Intercellular adhesion molecule-1 (ICAM-1, CD54) has been implicated as a cytoadhesion receptor for P. falciparum–infected erythrocytes. Characterization of domain deletion, human/murine chimeric ICAM-1 molecules, and amino acid substitution mutants localized the primary binding site for parasitized erythrocytes to the first amino-terminal immunoglobulin-like domain of ICAM-1. The ICAM-1 binding site is distinct from those recognized by LFA-1, Mac-1, and the human major-type rhinoviruses. Synthetic peptides encompassing the binding site on ICAM-1 inhibited malaria-infected erythrocyte adhesion to ICAM-1-coated surfaces with a K_i of 0.1–0.3 mM, whereas the K_i for soluble ICAM-1 is 0.15 μM. These findings have implications for the therapeutic reversal of malaria-infected erythrocyte sequestration in the host microvasculature.

Introduction

Erythrocytes infected with the human malaria parasite Plasmodium falciparum adhere to vascular postcapillary endothelium, and the sequestration of the malaria-infected erythrocytes is a primary event responsible for the clinical complications of severe and cerebral malaria. While immature ring-stage parasitized erythrocytes circulate unobstructed throughout the vasculature, adhesion of mature intraerythrocytic stages of the parasite to endothelium averts splenic clearance of infected red blood cells (IRBCs) and allows parasite maturation in a microenvironment of low oxygen tension. Two cell surface receptors with broad tissue distribution, intercellular adhesion molecule-1 (ICAM-1, CD54) (Berendt et al., 1989) and CD36 (GPIV) (Ockenhouse et al., 1989), have recently been identified as endothelial receptors for IRBCs. Laboratory-adapted IRBCs bind to purified ICAM-1-coated and CD36-coated surfaces, and the cytoadherent phenotype of these malaria-infected red cells can be modulated by successive panning on ICAM-1- or CD36-coated surfaces (Ockenhouse et al., 1991). In addition, IRBCs bind to COS cells transfected with cDNA of human ICAM-1 and to cytokine-activated human umbilical vein endothelial cells. We recently confirmed the in vivo relevance of these observations by demonstrating that infected erythrocytes obtained from naturally acquired uncomplicated and severe malaria infections bind to ICAM-1- and CD36-coated surfaces (Ockenhouse et al., 1991). Moreover, ICAM-1-specific and CD36-specific monoclonal antibody (MAb) staining of small capillary endothelium from postmortem brain tissue colocalizes with IRBC cytoadherence in patients who have died from complications of cerebral malaria (Barnwell et al., 1989; Aikawa et al., 1990).

ICAM-1, a member of the immunoglobulin-like superfamily, is a monomeric, unpaired 90–115 M_r glycoprotein composed of a bent extracellular domain containing five tandemly arranged immunoglobulin-like domains, a transmembrane region, and a cytoplasmic domain (Staunton et al., 1988, 1990; Simmons et al., 1988). ICAM-1 is a ligand for the leukocyte integrins, lymphocyte function–associated antigen-1 (LFA-1; CD11a/CD18) (Rothlein et al., 1986; Marlin and Springer, 1987) and Mac-1 (CD11b/CD18) (Smith et al., 1989; Diamond et al., 1990). The recognition, adhesion, and extravasation of lymphoid and myeloid blood cells through the vascular endothelium is an initial step of host immune response to tissue injury. The CD11/CD18 family of proteins is crucial for leukocyte and myeloid cell adhesion to endothelium, T cell activation, cytotoxic T cell killing, neutrophil chemotaxis, and homotypic aggregation (Larson and Springer, 1990). ICAM-1 is also subverted as a cellular receptor by the major group of human rhinoviruses (HRV), the etiologic agent of 40%–50% of common colds (Staunton et al., 1989b; Greve et al., 1989). A soluble form of ICAM-1 lacking the transmembrane and cytoplasmic domains binds HRV and inhibits rhinovirus infection (Marlin et al., 1990).

