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Binding of the Integrin Mac-1 (CD11b/CD18) to the Third Immunoglobulin-like Domain of ICAM-1 (CD54) and Its Regulation by Glycosylation

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Summary

Both the integrins LFA-1 and Mac-1 bind to ICAM-1, an immunoglobulin superfamily member. Previously, we localized the binding sites of LFA-1 and the major group of human rhinoviruses to the first NH_2–terminal immunoglobulin-like domain of ICAM-1. Here, we show that the binding site on ICAM-1 for Mac-1 is unexpectedly distinct from that for LFA-1 and maps to the third NH_2–terminal immunoglobulin-like domain. These findings provide a function for the tandem duplication of immunoglobulin-like domains in ICAM-1 and have implications for other immunoglobulin superfamily members. Mutations at two sites in the third domain that destroy consensus sequences for N-linked glycosylation enhance binding to purified Mac-1. Agents that interfere with carbohydrate processing provide evidence that the size of the N-linked oligosaccharide side chains on ICAM-1 affects binding to Mac-1 but not to LFA-1. Thus, we suggest that the extent of glycosylation on ICAM-1 may regulate adhesion to LFA-1 or Mac-1 in vivo.

Introduction

Lymphocyte function–associated antigen 1 (LFA-1, CD11a/CD18) and Mac-1 (CD11b/CD18) are members of a family of proteins, the leukocyte integrins, that are critical for adhesive functions in the immune system (Springer et al., 1987; Springer, 1990). These two proteins, along with a third member, p150,95 (CD11c/CD18), share a common β chain that is noncovalently associated with each unique α chain. LFA-1, which is expressed on all leukocytes, mediates lymphocyte adhesion to endothelial cells (Haskard et al., 1986; Dustin and Springer, 1988; Smith et al., 1989), facilitates killer T lymphocyte conjugation required for target cell lysis (Davignon et al., 1981), and promotes the adhesion required for T cell proliferation (Davignon et al., 1981) and natural killing (Krensky et al., 1983). Mac-1 is expressed primarily on myeloid and natural killer cells (Kishimoto et al., 1989) and is responsible for myeloid cell adhesion to the endothelium (Harlan et al., 1985; Smith et al., 1989; Lo et al., 1989), neutrophil homotypic aggregation and chemotaxis (Anderson et al., 1986), and the binding of iC3b-coated particles (Beller et al., 1982), fibrinogen (Altieri et al., 1988; Wright et al., 1988), and factor X (Altieri and Edgington, 1988). A congenital deficiency or absence of the leukocyte integrins results in a clinical syndrome, leukocyte adhesion deficiency, characterized by diminished pus formation, abnormal wound healing, and grave susceptibility to pyogenic infections as well as abnormalities of adhesion-dependent leukocyte functions in vitro (Anderson and Springer, 1987; Carlos and Harlan, 1990).

Intercellular adhesion molecule 1 (ICAM-1, CD54) is a cell surface adhesion protein that is a counterreceptor for LFA-1 (Rothlein et al., 1986; Marlin and Springer, 1987). It is a heavily glycosylated 85,000–110,000 M_r single chain molecule that has five tandem immunoglobulin-like domains (Dustin et al., 1986; Simmons et al., 1988; Staunton et al., 1988). It is expressed on a variety of hematopoietic and nonhematopoietic cells, up-regulated by inflammatory mediators (Dustin et al., 1986), and is partially responsible for lymphocyte, monocyte, and neutrophil adhesion to endothelial cells (Dustin and Springer, 1988; Smith et al., 1988, 1989; Diamond et al., 1990). ICAM-1 has also been suggested as a ligand for Mac-1 because a monoclonal antibody (MAb) to ICAM-1 blocks activated neutrophil adhesion to endothelial cells and to ICAM-1-containing planar membranes (Smith et al., 1988, 1989). Recently, we have confirmed in reciprocal adhesion assays that ICAM-1- or Mac-1-transfected COS cells bind to purified Mac-1 or ICAM-1 substrates (Diamond et al., 1990); thus, ICAM-1 is a counterreceptor for Mac-1. Discrepancies among previous reports as to whether Mac-1 binds to ICAM-1 (Smith et al., 1989; Lo et al., 1989) can be explained partially by the disparity among ICAM-1 MAbs used; four MAbs that block LFA-1–ICAM-1 interaction do not affect binding to Mac-1, one MAb that partially blocks adhesion to Mac-1 does not decrease attachment to LFA-1, and one MAb abrogates binding to both Mac-1 and LFA-1 (Diamond et al., 1990).

Here we examine the binding site on ICAM-1 for Mac-1. We show that adhesion of ICAM-1-expressing COS cells to Mac-1 can be abrogated by mutation or deletion of the third N-terminal immunoglobulin-like domain of ICAM-1. All previous mutagenesis studies on adhesion molecules of the immunoglobulin family, including ICAM-1 (Staunton et al., 1990), CD4 (Peterson and Seed, 1988; Arthos et al., 1989; Clayton et al., 1989), and CD2 (Peterson and Seed, 1987), have localized the ligand-binding site to the N-terminal immunoglobulin-like domain. Our results suggest that tandem duplication of immunoglobulin domains found in several members of the immunoglobulin superfamily (Williams and Barclay, 1988) may have functional importance for the creation of multiple binding sites. Unexpectedly, we also find that the level of glycosylation on ICAM-1 can modify the interaction between Mac-1 and ICAM-1, as Mac-1 binds with higher avidity to molecules of ICAM-1 that have smaller N-linked oligosaccharide side chains or that have been mutated to remove N-linked glycosylation sites in the third domain.

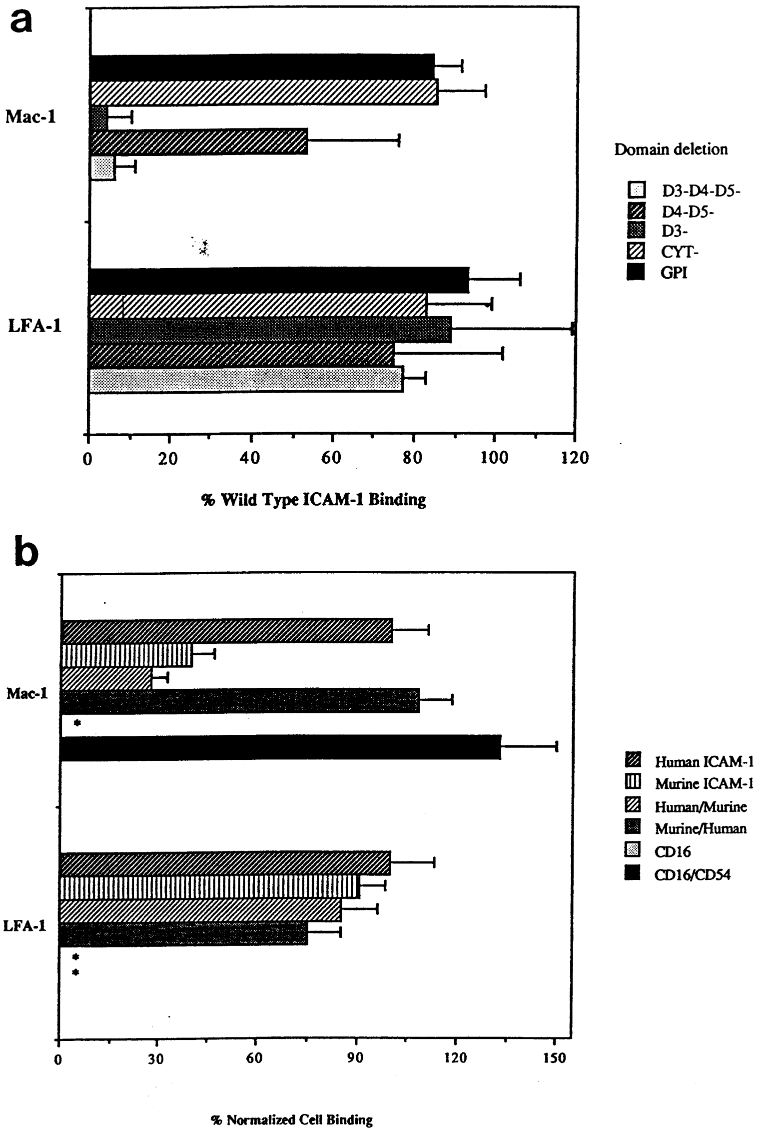

Figure 1. The Binding of Domain-Deleted and Chimeric Forms of ICAM-1 to LFA-1 and Mac-1

