ICAM-1 (CD54): A Counter-Receptor for Mac-1 (CD11b/CD18)

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Abstract. While the leukocyte integrin lymphocyte function-associated antigen (LFA)-1 has been demonstrated to bind intercellular adhesion molecule (ICAM)-1, results with the related Mac-1 molecule have been controversial. We have used multiple cell binding assays, purified Mac-1 and ICAM-1, and cell lines transfected with Mac-1 and ICAM-1 cDNAs to examine the interaction of ICAM-1 with Mac-1. Stimulated human umbilical vein endothelial cells (HUVECs), which express a high surface density of ICAM-1, bind to immunoaffinity-purified Mac-1 adsorbed to artificial substrates in a manner that is inhibited by mAbs to Mac-1 and ICAM-1. Transfected murine L cells or monkey COS cells expressing human ICAM-1 bind to purified Mac-1 in a specific and dose-dependent manner; the attachment to Mac-1 is more temperature sensitive, lower in avidity, and blocked by a different series of ICAM-1 mAbs when compared to LFA-1. In a reciprocal assay, COS cells cotransfected with the α and β chain cDNAs of Mac-1 or LFA-1 attach to immunoaffinity-purified ICAM-1 substrates; this adhesion is blocked by mAbs to ICAM-1 and Mac-1 or LFA-1. Two color fluorescence cell conjugate experiments show that neutrophils stimulated with fMLP bind to HUVEC stimulated with lipopolysaccharide for 24 h in an ICAM-I-, Mac-I-, and LFA-I-dependent fashion. Because cellular and purified Mac-1 interact with cellular and purified ICAM-1, we conclude that ICAM-1 is a counter receptor for Mac-1 and that this receptor pair is responsible, in part, for the adhesion between stimulated neutrophils and stimulated endothelial cells.

A primary event in the immune system's response to infectious agents is the recruitment of circulating neutrophils to the inflammatory site. Adhesion to the endothelium is the prerequisite physical step for extravasation to the peripheral site of injury. Neutrophil localization has been examined on a molecular level to define both the sequence of events that promotes neutrophil exit from the bloodstream and the cognate proteins on the surface of neutrophils and the endothelial cells that coordinate this interaction.

The CD11/CD18 family defines three high molecular weight, cell surface heterodimeric glycoproteins that have a broad distribution on leukocytes (53). This family, known as the leukocyte integrins, consists of lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18; α 175,000 M), Mac-1 (CD11b/CD18; α 160,000 M), and p150,95 (CD11c/CD18; α 150,000 M); the three proteins share a common β chain (95,000 M) that is noncovalently associated with each unique α chain. These proteins are critical for adhesive functions in the immune system (29): mAbs to LFA-1 block leukocyte adhesion to endothelial cells (16, 56) and inhibit conjugate formation that is required for antigen-specific cytotoxic T cell killing (29), T cell proliferation (14), and natural killer cell killing (30); mAbs to Mac-1 block binding of iC3b-coated particles (9), myeloid cell adhesion to endothelial cells (34, 56), neutrophil homotypic aggregation, and chemotaxis (3); mAbs to p150,95 block monocyte adhesion to endothelial cells (3, 25) and CTL conjugate formation with target cells (24). The importance of the leukocyte integrins was confirmed by the discovery of a clinical syndrome, leukocyte adhesion deficiency, that is characterized by a congenital deficiency or absence of the common β chain and presents with diminished pus formation, abnormal wound healing, and grave susceptibility to pyogenic infections (1, 2, 4) as well as abnormalities of adhesion-dependent leukocyte functions in vitro (1, 62). The CD11/CD18 family is related structurally and genetically to the larger integrin family of surface receptors that moderate embryogenesis, adhesion to extracellular substrates, and cell differentiation (23, 28, 29, 51).