MAb blocking studies have indicated that the binding sites for LFA-1 and HRV are proximal. Analysis of mutant ICAM-1 molecules has demonstrated that mutations in the amino-terminal domain have the strongest effect on LFA-1 and HRV binding (Staunton et al., 1990). Domains 1 and 2 demonstrate a close physical association and appear conformationally linked (Staunton et al., 1990). Amino acid substitution mutants demonstrate that while the LFA-1 and HRV contact sites overlap, they are distinct (Staunton et al., 1990). The integrin Mac-1 binds to the third amino–terminal immunoglobulin-like domain of ICAM-1, in contrast to LFA-1, and this binding is influenced by the extent of glycosylation on the ICAM-1 molecule (Diamond et al., 1991). The molecular basis for adhesion of malaria-infected erythrocytes to ICAM-1 is not known. MAbs RR1/1 and R6.5, which inhibit binding of LFA-1 and HRV to ICAM-1, have no effect on IRBC binding to purified ICAM-1-coated surfaces (Ockenhouse et al., 1991). Here, we examine the binding site on ICAM-1 for P. falciparum–infected erythrocytes. We demonstrate that IRBCs bind to the first amino–terminal domain of human but not murine ICAM-1, and that the binding sites within domain 1 reside

Table 1. Adhesion of P. falciparum–Infected Erythrocytes to Deletion Mutant ICAM-1 and ICAM-2 Molecules Expressed in COS Cells

	Malaria-Infected Erythrocytes (IRBCs Bound per 100 COS Cells)				
COS Cell Transfectants	LFI	ItG-ICAM-1	ItG-CD36	CY25	G15
Wild-type	14	2176 ± 258	64 ± 11	2203 ± 432	706 ± 190
MOCK	–	22 ± 8	30 ± 14	29 ± 14	21 ± 14
$D3^-$	3	903 ± 220	23 ± 13	1618 ± 351	600 ± 79
$D4^-$	11	1222 ± 307	43 ± 35	1383 ± 185	638 ± 122
$D4^-D5^-$	11	1293 ± 229	44 ± 24	1290 ± 193	515 ± 127
ICAM2	21	23 ± 7	45 ± 21	35 ± 18	24 ± 11

Laboratory-adapted (ItG) and naturally acquired (CY25, G15) IRBCs were assayed for adhesion to COS cells transfected with cDNAs from wild-type ICAM-1 and ICAM-2 or ICAM-1 domain deletion mutants as described in Experimental Procedures. The linear fluorescence intensity (LFI) of ICAM-1 or ICAM-2 monoclonal antibody binding to transfected COS cells was determined by indirect immunofluorescence and flow cytometry. Results represent the mean percentage ± SD of the determinations. Note: $D3^-$ is always expressed less than 50% that of other deletions (Staunton et al., 1990a).

spatially distant from the recognition sites for LFA-1 and HRV.

Results and Discussion

Previously, it has been determined, using amino acid substitution and domain deletion mutants of ICAM-1, that the binding sites for LFA-1 and HRV localized to the first amino–terminal domain of ICAM-1. The same and additional mutant molecules are used here to define the ICAM-1 binding site for malaria-infected erythrocytes. Mutant cDNA clones (Staunton et al., 1990) with one or two immunoglobulin-like domains deleted ($D3^-$, $D4^-$, and $D4^-5^-$) were expressed in COS cells and assayed for IRBC adhesion (Table 1). Erythrocytes infected with laboratory-adapted parasite strains selected in vitro by repeated panning of infected erythrocytes on ICAM-1-coated surfaces (ItG-ICAM) bound to COS cells expressing wild-type ICAM-1 but not to mock-transfected cells or to cells transfected with ICAM-2. IRBC adhesion to cells was retained after deletion of domains 3, 4, or 4 and 5 (Table 1). The somewhat decreased adhesion (up to 2-fold) of IRBCs to cells transfected with $D3^-$, $D4^-$, or $D4^-5^-$ may be attributed in part to decreased expression or to decreased accessibility of binding sites as a result of the shortening of the ICAM-1 molecules. Binding was specific, since IRBCs selected in vitro to bind to human CD36 (ItG-CD36) did not bind wild-type or mutant ICAM-1. IRBCs from individuals with uncomplicated malaria (CY25) or complicated severe cerebral malaria (GI5) were cultured in vitro for 24 hr to allow intraerythrocytic parasite maturation to the trophozoite stage of development. These infected erythrocytes bound similarly to COS cells expressing ICAM-1 of wild type and with domains 3, 4, or 5 deleted (Table 1).