(a) Adhesion of domain-deleted forms of ICAM-1 to LFA-1 and Mac-1. Domain deletion mutants were generated by oligonucleotide-directed mutagenesis (Staunton et al., 1990), and the cDNA was transfected into COS cells by the DEAE-dextran method. Cells were harvested 72 hr after transfection, assayed for surface expression by immunofluorescence with MAb to ICAM-1 (RR1/1 and CL203), and allowed to adhere to purified Mac-1 and LFA-1 adsorbed to plastic on 60 mm Petri dishes. The mutants lack the following domains: D3$^-$D4$^-$D5$^-$ (domains 3–5), D4$^-$D5$^-$ (domains 4 and 5), D3$^-$ (domain 3), cyt$^-$ (the cytoplasmic tail). GPI represents a form of ICAM-1 that has a phosphatidylinositol lipid anchor instead of the wild-type transmembrane and cytoplasmic regions. The data are the average of three independent experiments and are normalized for surface expression and wild-type and mock binding as described in Experimental Procedures. The bars represent standard deviations.

(b) Adhesion of chimeric forms of ICAM-1 to Mac-1 and LFA-1. Chimeric molecules were generated as described in the Results and transfected into COS cells. The cells were harvested 72 hr after transfection, assayed for surface expression by immunofluorescence with MAb to human ICAM-1 (R6.5 and CL203), murine ICAM-1 (YN1/1.7), and domain 1 of CD16 (B73.1), and allowed to adhere to purified Mac-1 and LFA-1 adsorbed to plastic on 60 mm Petri dishes. The three chimeric molecules are composed as follows: Human/Murine (human ICAM-1, domains 1 and 2; murine ICAM-1, domains 3–5); Murine/Human (murine ICAM-1, domains 1 and 2; human ICAM-1, domains 3–5); CD16/CD54 (CD16, domain 1; human ICAM-1, domains 3–5). One representative experiment of two is shown and the bars indicate standard deviations. The data are normalized for surface expression and wild-type and mock binding as described in Experimental Procedures. Asterisks indicate there is no binding above mock-transfected cells.

Results

Binding of ICAM-1 Mutants to Purified Mac-1 and LFA-1

Previously, we localized the binding site for LFA-1 to the first N-terminal immunoglobulin-like domain of ICAM-1 (Staunton et al., 1990). The same and additional ICAM-1 domain deletion and amino acid substitution mutants are used here to localize the binding site for Mac-1. Less stringent washing conditions have been employed in this study due to the apparent lower avidity for ICAM-1 of Mac-1 compared with LFA-1 (Diamond et al., 1990). Binding of transfected COS cells is measured in parallel to demarcated spots of purified LFA-1 and Mac-1 applied to the same Petri dish (Figure 1a). Binding to purified LFA-1 is unaffected by truncation of the cytoplasmic domain, reanchoring on glycophosphatidylinositol (GPI), removal of domain 3, removal of domains 4 and 5, or removal of domains 3, 4, and 5 of ICAM-1. This agrees with previous localization of the LFA-1-binding site to domain 1 (Staunton et al., 1990). However, shortening of ICAM-1 by domain deletion has less effect in this assay than in the more stringent washing procedure previously described; this is consistent with some amino acid substitutions that show a 2- to 3-fold effect in the previous system but a smaller effect in the current assay (see Figure 2). In contrast to results with LFA-1, deletion of domain 3 or domains 3–5 of ICAM-1 eliminates binding to Mac-1. Deletion of domains 4 and 5 has only a minor effect. These results suggest that Mac-1 binds to domain 3 of ICAM-1.

To confirm that domain 3 of ICAM-1 is an integral component of the ICAM-1 binding site for Mac-1, adhesion studies were initiated on chimeric molecules, using murine and human ICAM-1 and the low affinity Fcγ receptor type III (CD16) (Figure 1b). Murine ICAM-1 binds to both purified human LFA-1 (Staunton et al., 1990; Johnston et al., 1990) and Mac-1, although the binding is weaker to Mac-1 when compared with human ICAM-1. Chimeric forms of ICAM-1

Mutation	Mac-1 Binding (% wt (SD))	LFA-1 Binding (% wt (SD))	LFA-1 Binding* (% wt (SEM))
Domain 1			
Q1T/KA	121 (80)	150 (42)	119 (23)
K8/E	110 (59)	126 (33)	84 (6)
R13G/EA	109 (19)	**3** (2)	**2** (2)
D26QPK/ALPE	99 (24)	80 (52)	30 (13)
E34/A	109 (13)	**1** (0)	**0** (0)
K39KE/ERQ	73 (50)	46 (33)	99 (25)
G46NN/ASI	101 (60)	140 (51)	49 (15)
R49KV/EKL	105 (45)	115 (11)	123 (20)
Q58EDS/AKDI	75 (18)	**3** (4)	**3** (3)
D60S/KL	108 (25)	**0** (0)	**1** (1)
Q62PM/API	110 (1)	164 (19)	104 (48)
D71/N	75 (12)	97 (35)	79 (3)
Q73/H	125 (59)	**21** (19)	**12** (10)
Domain 2			
G101K/AN	121 (30)	96 (23)	97 (55)
N103/K	88 (29)	**0** (1)	**12** (6)
E111GGA/KAGS	98 (16)	84 (22)	103 (35)
N118/Q	122 (41)	164 (21)	54 (22)
R125/E	83 (37)	101 (41)	81 (27)
E127/R	88 (7)	109 (34)	82 (29)
K128/R	112 (36)	110 (12)	109 (52)
R149RD/EEG	102 (50)	130 (5)	81 (40)
H152HGA/EEGS	94 (41)	104 (52)	85 (52)
N156/E	124 (4)	150 (21)	60 (4)
R166PQ/EPA	94 (5)	82 (37)	75 (8)
N175/A	78 (41)	107 (35)	59 (1)
Domain 3			
A189T/SI	162 (61)	160 (22)	91 (3)
D203TQ/TAD	139 (33)	110 (14)	91 (53)
D213GL/HGV	122 (1)	145 (26)	90 (52)
D229QR/HLE	**2** (2)	103 (19)	90 (37)
Q230/H	179 (26)	86 (21)	ND
R231/I	74 (15)	87 (9)	ND
N240DS/KNA	205 (137)	144 (24)	147 (29)
E254DE/KEK	**29** (17)	114 (40)	122 (33)
E254/A	90 (47)	145 (47)	ND
N269QSQE/IQAEQ	**437** (207)	108 (32)	101 (9)
N269/D	**381** (44)	97 (13)	ND

Figure 2. The Binding of Amino Acid Substitution Mutants to LFA-1 and Mac-1

Mutants were constructed and assayed as described in Experimental Procedures and the legend to Figure 1. Mutants that are italicized and underlined disrupt the overall conformation of domains 1 and 2 (Staunton et al., 1990). Boldface mutants in domain 1 correspond to those that decrease binding to LFA-1 greater than 75%. Boldface mutants in domain 3 correspond to those that have a significant effect on binding to Mac-1 ($p < 0.05$). The data are the average of at least three independent experiments and the values in parentheses represent standard deviations.