Although mAb blocking studies have demonstrated convincingly a role for Mac-1 in neutrophil adhesion to both unstimulated and cytokine-stimulated endothelial cells (33, 34, 36, 56), the identification of the counter-receptor(s) on the endothelial cell surface remains less certain. Two candidate ligands for Mac-1 are intercellular adhesion molecule (ICAM)-1 and ICAM-2, adhesion molecules that were origi-
nally defined as counter-receptors for LFA-1 (41, 54, 58, 59). ICAM-1 is a 90,000–110,000 M, glycoprotein with a low RNA message level and moderate surface expression on unstimulated endothelial cells; lipopolysaccharide (LPS), interleukin (IL)-1, and tumor necrosis factor α strongly up-regulate ICAM-1 mRNA and surface expression with peak expression at ~18–24 h (16, 58). LFA-1-ICAM-1 interaction is responsible partially for lymphocyte (16, 42), monocyte (5, 43, 61), and neutrophil (34, 56) adhesion to endothelial cells. ICAM-2 is a 55,000 M, glycoprotein with high mRNA level (59) and surface expression (A. de Fougerolles and T. Springer, manuscript in preparation) in both unstimulated and stimulated endothelial cells. Unstimulated endothelial cells bind purified LFA-1 in a manner that is inhibited by a combination of ICAM-1 and ICAM-2 mAbs (A. de Fougerolles and T. Springer, manuscript in preparation).

Recent studies (55, 56) by one group suggest that LFA-1 and Mac-1 cooperate in neutrophil adherence to endothelial cells; unstimulated neutrophils bind primarily through surface LFA-1 while formyl methionine-leucine-phenylalanine (fMLP) activated neutrophils attach mostly through Mac-1. Mac-1 and LFA-1 both may be interacting with ICAM-1 since a mAb to ICAM-1 thoroughly blocks CD18-dependent, fMLP-stimulated neutrophil adhesion to unstimulated endothelial cells (55). Because mAbs to LFA-1 and Mac-1 also abrogate neutrophil attachment to planar membranes containing ICAM-1 (56), this group concludes that neutrophil adhesion to endothelial cells is partially ICAM-1-LFA-1- and ICAM-1-Mac-1-dependent. In contrast, a second group reports (34) that when phorbol ester–stimulated neutrophils adhere to unstimulated endothelial cells, while both Mac-1 and LFA-1 are involved, only LFA-1 interacts with ICAM-1. In addition, they find that when macrophages are plated on ICAM-1 substrates, only LFA-1 is down-modulated from the apical portion of the cell surface. This group concludes that Mac-1 does not bind ICAM-1, but rather, it interacts with an uncharacterized endothelial cell surface receptor.

To resolve this paradox, we use immunooaffinity-purified Mac-1 and ICAM-1, and transfected cells expressing ICAM-1, Mac-1, and LFA-1, to show in reciprocal adhesion studies that ICAM-1 is a counter receptor, not only for LFA-1, but also for Mac-1. Cell–cell binding studies demonstrate that neutrophil Mac-1 interacts with ICAM-1 expressed on human umbilical vein endothelial cells (HUVECs). These findings also suggest that Mac-1 interacts with at least one additional cellular ligand, besides ICAM-1, on the surface of endothelial cells.

Materials and Methods

mAbs

The following murine mAbs against human antigens were from ascetics: LPM19c (anti-CD1b, IgG2a, gift of Dr. K. Pehlman, Oxford) (63), W6/32 (anti-HLA A, B, C, IgG2a) (7), TSI/22 (anti-CD11a, IgG1) (52), YFC31.1 (anti-CD18, rat IgG2b, gift of Dr. H. Waldmann, Cambridge) (63), CBRC1/1 and CBRC1/2 (anti-ICAM-2, IgG2a; A. de Fougerolles and T. Springer, manuscript in preparation), and TS6/26 (anti-CD29, IgG1) (52). The following ICAM-1 mAbs were used as purified IgG: CL203 (37) (gift of Dr. S. Ferrone), LB-2 (12) (gift of Dr. E. Clark), and 84H10 (38). RR/1 Fab, (IgG1) (50) were prepared by papain digestion after Protein A affinity chromatography (48). R6.5 (IgG2a) (55) IgG and Fab were a generous gift of Dr. R. Rothlein, Boehringer Ingelheim (Ridgefield, CT). CBRC1/1, CBRC1/2, CBRC1/3, CBRC1/4 (anti-ICAM-1) mAb, S. Stacker and T. Springer, manuscript in preparation), MJ/42 (anti-H-2, rat IgG2a) (57), and X63 (nonbinding antibody, IgG1) were used as tissue culture supernatants. For inhibition assays ascites were used at 1:400 dilutions, purified IgG were used at 20–25 μg/ml, Fab, were used at 20 μg/ml, Fab were used at 50 μg/ml, and tissue culture supernatants were used at 1:2. In inhibition experiments, the difference between our binding mAb and media controls was <30%. Protein A–purified TSI/18 (anti-CD18, IgG1) (52) and LM2/1 (anti-CD11b, IgG) (44) were iodinated and used for site density measurements as described (17). TSI/18 recognizes only the intact αβ heterodimer.