To confirm that domains 1 and 2 of ICAM-1 contain the binding site for IRBC adhesion, human/murine chimeric ICAM-1 molecules were assayed for IRBC binding. The human (Simmons et al., 1988; Staunton et al., 1990) and murine ICAM-1 (Horley et al., 1989) amino acid sequences are 50% identical, and each molecule contains five immunoglobulin-like domains with a conserved BglII restriction site at residue 168 near the end of domain 2, enabling chimeric exchanges (Staunton et al., 1990). Human domains 1 and 2 (hmICAM-1) or murine domains 1 and 2 (mhICAM-1) were recombined at the BglII site with domains 3–5 of the other species. The chimeric cDNAs were expressed in COS cells, and IRBC binding was determined. The efficiency of expression was determined using two MAbs to human ICAM-1, RR/1 and CL203, and MAb YN1/1 (Takei, 1985), which recognizes an epitope confined to domains 1 and 2 of murine ICAM-1. COS cells that express human but not murine wild-type ICAM-1 bind IRBCs (Figure 1). Furthermore, IRBCs bind to hmICAM-1 but not to mhICAM-1 (Figure 1). Thus, the first 168 residues of human ICAM-1 are sufficient to support binding of an IRBC counter-receptor.

Amino acid substitution mutants of ICAM-1 have profound effects on LFA-1, Mac-1, and HRV binding (Staunton et al., 1990; Diamond et al., 1991). Similarly, the adhesion of IRBCs to single and multiple amino acid substitution mutants in domains 1 and 2 was examined. Amino acid substitutions are denoted by the one-letter code for the wild-type sequence, followed by a slash and the one-letter code for the mutant sequence (Table 2). The efficiency of mutant ICAM-1 expression on COS cells was determined using MAb CL203 by immunocytofluorometry and in adhesion assays by immunogold silver staining. MAb CL203 recognizes an epitope located within domain 4 and has no effect on IRBC binding.

We tested two mutations that disrupt the overall conformation of domains 1 and 2, based on loss of three independent MAb epitopes and the LFA-1 and HRV binding sites, and mutations that preserve overall conformation as shown by disruption of no more than one of the MAb epitopes (Staunton et al., 1990). The amino acid substitution mutants, D60S/KL and R13G/EA, which conformationally disrupt the structure of domains 1 and 2 (Staunton et al., 1990), abrogate IRBC adhesion (Table 2). Among the previously characterized mutations that do not disrupt structure, only the amino acid substitution mutant G15S/SA abrogated IRBC adhesion (Table 2). This mutation has no effect on LFA-1 binding or rhinovirus binding (Staunton et al., 1990). Gly-15–Ser-16 residues are conserved in human (Staunton et al., 1988; Simmons et al., 1988) and murine ICAM-1 (Horley et al., 1989) and human ICAM-2 (Staunton et al., 1989a) (Figure 2), whereas IRBCs do not

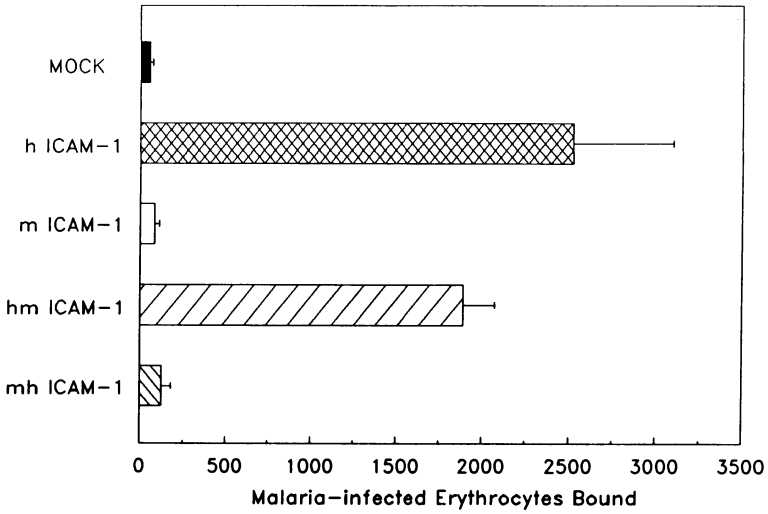

Figure 1. Binding of Malaria-Infected Erythrocytes to Chimeric Forms of ICAM-1

Chimeric molecules were generated as described elsewhere (Staunton et al., 1990) and transfected into COS cells. The two chimeric molecules are composed as follows: hmICAM-1 (human ICAM-1, domains 1 and 2; murine ICAM-1, domains 3–5) and mhICAM-1 (murine ICAM-1, domains 1 and 2; human ICAM-1, domains 3–5). Results represent the mean of three determinations ± SD. The level of expression of hICAM-1 (linear fluorescence intensity [LFI] = 11), mICAM-1 (LFI = 26), hmICAM-1 (LFI = 26), and mhICAM-1 (LFI = 29) does not account for the amount of IRBC binding.