*From Staunton et al. (1990).

were generated by exchanging sequences of murine and human ICAM-1 about a common BglII site at amino acid 168 (Staunton et al., 1990). The exchanged sequences encompass all of domains 1 and 2 except the 17 most C-terminal residues of domain 2. Both chimeric forms of ICAM-1 bind equally well to LFA-1, but adhesion to Mac-1 of the chimeric ICAM-1 is equivalent to human ICAM-1 when domains 3–5 of human ICAM-1 are present, and equivalent to the lower binding of murine ICAM-1 when domains 3–5 of murine ICAM-1 are present.

To distinguish further the binding sites on ICAM-1 for LFA-1 and Mac-1, a chimeric molecule, CD16–CD54, was generated by introducing silent SacII restriction sites between domains 1 and 2 of CD16 (residues 114–115) and domains 2 and 3 of human ICAM-1 (residues 197–198) (M. Hibbs, O. Carpen, and T. A. Springer, unpublished data). CD16 binds neither to Mac-1 nor LFA-1 despite being expressed strongly on the surface of transfected COS cells. Chimeric CD16–CD54, which contains domain 1 of CD16 and domains 3, 4, and 5 of ICAM-1, binds strongly to Mac-1 but not to LFA-1. Thus, domains 1 and 2 of ICAM-1 are not required for binding to Mac-1. These results confirm the mapping of the Mac-1-binding site to domain 3.

The binding site for Mac-1 was further examined with single or multiple amino acid mutations in each of the predicted β-turn loops of domains 1–3 (Figure 2). Two single amino acid substitution mutations in domain 1 strongly abrogate binding to LFA-1 without altering domain conformation: E34/A and Q73/H. These do not decrease adhesion to purified Mac-1. Three other mutations, R13G/EA, Q58EDS/AKDI, and D60S/KL, disrupt the overall conformation of domains 1 and 2, as judged by loss of three spatially distinct MAb epitopes (RR1/1, LB-2, R6.5) that map to domains 1 and 2 (Staunton et al., 1990). These mutations abolish binding to LFA-1, but show no effect on binding to Mac-1. Other mutations in domain 1 have little effect on adhesion of ICAM-1 to LFA-1 or Mac-1.

None of the mutations in domain 2 affects Mac-1 binding, including the N103/K mutation; this mutation disrupts the overall conformation of domains 1 and 2 as shown by the loss of three MAb epitopes (Staunton et al., 1990) and the reduction of binding to LFA-1. All other amino acid substitution mutants within domain 2 effect no significant changes in adhesion to either Mac-1 or LFA-1 (Figure 2).

In contrast to the findings for domains 1 and 2, several mutations in domain 3 profoundly inhibit adhesion to Mac-1. D229QR/HLE completely abrogates binding to Mac-1, while E254DE/KEK reduces binding approximately 71%. This effect is not caused by decreased surface levels of the mutant ICAM-1 molecules as both D229QR/HLE and E254DE/KEK are expressed at wild-type levels (Staunton et al., 1990; data not shown). Furthermore, these mutations sustain wild-type levels of binding to LFA-1. None of the other amino acid mutations in domain 3 decreases binding to Mac-1 or LFA-1.

Unexpectedly, two mutations that destroy N-linked glycosylation sites (N-X-S/T) in domain 3 augment binding to Mac-1. The mutation N240DS/KNA augments binding to Mac-1 more than to LFA-1. A more impressive 4.4-fold enhancement is observed with the mutation N269QSQE/IQAEQ. Asparagines 240 and 269 are two of eight consensus sites for N-linked glycosylation in ICAM-1 (Staunton et al., 1988) and are the only putative glycosylation sites in domain 3. ICAM-1 is known to be heavily glycosylated (Dustin et al., 1986), and enzymatic digestion with N-glycanase has demonstrated that at least seven of the eight sites are utilized (Tomassini et al., 1989). We hypothesized that the increase in adhesion may be caused by loss of oligosaccharide side chains that interfere with interaction with Mac-1. To test this, the mutation N269/D was engineered, which eliminates the recognition site for N-linked glycosylation but substitutes conservatively an aspartic acid for asparagine. N269/D effects a similar 3.8-fold enhancement over wild-type adhesion to Mac-1.

Effect of N-Linked Oligosaccharides on ICAM-1 Adhesion to Mac-1

The mutagenesis data suggest that Mac-1 binds to a distinct site in domain 3 of ICAM-1 and that this interaction may be altered by the extent of N-linked glycosylation on

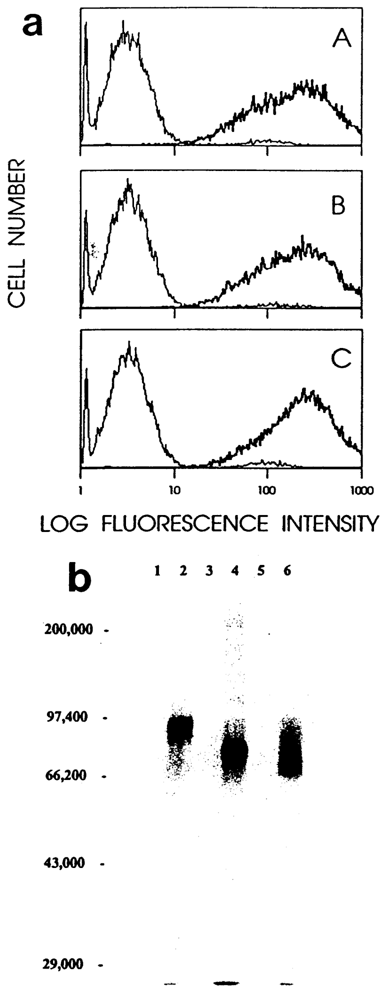

Figure 3. Surface Expression of ICAM-1 on L-ICAM-1$^+$ Cells after Treatment with Agents That Reduce N-Linked Glycosylation

(a) Flow cytometry profiles of ICAM-1 expression on L-ICAM-1$^+$ cells. L-ICAM-1$^+$ cells were incubated with a nonbinding MAb (X63, light line) or an ICAM-1 MAb (R6.5), FITC goat anti-mouse IgG, and subjected to flow cytometry. Prior to incubation with MAb, cells were untreated (A), treated with 0.2 U/ml neuraminidase for 1 hr at 37°C (B), or treated with 40 μg/ml deoxymannojirimycin for 3 days at 37°C (C).

(b) SDS–PAGE of ICAM-1 immunoprecipitated from L cells. L-ICAM-1$^+$ cells were untreated (lanes 1 and 2), or treated with neuraminidase (lanes 3 and 4) or deoxymannojirimycin (lanes 5 and 6) as described in Experimental Procedures. Cells were washed and surface labeled with ^{125}I, immunoprecipitated with MAb–Sepharose (R6.5) to ICAM-1 (lanes 2, 4, and 6), or MAb–Sepharose (TS1/18) to an antigen not present on L-ICAM-1$^+$ cells (CD18, lanes 1, 3, and 5). Material was boiled in SDS sample buffer with 5% β-mercaptoethanol, electrophoresed on an 8% polyacrylamide gel, and autoradiographed. Molecular weight markers are indicated on the left.