Protein Purification

Mac-1 is purified from leukocyte lysates by a modification of a previous procedure (17, 46). Briefly, to obtain 500 μg of the purified functional heterodimer, 10 g of frozen peripheral blood leukocytes are solubilized in 200 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl2, 1% Triton X-100, 5 mM iodoacetamide, 0.025% NaN3, 1 mM PMSF, 1 mM diisopropylfluorophosphate, 0.2 TIU/ml aprotinin) for 1 h at 4°C while stirring gently. The resultant lysate is centrifuged at 10,000 g for 2 h at 4°C; the supernatant is decanted and then ultracentrifuged at 100,000 g (Ti45; Beckman Instruments, Inc., Palo Alto, CA) for 1 h. The clarified lysate is prepared with human IgG coupled to Sepharose CL-4B, 40 μl of a 1:1 slurry of IgG-Sepharose is added per ml of lysate and rotated overnight at 4°C. The Sepharose is pelleted and the precipitated lysate is then passed over an LM2/1 (anti-CD11b, IgG) immunoaffinity column (bed volume 6 ml, 3 mg/ml of LM2/1) in the presence of using a combination of mAbs to ICAM-1 and LM2/1 to ion exchange–activated Sepharose (40). The column is pre-equilibrated with 10 bed volumes of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl2, 0.1% Triton X-100, and then 10 bed volumes of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl2, 0.1% n-octyl-2-p-glucopyranoside. Mac-1 is eluted with 5 bed volumes of 50 mM triethylamine (TEA), pH 10.0, 300 mM NaCl, 2 mM MgCl2, 1% n-octyl-β-D-glucopyranoside into tubes with neutralizing buffer (10% by volume 1 M Tris-HCl, pH 7.4). The peak fractions are pooled, aliquoted, and stored at ~80°C for 3–6 mo without loss of activity.

LFA-1 is purified by immunooaffinity chromatography as described previously (17) except peripheral blood leukocyte lysates were substituted. ICAM-1 is purified by immunooaffinity chromatography as described previously (41).

SDS-PAGE

Protein samples are run on reducing (5% β-mercaptoethanol) SDS 7–10% polyacrylamide gels (31) and silver stained (47).

Tissue Culture, Transfection, and Cell Preparation

COS cells are grown on 10- or 15-cm tissue culture–treated plates (Falcon Labware, Oxnard, CA) in RPMI 1640 with 10% FCS, 5 mM glutamine, and 50 μg/ml gentamycin. Cells are transfected at ~50–60% confluency in 10-cm plates with 4–6 μg of CaCl2-purified (39) ICAM-1 cDNA (60) or cotransfected with 6 μg Mac-1 α or LFA-α plus 6 μg β-CDNA (21, 32) by the DEAE-Dextran method for 4 h at 37°C (6, 26). After 72 h cells are eluted from the tissue culture plates before functional assays with PBS, 5 mM EDTA at 37°C for 5 min.