bind to murine ICAM-1 or human ICAM-2, as shown above. To characterize further the binding site, five additional amino acid substitution mutants were generated, including the substitution of murine for human residues at the nearby positions 7, 9, and 18. Replacement of Leu-18 (hICAM-1) with Gln-18 (mICAM-1) resulted in marked loss of IRBC binding to transfected COS cells (Table 2). In contrast, 37 other mutations in domain 1 and 13 mutations in domain 2, including two potential N-linked glycosylation sites, had no effect on IRBC adhesion.

X-ray crystallographic studies of the immunoglobulin-like molecules and secondary structure predictions based on the amino acid sequence (25,20) suggest that each immunoglobulin-like domain of ICAM-1 is composed of seven antiparallel β strands folded into a sandwich comprising two facing β sheets, connected by intramolecular disulfide bonds between β strands B and F (Figure 2). β strands A, B, E, and D form one sheet and strands C, F, and G fashion the opposing sheet. The contact site for P. falciparum–infected erythrocytes is predicted to be localized in domain 1 to a loop between β strands A and B and to extend into strand B, in contrast to the binding sites for LFA-1 and HRV (Figure 2). There is another important contrast between the current findings for the P. falciparum sequestration binding site and previous findings for LFA-1 and rhinovirus. The malaria-infected erythrocyte binding site is highly localized within the sequence, whereas the sites affecting LFA-1 and rhinovirus are noncontiguous within the sequence, suggesting that different segments of the polypeptide chain are folded together to form the contact surface.

The localization of the IRBC binding site to a specific polypeptide segment within domain 1 suggested that peptides within this region might be effective inhibitors. To test this hypothesis and to define further the IRBC binding site, a synthetic peptide spanning amino acids Pro-12–Thr-23 and overlapping hexapeptides were assayed for inhibition of IRBC binding to ICAM-1- or CD36-coated surfaces. The inhibitory effect of these peptides was compared to the effect that recombinant soluble ICAM-1 (domains 1–5) (Marlin et al., 1990) has on IRBC binding to immobilized ICAM-1-coated surfaces. Hexapeptides spanning Gly-14–Ser-22 effectively inhibited the binding of ItG-CAM-infected erythrocytes to ICAM-1-coated plates, while overlapping peptides flanking these regions did not inhibit binding (Figure 3A). The peptide Pro-12–Thr-23 and the hexapeptide GSVLVT inhibited IRBC binding in a dose-dependent manner with 50% inhibition at approximately 0.125 and 0.3 mM, respectively (Figure 3B). The inhibitory effect of these peptides was three orders of magnitude less than that observed using sICAM-1 as an inhibitor of IRBC binding (0.15 μM) (Figure 3B). This suggests either that other regions also participate in the contact with IRBCs, or that the conformation of the sequence is important, as suggested above by the inhibitory effect of mutations that disrupt overall conformation of ICAM-1 domains 1 and 2. The inhibition by the ICAM-1 peptides was highly specific, since parasitized red cells that bind to CD36 but not to ICAM-1 were not inhibited from binding to immobilized CD36 (Figure 3B). The inhibition of IRBC binding to ICAM-1 by soluble ICAM-1 or synthetic peptides suggests a potential therapeutic use for ICAM-1 analogs in severe and complicated malaria that spare important adhesive interactions between ICAM-1 and its counter-receptors, LFA-1 and Mac-1. ICAM-1 has a restricted distribution in vivo, and its expression is regulated by lipopolysaccharide and the cytokines interleukin-1, tumor necrosis factor, and interferon-γ (Dustin et al., 1986; Pober et al., 1986; Pober and Cotran, 1990). Bacterial products and/or inflammatory mediators released at sites of local tissue injury induce ICAM-1 mRNA and protein expression in a wide variety of cells. In vitro, human umbilical endothelial cells induced with tumor necrosis factor upregulate the surface expression of ICAM-1 and support adhesion of malaria-infected erythrocytes (Berendt et al., 1989). In vivo, individuals with cerebral malaria have higher levels of plasma tumor necrosis factor than individuals with uncomplicated malaria or uninfected controls. Paradoxically,