ICAM-1. To determine how the size of the core N-linked oligosaccharide affects Mac-1 binding to ICAM-1, untransfected (control) L cells or L cells that express human ICAM-1 (L-ICAM-1$^+$) (Diamond et al., 1990) were treated with deoxymannojirimycin, a specific inhibitor of the Golgi α mannosidase I that prevents the processing of high mannose to complex-type oligosaccharides in cultured cells so N-linked sugars remain in a $Man_8GlcNAc_2$ form (Fuhrmann et al., 1984; Bischoff et al., 1986), or with Vibrio cholera neuraminidase, an enzyme that hydrolyzes O-ketosidic linkages of terminial sialic acid so treated glycoproteins lose their terminal sialylation. When L-ICAM-1$^+$ cells are treated with these agents, there is little change in cell surface expression as judged by indirect immunofluorescence with two MAbs to ICAM-1 whose epitopes localize to domains 1 and 4 (Figure 3a; data not shown). Thus, the biosynthetic inhibitor and neuraminidase do not alter protein synthesis, gross tertiary structure, or the level of expression of the protein on the cell surface.

To confirm that these agents effectively produce underglycosylated forms of ICAM-1, L-ICAM-1$^+$ cells were surface labeled with ^{125}I, immunoprecipitated with a MAb to ICAM-1, and resolved by SDS–PAGE. Under reducing conditions, ICAM-1 from untreated L-ICAM-1$^+$ cells migrates at M_r 95,000, ICAM-1 from neuraminidase-treated L-ICAM-1$^+$ cells migrates at M_r 82,000, and ICAM-1 from deoxymannojirimycin-treated L-ICAM-1$^+$ cells migrates at M_r 71,000 (Figure 3b). When L-ICAM-1$^+$ cells are treated with these agents and assayed for binding to purified Mac-1, treatment with deoxymannojirimycin, but not neuraminidase, shifts the dose-response curve and improves adhesion (Figure 4a). The following suggest this effect is specific: untransfected L cells do not show significantly increased adhesion to purified Mac-1 after treatment (Figure 4a); the binding of deoxymannojirimycin-treated L-ICAM-1$^+$ cells to Mac-1 is inhibited by a blocking ICAM-1 MAb (R6.5) but unaffected by a control ICAM-2 MAb (CBRIC2/1) or a nonblocking ICAM-1 MAb (CL203) (Figure 4b); and these agents do not promote similar effects on adhesion of L-ICAM-1$^+$ cells to purified LFA-1 (Figure 4c), although we do observe a slight shift in the dose-response curve in binding to LFA-1 after cells are treated with neuraminidase.

Neutrophil Adhesion to Different Forms of Soluble ICAM-1

To confirm that Mac-1 interacts preferentially with an ICAM-1 that is less glycosylated, we tested how neutrophils, which express both Mac-1 and LFA-1, bind to forms of ICAM-1 with different N-linked oligosaccharide side chains. Previously, neutrophils have been shown to bind a transmembrane form of ICAM-1 in planar membranes (Smith et al., 1989) or adsorbed to plastic (M. S. Diamond and T. A. Springer, unpublished data) in a manner that involves both LFA-1 and Mac-1. A soluble form of ICAM-1 (sICAM-1), truncated just before the hydrophobic transmembrane segment (Y452E/F*), has been expressed and purified from a variety of cell types (Staunton et al., 1990; Marlin et al., 1990). To create differentially glycosylated forms of sICAM-1, several strategies were employed: immunoaffinity-purified sICAM-1 secreted by CHO cells (Y452-CHO) was treated with neuraminidase to cleave sialic acid residues (Y452-NEUR); CHO cells expressing sICAM-1 were cultured in the presence of tunicamycin to generate a nonglycosylated form of sICAM-1 (Y452-TUNIC); and the Y452 sICAM-1 was expressed in a baculovirus-insect cell expression system. High levels of sICAM-1 were generated that lack complex or hybrid oligo-

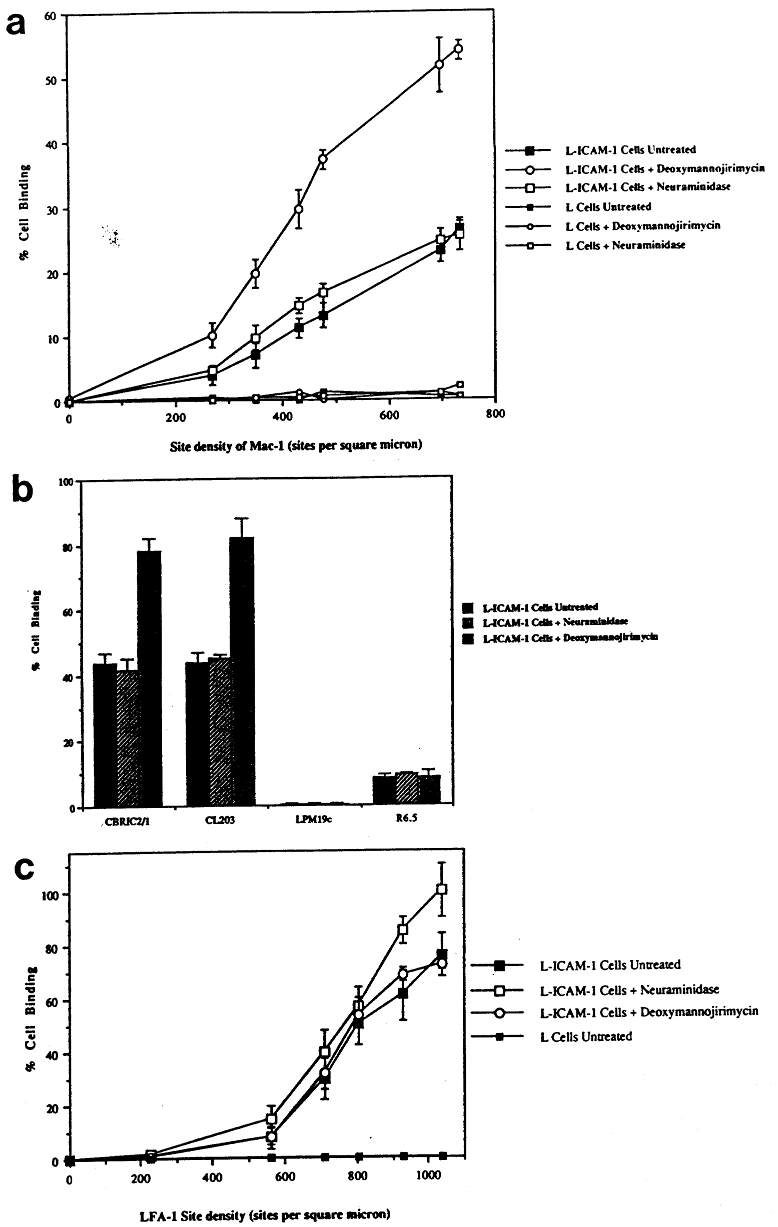

Figure 4. Adhesion of Treated L-ICAM-1$^+$ Cells to Purified Mac-1 and LFA-1

(a) Adhesion to purified Mac-1. Untransfected L or L-ICAM-1$^+$ cells were untreated (shaded squares), or treated with neuraminidase (open squares) or deoxymannojirimicin (open circles) as described in Experimental Procedures. Cells were resuspended (1.5 × 10^6 cells/ml) and added to 60 mm Petri dishes coated with spots of Mac-1 at the indicated site density (as determined by saturation equilibrium binding with ^{125}I-labeled LM2/1) for 60 min at 37°C. Unbound cells were removed by five washes with a Pasteur pipette. Bound cells were quantitated by visually scoring the number of cells in four to five microscopic fields (100 × magnification) for each experimental point. This number was divided into the input number of cells, which was determined in parallel to obtain the percentage of cell binding. One representative experiment of four is shown and the bars indicate standard error of the mean.