ICAM-1+ L cell stables are generated as described (1). Briefly, murine L cells (thymidine kinase [tk]-) are seeded onto 6-cm tissue culture dishes and grown to 10–20% confluence over 2 d. ICAM-1 cDNA in the plasmid CD8 (60) (10 μg) and plasmid containing a thymidine kinase selection marker (100 ng) are mixed with 0.2 ml CaCl2 and 0.2 ml of 50 mM N2-[N-bis-(2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na2HPO4, pH 6.95, and incubated at room temperature for 10–20 min. The calcium phosphate-DNA solution (0.4 ml) is added dropwise to the plated cells and incubated at 35°C in a 3% CO2 incubator. 24 h later, the cells are washed with regular tissue culture medium and grown at 37°C in a 5% CO2 incubator until day 3 when HAT (100 μM hypoxanthine, 400 mM aminopterin, 16 μM thymidine) selection is initiated. Cells with high ICAM-1 expression are identified by flow cytometry after single colony picks with a cloning cylinder. ICAM-1+ L cells are maintained in a selection media that consists of DME with 10% heat inactivated FCS, 5 mM glutamine, 50 μg/ml gentamycin, and supplemented with HAT. Untransformed L cells are maintained in DME without HAT.

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HUVECs are cultured to low passage number (2–5) on tissue culture plates precoated with fibronectin (50 μg/ml) in M199 media supplemented with 20% heat-inactivated FCS, 100 μg/ml heparin, 100 μg/ml endothelial cell growth factor, 10 mM HEPES, pH 7.3, 5 mM glutamine, and 50 μg/ml gentamicin (16). LPS treatment is performed by adding 1 μg/ml of E. coli lipopolysaccharide (endotoxin) 24 h before harvesting of cells. Cytokine stimulation is performed by addition of 5 μg/ml of rIL-1β (Genzyme, Cambridge, MA). To elute HUVEC for the two color fluorescence studies, cells are treated with PBS, 5 mM EDTA at 37°C for 5–10 min.

Neutrophils are isolated from the whole blood of healthy volunteers by dextran sedimentation, Ficoll gradient centrifugation, and hypotonic lysis (19). Before experimental manipulation, neutrophils are stored at room temperature in HBSS, 10 mM HEPES, pH 7.3, 0.5% human serum albumin (HBSS/HSA).

**Cell Conjugation Experiments**

The dual fluorescence cell conjugate assay is performed by a modification of a published protocol (35). Briefly, 106 neutrophils are washed twice in HBSS/HSA, resuspended in 10 ml of a filtered (0.2 μm) solution of 40 μg/ml hydroethidine (HE; Polysciences, Inc., Warrington, PA) in HBSS/HSA, and incubated for 35 min at 37°C while rocking gently. HUVECs (2 × 104) are eluted from T-150-cm² flasks with PBS, 5 mM EDTA, washed thrice in RPMI 1640, 20 mM HEPES pH 7.3, 5% heat-inactivated FCS, resuspended in 10 ml of a 200-μM solution of sulfofluorescein diacetate (SFDA, Molecular Probes Eugene, OR) and incubated also for 30 min at 37°C. Subsequently, cells are washed four times in RPMI 1640, 10 mM HEPES, pH 7.3, 0.5% HSA, 5% heat-inactivated human serum and resuspended as follows: neutrophils, 1 × 105 cells/ml; and HUVECs, 2 × 106 cells/ml. The assay is performed in the presence of heat-inactivated human serum (5%) to prevent Fc receptor cross-linking with the mAbs. The appropriate antibodies are added to both cell types and preincubated for 25 min at room temperature. Neutrophils (150 μl) and HUVECs (250 μl) are added to a 24-well plate (Falcon Labware) and the following experimental protocol is used (all incubations are at 37°C while shaking at 75 rpm on a gyratory shaker (New Brunswick Scientific Co. Inc., Edison, NJ)): 5 min preincubation, 1 × 10^{-13} M FMLP addition, 10 min incubation. Conjugates are recovered after pipetting the solution thrice (small aggregates may settle) and quantitated as the percentage of HUVEC found in two color conjugates by flow cytometry with either an EPICS V ( Coulter Electronics Inc., Hialeah, FL) or a FACScan (Becton Dickinson and Company, Paramus, NJ).