Table 2. Adhesion of P. falciparum–Infected Erythrocytes to ICAM-1 Amino Acid Substitution Mutants Expressed in COS Cells

Mutation	IRBC Binding (% WT ± SD)	LFA-1 Binding (% WT ± SD)	HRV14 Binding (% WT ± SD)
Domain 1			
Q1T/KA	102 ± 13		
Q1/E	146 ± 12		
S3/T	126 ± 8		
S5/T	137 ± 29		
S7KV/RKA	102 ± 20	71 ± 23	103 ± 6
K8/E	138 ± 12		
R13/K	120 ± 7		
R13G/EA[a]	6 ± 6		
G15S/SA	**2 ± 0.2**		
L18/Q	**15 ± 3**	132 ± 26	126 ± 22
T20CS/ACT	118 ± 6		
S24/A	127 ± 37		
D26QPK/ALPE	135 ± 5		
E34/A	109 ± 19		
L37/S	102 ± 18	82 ± 19	39 ± 3
K39KE/ERQ	88 ± 15		
K40/A	116 ± 5		
L43LPGN/RLPGP	109 ± 17	142 ± 38	145 ± 13
G46NN/ASI	69 ± 17		
N48/H	122 ± 22		
R49KV/EKL	81 ± 11		
Y52/F	117 ± 15		
Y52E/FA	104 ± 22		
N56V/HM	110 ± 13		
Q58/H	98 ± 13		
E59/K	97 ± 17		
D60/N	100 ± 19		
D60S/KL[a]	2 ± 1		
S61/I	95 ± 15		
Q62PM/API	97 ± 28		
Y66/T	124 ± 20		
N68/K	106 ± 26		
D71/E	85 ± 15		
D71/N	109 ± 12		
Q73/T	123 ± 24		
S74/A	98 ± 26		
T75/A	106 ± 30		
K77T/ES	80 ± 6		
T78F/SL	122 ± 13	137 ± 46	142 ± 54
R88V/EA	100 ± 11		
E90/Q	105 ± 17		
Domain 2			
G101K/AN	94 ± 6		
E111GGA/KAGS	98 ± 23		
N118/Q	127 ± 26		
R125/E	97 ± 6		
E127/R	96 ± 20		
K128/R	90 ± 14		
V136GE/GVK	100 ± 9		
R149RD/EEG	130 ± 20		
H152HGA/EEGS	121 ± 19		
N156/E	150 ± 15		
R166PQ/EPA	107 ± 13		
N175/A	98 ± 29		
S177/G	117 ± 12		

ICAM-1 amino acid substitution mutants were generated by oligonucleotide-directed mutagenesis (Staunton et al., 1990a). Wild-type (WT) residues precede the slash and are followed by the residues in the mutant. IRBC (ItG-ICAM-1) adhesion to COS cells expressing mutant ICAM-1 was assessed by concurrent MAb CL203 staining and IRBC adhesion and expressed as the mean percentage ± SD of binding of IRBCs to wild-type ICAM-1-transfected cells. The values for LFA-1 binding and HRV14 binding to the new mutants generated for these studies are shown in the columns within the table. All new mutants bind ICAM-1 MAbs RR1/1, R6.5, LB-2, and CL203 equivalently to wild type, with the exception of L43LPGN/RLPGP and L37/S, which demonstrate reduced LB-2 and RR1/1 binding, respectively (data not shown).

[a] Disrupts overall conformation of domains 1 and 2.