(b) Specificity of adhesion to purified Mac-1. L-ICAM-1$^+$ cells were untreated, or treated with neuraminidase or deoxymannojirimycin as described. Cells were resuspended (1.5 × 10^6 cells/ml), preincubated with MAbs, added to 60 mm Petri dishes coated with spots of Mac-1 (1170 sites per μm^2), and incubated for 60 min at 37°C. Unbound cells were removed and bound cells quantitated as described above. Antibody blocking was performed by preincubating the cells and the Petri dishes at room temperature for 20 min with the following MAbs: CBRIC2/1 (anti-ICAM-2), CL203 (anti-ICAM-1, domains 4 and 5), LPM19c (anti-Mac-1), and R6.5 (anti-ICAM-1, domain 2). One representative experiment of two is shown and the bars indicate standard error of the mean.

(c) Adhesion of treated L-ICAM-1$^+$ cells to purified LFA-1. L-ICAM-1$^+$ cells were untreated (open squares), or treated with neuraminidase (shaded circles) or deoxymannojirimtcin (open circles) as described in Experimental Procedures. Cells were resuspended (1.5 × 10^6 cells/ml) and added to 60 mm Petri dishes coated with spots of LFA-1 at the indicated site density (as determined by saturation equilibrium binding with ^{125}I-labeled TS1/18) for 45 min at room temperature. Unbound cells were removed and bound cells quantitated as described in (a). This number was divided into the input number of cells, which was determined in parallel to obtain the percentage of cell binding. One representative experiment of three is shown and the bars indicate the standard error of the mean.

saccharides, but contain $Man_3GlcNAc_2$ as the N-linked core sugar (Y452-BAC) (Hsieh and Robbins, 1984; Paul et al., 1990). Underglycoslyation of these sICAM-1 glycoforms is confirmed by faster migration on SDS–PAGE: Y452-CHO, Y452-NEUR, Y452-TUNIC, and Y452-BAC migrate at 79,400, 73,300, 48,600, and 63,600 M_r, respectively (Figure 5).

Neutrophils were tested for their ability to adhere to these forms of sICAM-1 adsorbed to plastic. In this system, after a short 2.5 min incubation at room temperature in the absence of stimulation, neutrophils do not adhere to any form of sICAM-1 (data not shown). However, upon stimulation with the chemotactic peptide fMLP (10^{-7} M), neutrophils bind all forms of sICAM-1. The adhesion is specific as it is blocked by Fab fragments (R6.5) to ICAM-1 (85%–90%) and by a combination of MAbs (LPM19c + TS1/22) to Mac-1 and LFA-1 α subunits (95%–100%). The adhesion is not reduced by a nonbinding control MAb (anti-ICAM-2, CBRIC2/1) nor a MAb to Mac-1 (VIM-12) that binds an epitope in the C-terminal region of Mac-1 that is distinct from the blocking LPM19c epitope (data not shown; J. Garcia-Aguilar and T. A. Springer, unpublished data). However, the nature of LFA-1 or Mac-1 dependency of neutrophil adhesion changes depending on the level of glycosylation of sICAM-1 (Figure 6). Adhesion to forms of sICAM-1 with larger, complex carbohydrate side chains (Y452-CHO, Y452-NEUR) is primarily LFA-1 dependent as it is inhibited more by the MAb to LFA-1, TS1/22 (69%–

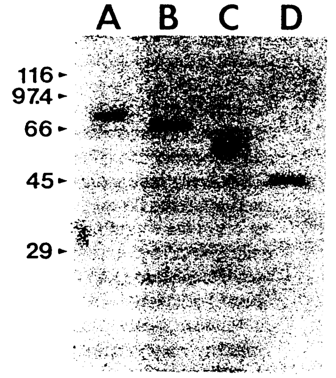

Figure 5. SDS–PAGE of Different Forms of Purified sICAM-1

Purified forms of sICAM-1 were electrophoresed on 10%–15% gradient SDS–PAGE under reducing conditions and subjected to silver staining. The migration of the molecular weight markers is represented on the left. Lane A, Y452-CHO (CHO cell sICAM-1); lane B, Y452-NEUR (neuraminidase-treated CHO cell sICAM-1); lane C, Y452-BAC (sICAM-1 expressed in insect cells); lane D, Y452-TUNIC (sICAM-1 from tunicamycin-treated CHO cells).

84%), than by the blocking MAb to Mac-1, LPM19c (22%–24%). In contrast, the adhesion to forms of sICAM-1 that lack N-linked carbohydrate addition (Y452-TUNIC) or have simple mannose side chains (Y452-BAC) is primarily Mac-1 dependent as it is inhibited more by the blocking MAb to Mac-1, LPM19c (61%–81%), than by the MAb to LFA-1, TS1/22 (4%–24%).

Discussion

The domain deletion and amino acid substitution experiments presented here suggest that LFA-1 and Mac-1 bind to discrete regions of ICAM-1, in domains 1 and 3, respectively. The binding site for LFA-1 has previously been mapped to domain 1 (Staunton et al., 1990). Our evidence for a distinct Mac-1-binding site in domain 3 is as follows. First, domain-deleted forms of ICAM-1 that lack domain 3 do not bind to Mac-1 but retain binding to LFA-1. Second, chimeric CD16–CD54 molecules, which lack domains 1 and 2 of ICAM-1, bind to Mac-1 but not to LFA-1. Third, point mutations in domain 1 (E34/A, Q73/H) that abolish or reduce binding to LFA-1 do not affect adhesion to Mac-1. Fourth, mutations (R13G/EA, Q58EDS/AKDI, D60S/KL, N103/K) that disrupt the overall structure of domains 1 and 2 as judged by loss of MAb epitopes in both domains result in loss of binding to LFA-1 but do not affect binding to Mac-1. Fifth, two amino acid substitution mutations in domain 3 (D229QR/HLE, E254DE/KEK) markedly reduce binding to Mac-1 but have no effect on adhesion to LFA-1. Finally, other amino acid mutations in domain 3 that eliminate N-linked glycosylation sites enhance binding to Mac-1 but do not affect adhesion to LFA-1.

The mutagenesis experiments explain the apparent discrepancy in the pattern of blocking observed with different MAbs to ICAM-1 (Diamond et al., 1990). MAbs that map to domain 1 on ICAM-1 (RR1/1, LB-2, 84H10) (Staunton et al., 1989, 1990) reduce adhesion to LFA-1 but do not block ICAM-1 binding to Mac-1 (Diamond et al., 1990). ICAM-1 interactions with Mac-1 and LFA-1, however, are blocked by MAb R6.5 (Smith et al., 1989; Diamond et al., 1990), which recognizes an epitope in domain 2 that includes a predicted β turn at residues 111–114 (Staunton et al., 1990). The mutation of these residues (E111GGA/KAGS) knocks out the R6.5 epitope completely (Staunton et al., 1990), but does not reduce adhesion to Mac-1 or LFA-1. Thus, R6.5 probably does not bind to the contact residues directly, but instead may reduce adhesion by sterically blocking the binding sites in domains 1 and 3. This hypothesis is consistent with our mutagenesis experiments in domain 2, which show that substitutions that retain overall conformation do not decrease binding to Mac-1 and have little effect on binding to LFA-1.