**Cell Binding to Purified Proteins**

Purified Mac-1, LFA-1, and ICAM-1 are diluted from 1/8-1/120 depending on protein content (judged by SDS-PAGE and RIA; 17) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl2, with an ocycl glucoside detergent concentration ranging from 0.1% to 0.01% and plated over the entire surface of 35-mm Petri dishes, as a 40-μl demarcated spot on 60-mm Petri dishes, blocked subsequently by three washes with and subsequent incubation in PBS, 0.5% HSA, 5% heat-inactivated human serum and resuspended as follows: neutrophils, 1 × 105 cells/ml; and HUVECs, 2 × 106 cells/ml. The assay is performed in the presence of heat-inactivated human serum (5%) to prevent Fc receptor cross-linking with the mAbs. The appropriate antibodies are added to both cell types and preincubated for 25 min at room temperature. Neutrophils (150 μl) and HUVECs (250 μl) are added to a 24-well plate (Falcon Labware) and the following experimental protocol is used (all incubations are at 37°C while shaking at 75 rpm on a gyratory shaker (New Brunswick Scientific Co. Inc., Edison, NJ)): 5 min preincubation, 1 × 10^{-13} M FMLP addition, 10 min incubation. Conjugates are recovered after pipetting the solution thrice (small aggregates may settle) and quantitated as the percentage of HUVEC found in two color conjugates by flow cytometry with either an EPICS V (Coulter Electronics Inc., Hialeah, FL) or a FACScan (Becton Dickinson and Company, Paramus, NJ).

For the 35- and 60-mm Petri dish assays, transfected COS cells or L cells are eluted from tissue culture plates with PBS, 5 mM EDTA or RPMI, 10 mM EDTA. COS cells are washed twice in HBSS/HSA, resuspended in HBSS/HSA to 8 × 105 cells/ml, and labeled with 50 μCi/ml 32P for 1 h. Excess label is washed out and the COS cells are resuspended to 8 × 105/ml in PBS, 1.5 mM MgCl2, 0.5 mM CaCl2, 0.2% glucose, 0.5% HSA or 20 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1.5 mM MgCl2, 0.25 mM MnCl2, 0.2% glucose, 0.5% HSA. Cells are preincubated with appropriate antibodies for 30 min at room temperature, added to Petri dishes, and incubated for 50 min at 37°C. Unbound COS or L cells are removed by three successive washes with 1 ml of binding buffer that is added and then swirled gently across the plate. With COS cell experiments, after washing, the plates are inspected visually for bound cells, assessed qualitatively, and the adherent population is eluted with 1 ml PBS, 25 mM EDTA (15 min at 37°C) and quantitated by γ emission. For L cell experiments, after washing, bound cells are quantitated by visually scoring the number of cells in four to five high power light microscope fields for each experimental point. This number is divided into the input number of cells per field to obtain the percentage of cells binding.

**Flow Cytometry**

0.5–1.0 × 103 cells in 50 μl in HBSS/HSA are added to V-bottom microtiter plates containing 50 μl of antibody supernatants or 50 μl of a 1/20 dilution of antibody ascites. The plate is sealed with tape and shaken on a Dytatech plate shaker for 45 min at 4°C. Cells are pelleted (2,500 rpm, 2 min, 4°C) and washed thrice with 50 μl with HBSS/HSA, and resuspended in 100 μl of a 1/20 dilution of purified FITC-conjugated goat anti-mouse IgG light and heavy chain antiserum (Zymed, CA) in HBSS/HSA. The plate is resealed, and shaken for 30 min at 4°C. Cells are washed twice in HBSS, once in PBS 5% FCS, and resuspended in 200 μl of PBS, 2.5% FCS, 1% paraformaldehyde. The samples are analyzed on an EPICS V flow cytometer.

**Reagents**

All chemical reagents were of highest grade and purchased from Sigma Chemical Co. (St. Louis, MO) except the following: rIL-1β (Genzyme), HE (Polysciences, Inc.), SFDA (Molecular Probes), Na235CrO4 and Na231P (Amersham Corp., Arlington Heights, IL), Dextran-500 (Pharmacia, Sweden), FCS (Flow Laboratories, Inc., McLean, VA or JR Scientific, Woodland, CA), endothelial cell growth factor (Chemicon International, Los Angeles, CA), HSA (Alpha Corporation, Los Angeles, CA), FITC goat antimouse Ab (Zymed, San Francisco, CA), Protein A (Pharmacia), Iodogen (Pierce Chemical Co., Rockford, IL), Triton X-100 (DuPont, Wilmington, DE), CCl3 (BRL, Bethesda, MD), hypoxanthine (Gibco Laboratories, Grand Island, NY).