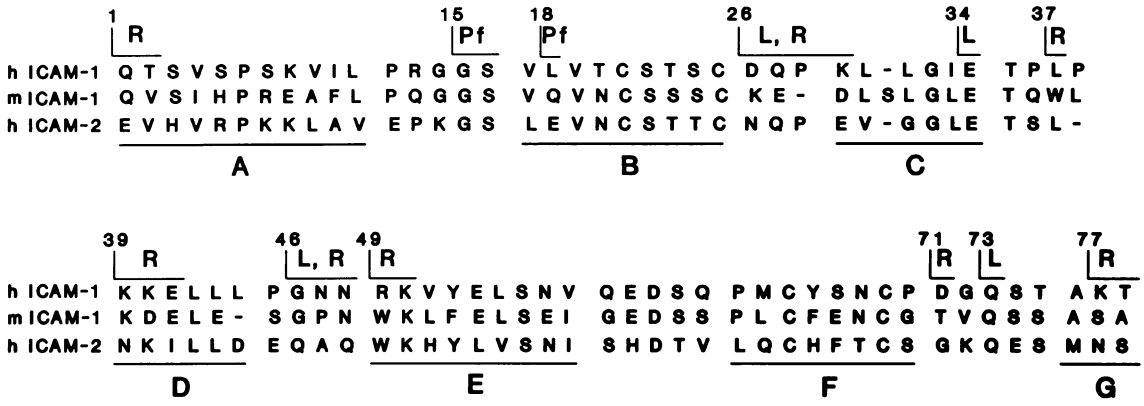

Figure 2. Alignment of Amino Acids in First Amino–Terminal Domain of Human ICAM-1, Murine ICAM-1, and Human ICAM-2

Amino acid substitution mutations within human ICAM-1 affecting binding of P. falciparum IRBCs (Pf), LFA-1 (L), and HRV (R) are indicated by the solid line. The alignment of sequences by predicted secondary structure is indicated by strands A–G.

an inflammatory response initiated in response to malarial infection may be used to the parasites' advantage by selectively modulating the expression of receptors to which parasitized erythrocytes would attach.

In principle, the receptor-binding site on IRBC surfaces should be conserved and selective pressure exerted to maintain minimal structural variation unless compensatory binding to alternate receptors occurs. Sequestration of malaria-infected erythrocytes to host endothelium occurs in all persons infected with P. falciparum, regardless

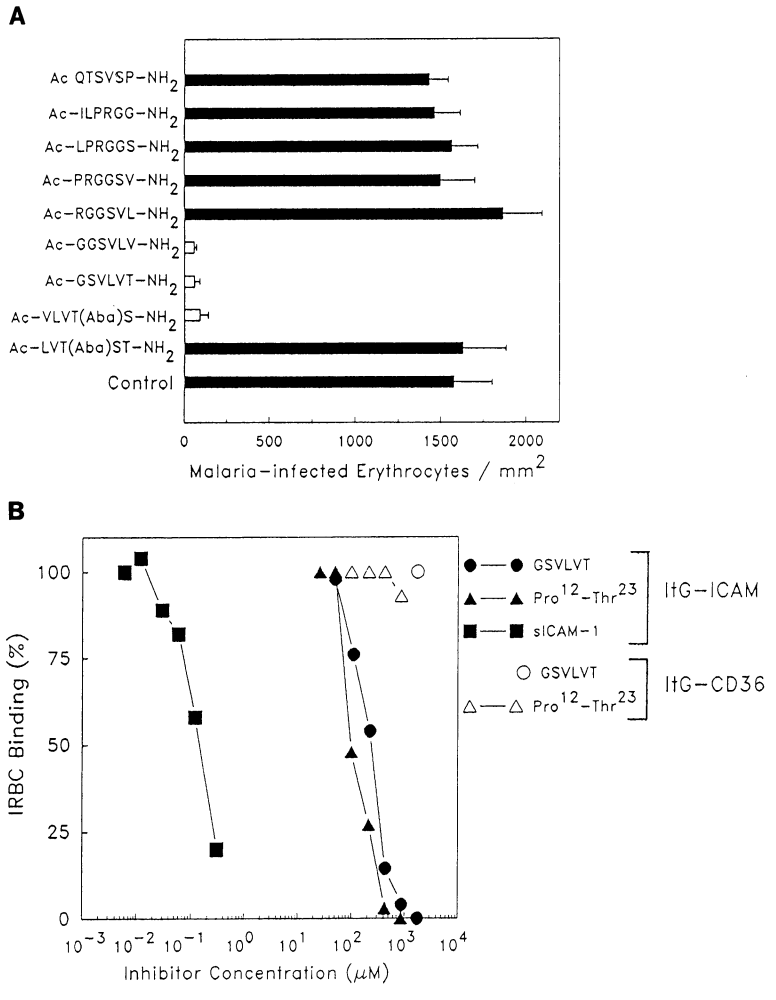

Figure 3. Effect of ICAM-1 Peptides on IRBC Binding

(A) Inhibition of binding of malaria-infected erythrocytes to ICAM-1 by overlapping synthetic hexapeptides. ItG-ICAM IRBCs and ICAM-1 hexapeptides (500 mg/ml) were added to ICAM-1-coated plates for 60 min. The peptides were acetylated at the amino terminus and amidated at the carboxyl terminus. Aba is α-aminobutyric acid and is substituted in sequence for Cys. Results represent the mean ± SD of three determinations and are compared to control IRBC binding to ICAM-1 in the absence of peptides.