The majority of immunoglobulin superfamily members

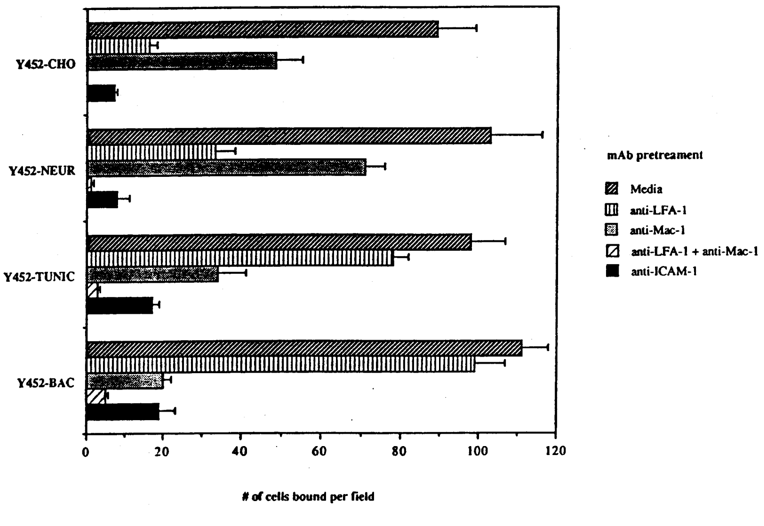

Figure 6. Neutrophil Adhesion to Different Forms of sICAM-1

Peripheral blood neutrophils were resuspended (1.5 × 10^6 cells/ml), preincubated with MAbs, and added to 60 mm Petri dishes coated with spots of Y452-CHO (CHO cell sICAM-1), Y452-NEUR (neuraminidase-treated CHO cell sICAM-1), Y452-TUNIC (sICAM-1 from tunicamycin-treated CHO cells), and Y452-BAC (sICAM-1 expressed in insect cells) in the presence of 10^{-7} M fMLP. The cells were allowed to bind for 2.5 min at room temperature, and unbound cells were removed by ten washes with a Pasteur pipette. Bound cells were scored by microscopy as described in Figure 4. Antibody blocking was performed by preincubating the cells and the Petri dishes at room temperature for 20 min with the following MAbs: Media (no MAb), anti-LFA-1 α (TS1/22), anti-Mac-1 α (LPM19c), anti-LFA-1 α + anti-Mac-1 α (TS1/22 + LPM19c), and anti-ICAM-1 (R6.5 Fab fragments). One representative experiment of four is shown and the bars indicate standard error of the mean.

have multiple, tandemly duplicated domains (Williams and Barclay, 1988; Hunkapiller and Hood, 1989). Previous studies on the adhesion molecules ICAM-1, CD2, and CD4 (Staunton et al., 1990; Peterson and Seed, 1987; Clayton et al., 1989) have shown that the ligand-binding site is essentially contained within a single immunoglobulin-like domain located at the N-terminus. It appeared as if this might be the general rule as even viruses that subvert ICAM-1 and CD4 utilize the N-terminal immunoglobulin-like domain (Staunton et al., 1990; Peterson and Seed, 1988; Arthos et al., 1989), although the membrane proximal domains may contribute to the accessibility of the ligand-binding site in the first immunoglobulin-like domain (Staunton et al., 1990).

We have demonstrated, however, that ICAM-1 has multiple binding interactions with distinct cell surface proteins in separate immunoglobulin-like domains. Thus, this report provides a function for the duplicated domains of these molecules, which is not unlike the one active site per domain model that was originally hypothesized for immunoglobulins (Edelman, 1970). Tandem domain duplication in the immunoglobulin superfamily may be an evolutionary mechanism that creates multiple and independent binding sites, and in the case of ICAM-1, these sites have diverged enough to bind distinct integrins. It is tempting to hypothesize that the sister integrins recognize homologous regions within domains 1 and 3 of ICAM-1; consistent with this, E34 of domain 1 and D229 of domain 3 both align to the turn predicted between β strands C and D of an immunoglobulin domain (Staunton et al., 1988). We speculate that other adhesion proteins in the immunoglobulin superfamily may prove to bind more than one counterreceptor by a similar mechanism, using distinct immunoglobulin-like domains to confer separate ligand specificity.

Mutations at two residues in domain 3, N240 and N269, augment binding to Mac-1 but have little effect on binding to LFA-1. These residues are both part of the N-X-S/T consensus sequences for N-linked glycosylation. Loss of the consensus sequence may eliminate branched or charged oligosaccharide groups that inhibit binding to Mac-1 either sterically or electrostatically. Two independent experiments suggest that the size of the N-linked oligosaccharide chain affects adhesion between Mac-1 and ICAM-1. First, when L cells expressing ICAM-1 are treated with deoxymannojirimycin, an agent that disrupts Golgi-associated N-linked carbohydrate addition, the adhesion to purified Mac-1 but not LFA-1 is improved; the enhancement is specific as it is blocked by MAb to ICAM-1. Deoxymannojirimycin causes N-linked oligosaccharides to accumulate in a high mannose ($Man_8GlcNAc_2$) form (Fuhrmann et al., 1984; Bischoff et al., 1986). Second, the size of the carbohydrate side chain affects the specificity of neutrophil interaction with sICAM-1. Adhesion depends primarily on Mac-1 if ICAM-1 has a smaller N-linked side chain (Y452-TUN, Y452-BAC) but on LFA-1 if ICAM-1 has a more complex carbohydrate side chain (Y452-CHO, Y452-NEUR). Treatment with neuraminidase, which removes terminal charged sialic acid residues, has no effect on Mac-1–ICAM-1 interaction and little, if any, effect on LFA-1–ICAM-1 adhesion.

ICAM-1 isolated from different cell types has been shown to differ in the extent of N-linked carbohydrate processing, since its size in SDS–PAGE varies from an M_r of 90,000 on B lymphoblastoid cells to an M_r of 114,000 on myelomonocytic cells, yet in all cases ICAM-1 is converted to a 55 kd protein after treatment with N-glycanase (Dustin et al., 1986). Thus, the level and type of N-linked glycoslation on ICAM-1 may regulate biological interaction with cells bearing specific leukocyte integrins: myeloid cells may accumulate if ICAM-1 is relatively deglycosylated, whereas lymphocytes may localize if ICAM-1 is more fully glycosylated. Such heterogeneity in glycosylation may itself be regulated developmentally as has been described for embryonic and adult forms of N-CAM (Edelman, 1986; Rutishauser et al., 1988). N-linked glycosylation of ICAM-1 appears to specifically shield the ligand-binding site for Mac-1. Polysialylation of N-CAM inhibits cell interactions involving N-CAM (Hoffman and Edelman, 1983), but whether this is due to charge repulsion or a shielding of a specific binding site is unclear. In contrast, carbohydrates also may serve as direct ligands, as has been shown recently for the selectin family of adhesion proteins (reviewed in Springer and Lasky, 1991).

Since our findings strongly suggest that Mac-1 and LFA-1 bind to ICAM-1 in disparate regions of the molecule, it is tempting to speculate that LFA-1 and Mac-1 on the same cell surface can bind a single ICAM-1 molecule. This hypothesis is structurally feasible if we consider the molecular dimensions of an interaction between an integrin and an immunoglobulin-like domain. Electron micrographs show that ICAM-1 is an 18.7 nm bent rod (Staunton et al., 1990), and this is compatible only with a model in which the five immunoglobulin-like domains are unpaired and arranged end to end, each with an approximate dimension of 4 nm parallel or at a slight angle to the rod axis and 2.5 nm in the two perpendicular dimensions as has been described by X-ray crystallography studies of immunoglobulin domains in HLA, immunoglobulin, and CD4 molecules (Bjorkman et al., 1987; Alzari et al., 1988; Wang et al., 1990; Ryu et al., 1990). In addition, electron micrographs show that integrin heterodimers (VLA-5, gpIIb-IIIa) are approximately 30 nm long with an 8–12 nm globular head and an 18–20 nm tail that is 2 nm thick (Carrell et al., 1985; Nermut et al., 1988). Mac-1 and LFA-1 globular heads could bind simultaneously to distinct immunoglobulin domains of ICAM-1, as the center of the domains are spaced approximately 8 nm apart and appear to have a molecular hinge between domains 2 and 3 (Staunton et al., 1988).