**Results**

**Adhesion of Mac-1 and LFA-1 Transfectants to Purified ICAM-1**

Previous studies (56) which suggest that Mac-1 on neutrophils binds to ICAM-1 are complicated by the presence of multiple adhesion receptors on neutrophils, e.g., neutrophils express significant quantities of both LFA-1 and Mac-1. We utilized a purified protein-transfectant cell binding assay where each of the ligand pairs could be examined independently for adhesion (32). COS cells cotransfected with the cDNAs for the common β subunit and either the Mac-1 or LFA-1 α subunit were assayed for binding to purified ICAM-1 coated on a plastic substrate. Surface expression averaged 30% for Mac-1 and 40% for LFA-1 as determined by immunofluorescence flow cytometry (Fig. 1 B). Transfected COS cells expressing Mac-1 and LFA-1 bind purified ICAM-1 (Fig. 1 A), while cells transfected with the β chain alone do not bind (data not shown; 32). Adhesion is specific as mAbs to Mac-1 or LFA-1 α subunit were not inhibitory. The adhesion of Mac-1 transfectants is weaker (44% binding compared to 75% for LFA-1 transfectants) and...
A

Control mAb
LFA-1 or Mac-1 mAb
ICAM-1 mAb

% Positive Cells Binding

COS Cell Transfectant

LFA-1
Mac-1

B

Mac-1

LFA-1

CELL NUMBER

FLUORESCENCE INTENSITY

FLUORESCENCE INTENSITY

Figure 1. Adhesion of LFA-1 and Mac-1 COS cell transfectants to purified ICAM-1. COS cells cotransfected with cDNAs of LFA-1 or Mac-1 α chains and the common β chain are labeled with 51Cr and then both the cells and the ICAM-1-coated substrates are pretreated with the following mAbs: control (TS2/16), LFA-1 (TS1/22), Mac-1 (LPM19c), and ICAM-1 (a mixture of R6.5 and RR1/1). Subsequently, the cells are allowed to settle on ICAM-1-coated 35-mm Petri dishes for 1 h at 37°C. Plates are washed four times by pipetting and bound cells are removed and quantitated for gamma emission. The data is normalized for surface expression by dividing the percentage of bound cells by the percentage of positive cells. Without normalization COS cells transfected with the β chain alone, Mac-1, and LFA-1 were positive for expression in 7, 30, and 40% of the cells, respectively. The data is the average of three separate experiments and the error bars represent the standard errors of the mean. (B) Surface expression of Mac-1 and LFA-1 on COS cell transient transfectants. (top) COS cells transfected with vector alone (light line) or Mac-1 α and β cDNA (bold line) were incubated with Mac-1 α mAb (LM2/1), FITC goat-anti-mouse IgG, and subjected to flow cytometry. (bottom) COS cells transfected with vector alone (light line) or LFA-1 α and β cDNA (bold line) were stained with TS1/22, FITC goat-anti-mouse IgG and subjected to flow cytometry. The profiles are from a single representative experiment. The data is expressed as the number of cells (ordinate) versus the log fluorescence intensity (abscissa).

Figure 2. SDS-PAGE of purified Mac-1. Purified Mac-1 (10 μl of a 2-ml fraction from an LM2/1 mAb affinity column) is subjected to reduction, SDS 7.5% PAGE, and silver staining. Molecular weight standards are marked to the left.

occurs at 37°C but not at 23°C, whereas COS cells transfected with LFA-1 bind to ICAM-1 at 23°C (data not shown).