(B) Dose-dependent inhibition of IRBC binding to ICAM-1 by peptides Pro-12–Thr-23 and GSVLVT and sICAM-1. ItG-ICAM-1 IRBCs (closed bars) and ItG-CD36 IRBCs (open bars) and sICAM-1 or synthetic peptides at concentrations indicated were incubated on plates previously coated with 10 μg/ml ICAM-1 or 1 μg/ml CD36, respectively. Binding of IRBCs to adhesion receptors was determined, and the results represent the mean percent binding compared to control samples incubated in PBS alone. Control binding of ItG-ICAM-IRBC to purified ICAM-1 is 1578 ± 225 IRBCs per mm², and binding of ItG-CD36 to purified CD36 is 860 ± 108 IRBCs per mm².

of clinical severity. A small percentage of infected individuals, independent of parasitemia, progress to complicated and severe forms of the disease. The precise factors and mechanisms responsible for severe malaria are unknown. While the majority of parasitized erythrocytes from naturally acquired infections bind only to CD36 in vitro, a smaller subpopulation of parasitized erythrocytes from some isolates binds to ICAM-1 and CD36. IRBCs may bind to different receptors in different tissues, depending upon the genetic regulation of host cellular receptors and the parasite cytoadherent phenotype as expressed by single or multiple counter-receptors. Deleterious effects to the host may result from the sequestration of a numerically smaller proportion of IRBCs expressing the pertinent counter-receptor within a population of parasitized red cells directing the binding of IRBCs to capillary endothelium within the brain, leading to cerebral malaria.

Antigenically diverse, naturally acquired malaria isolates demonstrate serologically defined, infected erythrocyte surface epitopes. Immune serum inhibits IRBC adhesion to human umbilical vein endothelial cells in a strain-specific manner (Udeinya et al., 1983), and no panspecific serum that inhibits IRBC adhesion of geographically diverse malaria isolates has been identified. A therapeutic strategy directed toward reversing parasite sequestration may ultimately protect infected individuals from the deleterious complications of vascular occlusion. The development of antireceptor-soluble ICAM-1 analogs based upon the critical contact residues for IRBCs may be engineered to bind, lyse, and kill sequestered intraerythrocytic parasites in cases of severe and complicated falciparum malaria.

Experimental Procedures

Generation of ICAM-1 Mutants

Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to generate ICAM-1 domain deletion and amino acid substitution mutants and chimeras as described (Staunton et al., 1990). The sequence of the five new amino acid substitution mutants (Table 2) was determined by DNA sequencing and found to be as indicated.

Transfection of COS Cells

COS cells at 50% confluency were transfected by the DEAE dextran method using vector alone or vector containing wild-type or mutant ICAM-1 cDNA. COS cells were harvested 72 hr after transfection, and the efficiency of transfection of ICAM-1 constructs was analyzed by indirect immunofluorescence and flow cytometry using anti-human ICAM-1 MAbs CL203 (Maio et al., 1989) (a gift of Dr. S. Ferrone) and RR1/1 (Dustin et al., 1986) and anti-murine MAb YN1/1 (Takei, 1985) (a gift of Dr. F. Takei, Vancouver, B.C.) as previously described (Staunton et al., 1990).

Parasites

A P. falciparum cloned parental line, ItG-2F6, was selected for increased adhesion to purified ICAM-1 (ItG-ICAM) or to purified CD36 (ItG-CD36) by panning the parasitized erythrocytes on ICAM-1- or CD36-coated surfaces (Ockenhouse et al., 1991). Parasites were maintained in continuous culture, synchronized, and enriched for mature trophozoite and schizont stages (35%–50% parasitemia) by gelatin flotation. Two naturally acquired isolates obtained from Thai patients with uncomplicated malaria (CY25) or severe cerebral malaria (GI5) were adapted to continuous culture and used within 10 cycles of multiplication.