The extracellular matrix components fibronectin and laminin also can bind more than one integrin (Hemler, 1990). The binding of multiple integrins to the same ligand may be functionally important if attachment of a single integrin independently or two integrins simultaneously transmits distinct signals to the cell. Furthermore, cells can regulate the number of integrin-binding sites that are present on specific ligands. In fibronectin, the CS-1-binding site for VLA-4 can be alternatively spliced so that some fibronectin molecules contain only the RGDS-binding site for VLA-5 (Hemler, 1990). ICAM expression is regulated during inflammation; ICAM-2, which binds to LFA-1 but not to Mac-1 (Diamond et al., 1990), is expressed constitutively on endothelial cells, whereas ICAM-1, which binds to both LFA-1 and Mac-1, is induced by inflammatory stimuli.

MAb R6.5, which blocks ICAM-1 interaction with both Mac-1 and LFA-1, has been demonstrated to have potential therapeutic applications. Trials in cynomologus monkeys show that administration of R6.5 prolongs renal (Cosimi et al., 1990) and cardiac (Flavin et al., 1991) allografts, reduces granulocytic infiltration into the inflamed lung (Barton et al., 1989), and attenuates the airway eosinophilia and hyperresponsiveness that are associated with asthma (Wegner et al., 1990). Since Mac-1 and LFA-1 bind to discrete domains on ICAM-1, therapeutic intervention could be refined by administration of ICAM-1 MAbs or structural analogs that interfere selectively with Mac-1–ICAM-1 or LFA-1–ICAM-1 function in vivo. Antibodies that selectively block the interaction of LFA-1 with ICAM-1 have already been described (Diamond et al., 1990). We predict that it should be possible to generate a MAb to ICAM-1 or analogs of domain 3 of ICAM-1 that selectively block ICAM-1 interaction with Mac-1. Such agents could be used in vivo, for example, to inhibit neutrophil emigration through inflamed endothelium but should not affect lymphocyte emigration or leukocyte adhesive events that require LFA-1–ICAM-1 interaction and are critical to the immune response.

Experimental Procedures

MAbs

The following murine MAbs against human antigens are from ascites: LPM19c (anti-CD11b, IgG2a, gift of Dr. K. Pohlford, Oxford; Uciechowski and Schmidt, 1989), VIM12 (anti-CD11b, IgG1, gift of Dr. J. Buyon, NY; Bernstein and Self, 1986), TS1/22 (anti-CD11a, IgG1; Sanchez-Madrid et al., 1982), CBRIC2/1 (anti-ICAM-2, IgG2a; de Fougerolles et al., 1991), TS2/16 (anti-CD29, IgG1; Sanchez-Madrid et al., 1982). CL203 (anti-CD54, IgG1, a gift of Dr. S. Ferrone; Maio et al., 1989) was used as purified IgG. R6.5 (anti-CD54, IgG2a, a gift of Dr. R. Rothlein, Ridgefield, CT) was used as a purified Fab. Protein A–purified TS1/18 (anti-CD18, IgG1; Sanchez-Madrid et al., 1982) and LM2/1 (anti-CD11b, IgG1; Miller et al., 1986) were iodinated and used for site density measurement as described (Dustin and Springer, 1989). Protein A–purified TS1/18, LM2/1, TS2/4 (anti-CD11a, IgG1; Sanchez-Madrid et al., 1982), and R6.5 were coupled to cyanogen bromide–activated Sepharose for immunoprecipitation and immunopurification as described (March et al., 1974). YN1/1 (anti-murine ICAM-1, rat IgG2a, gift of Dr. F. Takei, Vancouver, B.C.; Takei, 1985) and B73.1 (anti-CD16, IgG1; Fleit et al., 1989) were used as culture supernatants.

Generation of ICAM-1 Mutants

Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to generate ICAM-1 deletion, chimeric, and amino acid substitution mutants as described (Staunton et al., 1990).

Protein Purification

Mac-1 and LFA-1 were purified from leukocyte lysates by immunoaffinity chromatography after detergent solubilization as described (Diamond et al., 1990; Dustin et al., 1989).

Soluble ICAM-1 (sICAM-1) was purified from the supernatants of a stable line of ICAM-1-transfected CHO cells designated CHO118A (Marlin et al., 1990). The clone Y452E/F*, which encodes a soluble form of ICAM-1 that possesses all five immunoglobulin-like domains, was previously described (Staunton et al., 1990). To produce the nonglycosylated material (Y452-TUN), the CHO118A cells were pretreated for 18 hr at 37°C with 2 μg/ml tunicamycin (Sigma T-7765, St. Louis, MO) in MEM-α without ribo- and deoxyribonucleosides containing 4 g/l glucose and 5% FCS (tunicamycin medium) to allow residual pools of glycosylated sICAM-1 to be secreted into the medium. The medium was aspirated, cells were washed twice with medium alone, and fresh tunicamycin medium was added. After 48 hr at 37°C, the cell-free supernatants were harvested for purification. Glycosylated sICAM-1 (Y452-CHO) was harvested from parallel cultures that were not treated with tunicamycin.

sICAM-1 in the supernatants of untreated and tunicamycin-treated CHO118A cells was purified at 4°C by sequential immunoaffinity, lectin affinity, and size exclusion chromatography (SEC). Protease inhibitors (0.2 U/ml aprotinin, 1 mM PMSF) were included in all buffers used prior to SEC. sICAM-1 was detected and quantitated by "sandwich" ELISA using MAbs R6.5 and R6.1 as previously described (Marlin et al., 1990). Immunoaffinity chromatography was performed with the ICAM-1-specific MAb R6.5 covalently coupled to CNBr-activated Sepharose 4B. Pooled eluate fractions from the R6.5 column containing approximately 2 mg of glycosylated or nonglycosylated sICAM-1 were loaded onto a 5 ml bed volume wheat germ lectin–Sepharose 6MB column (Sigma). Unbound material was washed out with 50 ml of Dulbecco's phosphate-buffered saline (D-PBS; GIBCO, NY). As detected by ELISA and SDS–PAGE, glycosylated sICAM-1 was retained on the column, and nonglycosylated sICAM-1 from tunicamycin-treated cells remained in the flowthrough. The bound sICAM-1 was then eluted with 50 ml of 0.5 M N-acetyl-D-glucosamine in D-PBS. Fractions containing sICAM-1 as detected by ELISA were desalted and concentrated 10-fold in Centricon-30 microconcentrators (Amicon, Beverly, MA). Final purification was performed by FPLC–SEC using a 1 cm × 30 cm Superose 12 HR column with D-PBS as the mobile phase at a flow rate of 0.5 ml/min. Fractions containing sICAM-1 were concentrated, quantitated by the BCA protein assay (Pierce, Rockford, IL), and analyzed by SDS–PAGE.