Peptides

Peptides were synthesized using 4 (2′, 4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink, 1987) on an Advanced Chemtech model 350 multiple peptide synthesizer (Schnorrenberg and Gerhardt, 1989). The side chain protection was as follows: Asn(Trt), Gln(Trt), Arg(Pmc), Lys(Boc), Ser(tBu), Thr(tBu). In the hexapeptide series, cysteine was replaced by 2-aminobutyric acid (Aba), which is isosteric with cysteine. Couplings were carried out using 10-fold excess of Fmoc amino acids–1,3 diisopropylcarbodiimide–1-hydroxybenzotriazole. Peptide cleavage from the resin as well as side chain deprotections were carried out by treating the resin with the cocktail trifluoroacetic acid–anisole–1,2 ethanedithiol–ethyl methyl sulfide (v/v 95:3:1:1) for 2.5 hr. After evaporating trifluoroacetic acid, peptide was precipitated with cold ether and centrifuged. The pellet was washed with cold ether to remove residual scavengers, dissolved in water, and lyophilized. The crude peptides were found to be 75%–95% pure by reverse phase high pressure liquid chromatography, and expected molecular weights were observed by fast atom bombardment mass spectroscopy. The 12-mer was synthesized using 4-methylbenzhydrylamine resin on an Applied Biosystems 430A peptide synthesizer. The t-boc chemistry was used for the synthesis, the crude peptide was purified by reverse phase high pressure liquid chromatography on a C18 column, and the purified peptide was characterized by fast atom bombardment mass spectroscopy.

IRBC Binding Assay

Transfected COS cells in RPMI 1640 plus 10% fetal bovine serum were reseeded (2.5–4 × 10^4 cells per well) 24–48 hr prior to assay into 24-well tissue culture plates at 37°C in 5% CO_2. Malaria-infected erythrocytes (400 μl per well; 2% hematocrit; 20%–35% parasitemia) were added to COS cells and incubated for 1 hr at 37°C with occasional rocking. Unattached erythrocytes were removed by rinsing the wells with RPMI 1640. To distinguish those cells expressing wild-type or mutant ICAM-1 from untransfected cells, the anti-ICAM-1 MAbs CL203 or RR1/1 (5 μg/ml) were added to each well. After 45 min of incubation at room temperature, the wells were washed twice with RPMI 1640, and the cells were fixed with an ice-cold acetone–methanol (50% v/v) mixture for 1 min. Cells were rinsed with phosphate-buffered saline (PBS), and colloidal gold-labeled anti-mouse antibody (Amersham, Arlington, IL) was added to each well for 30 min, followed by three washes with PBS. A silver enhancement reagent (IntenSEM, Amersham, Arlington Heights, IL), which amplifies the colloidal gold signal, was added, and the reaction was terminated after 20 min. Cell-bound IRBCs and surface ICAM-1 were easily identified under phase–contrast microscopy. Cells were fixed with 2% glutaraldehyde and stained with Giemsa, and bound IRBCs were quantitated under light microscopy by an unbiased observer. Binding of IRBCs to ICAM-1 mutants was expressed as the number of IRBCs bound per 100 COS cells expressing ICAM-1, or as follows:

$$\frac{\text{(number of IRBCs bound per 100 COS cells expressing mutant ICAM-1)} \times 100}{\text{(number of IRBCs bound per 100 COS cells expressing wild-type ICAM-1).}}$$

IRBC binding to ICAM-1- or CD36-coated surfaces was performed as follows. Soluble ICAM-1 (10 μg/ml) (Marlin et al., 1990) or CD36 (1 μg/ml) (Tandon et al., 1989) was coated onto plastic petri dishes overnight at 40°C. PBS containing bovine serum albumin (1%) was added for 60 min to block nonspecific binding. Malaria-infected erythrocytes (final concentration 0.5%) ItG-ICAM-1 or ItG-CD36, which bind to ICAM-1 or CD36, respectively, were added to the receptor-coated plates for 1 hr, rinsed carefully to remove unattached erythrocytes, fixed with 2% glutaraldehyde–PBS, and stained with Giemsa stain. In some experiments ICAM-1 peptides were preincubated for 30 min with the IRBCs prior to addition to receptor-coated plates. The number of IRBCs bound per mm^2 surface area was quantitated by light microscopy.

LFA-1 and HRV Binding Assays

The binding of ICAM-1 mutants to petri dishes coated with immunoaffinity-purified LFA-1 was performed as previously described (Diamond et al., 1990). HRV type 14 binding to COS cells transfected with mutant ICAM-1 was as described (Staunton et al., 1990).

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