To produce a sICAM-1 in insect cells, the mutant cDNA clone (Y452E/F*) that codes for sICAM-1 was expressed in SF9 cells as a recombinant baculovirus. The entire coding sequence of Y452E/F* was subcloned into the NheI site of the baculovirus transfer vector pBluebac (Invitrogen, San Diego, CA). A plasmid that contains the cDNA in the correct orientation relative to the polyhedron promoter was isolated and introduced with the wild-type baculovirus (AcPNV) into SF9 cells by $CaPO_4$ precipitation (Summer and Smith, 1988). Culture supernatants containing wild-type and recombinant virus were harvested 6 days after transfection, serially diluted, and used to infect SF9 cells. SF9 cells were then overlaid with 0.6% agarose and 150 μg/ml of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Recombinant viral plaques were picked, and after a second cycle of cloning, virus stocks were prepared from occlusion-negative cultures that produced sICAM-1 (Y452-BAC) as determined by sandwich ELISA. Material was subsequently purified by immunoaffinity chromatography and processed as described above.

All purified derivatives of sICAM-1 were analyzed on 10%–15% gradient SDS-polyacrylamide gels under reducing conditions using the PhastGel system (Pharmacia, Sweden). Proteins were visualized by silver staining using a PhastGel silver staining kit (Pharmacia, Sweden).

Tissue Culture, Transfection, and Cell Preparation

Neutrophils were purified from whole blood after dextran sedimentation, Ficoll gradient centrifugation, and hypotonic lysis as previously described (Diamond et al., 1990). Neutrophils (1.5 × 10^6 cells/ml) were resuspended in Hank's balanced salt solution (HBSS), 10 mM HEPES, and 2 mM $MgCl_2$ and stored at room temperature.

COS cells were grown on 15 cm tissue culture–treated plates in RPMI 1640 supplemented with 10% FCS, 5 mM glutamine, and 50 μg/ml gentamycin. COS cells at 50% confluency were transfected by the DEAE–dextran method (Kingston, 1987) using 6 μg of vector alone or vector containing the wild-type or mutated form of ICAM-1 per 10 cm plate. COS cells were suspended using trypsin-EDTA 1 day before assay and reseeded (Staunton et al., 1990).

L cells were grown on 15 cm tissue culture–treated plates in DMEM containing 20% FCS, 5 mM glutamine, 1 mM sodium pyruvate, and 50 μg/ml gentamycin. L cells expressing human ICAM-1 (L-ICAM-1^+ cells) (Diamond et al., 1990) were grown in the same DMEM but supplemented additionally with HAT (100 μM hypoxanthine, 400 nM aminopterin, 16 μM thymidine) to maintain plasmid selection conditions. For glycosylation inhibitor experiments, L cells were cultured in normal media but supplemented with deoxymannojirimycin (40 μg/ml; Calbiochem Corporation, San Diego, CA) for 48 hr. At this time, cells were detached from the plates with trypsin–EDTA and replated and cultured on fresh plates for an additional 24 hr in the presence of deoxymannojirimycin.

Enzymatic Treatment

Untransfected L cells or L cells expressing ICAM-1 were removed from tissue culture plates with PBS, 5 mM EDTA for 10 min at 37°C. Cells ($4–6 \times 10^6$) were washed twice in PBS, 1 mM $MgCl_2$, and 0.3 mM $CaCl_2$ and then resuspended in the same buffer containing 0.2 U/ml Vibrio cholera neuraminidase (Calbiochem Corporation, San Diego, CA) for 75 min at 37°C while gently rocking. Cells were washed three times in PBS, 2 mM $MgCl_2$, and 1% heat denatured BSA, resuspended (1.5×10^6 cells/ml), and used for immunofluorescence and adhesion assays, or washed in PBS for cell surface labeling.

To produce a desialyated form of sICAM-1 (Y452-NEUR), 1 mg of glycosylated sICAM-1 (as described above) in 0.5 ml of D-PBS was incubated with 1000 mU of Clostridium perfringens neuraminidase (type X; Sigma) at 37°C for 2 hr. The sample was chilled to 4°C, purified by FPLC–SEC, and processed as described above.

Cell Adhesion Assays

For the COS cell binding assay to purified Mac-1 or LFA-1, the transfected cells were eluted with PBS, 5 mM EDTA (5 min, 37°C) and washed three times in PBS, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2% glucose, and 1% heat-treated BSA or 0.5% HSA (PBS^{++}). COS cells were resuspended (2.5×10^5/ml) and added to 60 mm Petri dishes coated with demarcated spots of immunoaffinity-purified Mac-1 and LFA-1 (Diamond et al., 1990) and allowed to adhere for 50 min at 37°C. Unbound cells were removed with four washes with a transfer pipette, the plates were coded, and bound cells were counted by three independent observers. Binding of ICAM-1 mutants to LFA-1 and Mac-1 was corrected for binding to vector only (mock) transfected cells and was normalized to the percentage of COS cells staining with the MAb RR1/1 or CL203 (depending on the mutation) and for the percentage of binding obtained in the wild type (Staunton et al., 1990):

$$\%\ \text{binding} = \frac{(\%\ \text{mutant binding} - \%\ \text{mock binding})/(\%\ \text{mutant MAb staining})}{(\%\ \text{wild-type binding} - \%\ \text{mock binding})/(\%\ \text{wild-type MAb staining})} \times 100$$

For the L cell binding assay, untransfected or L cells expressing human ICAM-1 were eluted from tissue culture plates in PBS, 5 mM EDTA for 10 min at 37°C. Cells were washed three times in PBS, 2 mM $MgCl_2$, and 1% heat-treated BSA and resuspended (1.5×10^6 cells/ml). Cells (1 ml) were added to 60 mm Petri dishes coated with demarcated spots of Mac-1 and LFA-1 at various site densities that had their nonspecific sites blocked with heat-treated BSA (Diamond et al., 1990) and allowed to adhere for 60 min at 37°C (to purified Mac-1) or 45 min at room temperature (to purified LFA-1). Unbound cells were removed by four washes with a transfer pipette, and the bound cells were determined by counting the number of cells in four different fields at high magnification. The number was divided into the input number of cells per field to obtain the percentage of cells binding.

For the neutrophil binding assay, 60 mm Petri dishes were coated with demarcated spots (25 μl) of the following forms of soluble ICAM-1 (200 μg/ml): untreated CHO cell sICAM-1 (Y452-CHO), insect cell sICAM-1 (Y452-BAC), neuraminidase treated (Y452-NEUR), and that harvested from tunicamycin-treated CHO cells (Y452-TUNIC). These different forms of ICAM-1 were incubated for 90 min at room temperature, blocked with PBS, 1% Tween 20 for 2 min at room temperature, and washed with PBS six times. Purified neutrophils (1.5×10^5 cells in 1 ml) in HBSS, 10 mM HEPES (pH 7.3), and 2 mM $MgCl_2$ and protein-coated plates (1 ml) were preincubated with MAbs (TS2/16, VIM12, TS1/22, LPM19c, and R6.5) for 20 min at room temperature. Subsequently, cells were added to plates containing antibodies and fMLP added to a final concentration of 10^{-7} M and incubated for 2 min and 30 s at room temperature. Unbound cells were removed with ten washes of HBSS, 10 mM HEPES (pH 7.3), 2 mM $MgCl_2$, and 0.1% BSA. Bound cells were determined by counting at least five fields at high power magnification.

Flow Cytometry

Indirect immunofluorescence and flow cytometry were performed as decribed previously (Diamond et al., 1990).

Surface Iodination, Immunoprecipitation, and SDS–PAGE

L-ICAM-1^+ cells either grown in normal or deoxymannojirimycin-supplemented medium, or treated subsequently with neuraminidase as detailed above, were labeled with $Na^{125}I$ by the iodogen method (Fraker and Speck, 1978), subjected to immunoprecipitation (Springer, 1987), and analyzed on 8% vertical slab SDS–PAGE (Laemmli, 1970) using autoradiography (Laskey and Mills, 1977) as previously described.

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