Immunofluorescence Purification of Functional Mac-1

To gain additional evidence for an interaction between Mac-1 and ICAM-1, we wanted to test the reciprocal assay, whether cells bearing ICAM-1 bound to purified Mac-1. To purify Mac-1, we modified a procedure used to obtain LFA-1 in a form that was functional and in which the α and β chains remained noncovalently associated (17). Peripheral blood leukocytes were lysed in Triton X-100 in the presence of Mg2+, and the Mac-1 α/β complex was bound to an LM2/1 mAb affinity column. Triton X-100 was exchanged with the dialyzable detergent n-octyl-β-D-glucopyranoside. Elution conditions of varied salt, divalent cation concentration, and pH were tested and optimized for yield and the ability to reprecipitate the heterodimer. The optimum buffer contained 50 mM triethylamine, pH 10.0, 300 mM NaCl, and 2 mM Mg2+. Mac-1 obtained by this procedure was substantially pure, and migrated on reducing SDS-PAGE as two bands with Mr of 160,000 and 95,000 (Fig. 2), consistent with our previous observations (46). Immunoprecipitation with the
Adhesion of Cells Transfected with ICAM-1 cDNA to Purified Mac-1 and LFA-1

To test whether cells bearing ICAM-1 interact with Mac-1, COS cells were transfected with the ICAM-1 cDNA and allowed to settle on Mac-1-coated substrates. Surface expression of ICAM-1 on transfected COS cells averaged 50-60% (Fig. 3 B), while vector-transfected COS cells showed no expression (Fig. 3 A). COS cells expressing ICAM-1 adhere to purified Mac-1 (Fig. 4 A), whereas cells transfected with vector alone do not adhere (Fig. 4 D). The binding is specific as it is blocked completely with mAbs to Mac-1 α (LPM19c) or ICAM-1 (a mixture of R6.5 and RR1/1) (Fig. 4, B and C).

To assess more quantitatively the strength of the interaction between Mac-1 and ICAM-1, we performed a dose response analysis. Because of the variation between experiments in the percent of COS cells expressing ICAM-1 and the heterogeneity in the level of ICAM-1 expression between cells (Fig. 3 B), stable transfecants expressing human ICAM-1 in murine l. cells were generated as described in Materials and Methods (11). Colonies were selected in which 100% of the l. cells expressed high amounts of human ICAM-1 (Fig. 3 D).

Titration experiments were performed in which ICAM-1+ l. cells were allowed to bind to substrates coated with a wide range of Mac-1 site densities (Fig. 5). l. cells expressing ICAM-1 adhered to Mac-1 in a dose-dependent fashion with a threshold of ~250 sites/μm² before significant adhesion is seen. The adhesion is specific as plates pretreated with mAb to Mac-1 α (LPM19c), cells pretreated with mAbs to ICAM-1 (a mixture of RR1/1 and R6.5), and untransfected l. cells did not attach.

Parallel experiments were performed on LFA-1- and Mac-1-coated substrates to determine the difference in relative avidity for ICAM-1 using a lower stringency wash protocol (Fig. 6). The amount of Mac-1 and LFA-1 bound to substrate was determined by RIA with a mAb to the common β subunit. ICAM-1+ l. cells adhered to LFA-1 substrates with a slightly higher avidity as the binding isotherm for purified Mac-1 is shifted to a higher site density. The difference in avidity is consistent with the observation that when COS cells express ICAM-1 at low levels, they adhere to LFA-1 but not Mac-1 substrates (data not shown). Binding of ICAM-1+ l. cells to Mac-1 is more temperature sensitive than binding to LFA-1 (Fig. 7). Despite equivalent site densities of LFA-1 and Mac-1 on the substrate, cells adhere strongly to LFA-1 but weakly to Mac-1 at room temperature, while there is little or no difference in the quantitative adhesion at 37°C.

Adhesion of HUVEC to Purified Mac-1

To confirm that stimulated HUVECs adhere to Mac-1 in an ICAM-1-dependent fashion, we tested their ability to bind Mac-1 substrates. HUVECs, when stimulated for 18-24 h with IL-1β or LPS, bind purified Mac-1 under high stringency wash conditions (Fig. 8). Adhesion is specific and primarily ICAM-1 dependent since it is inhibited by mAbs to ICAM-1 (82% for LPS, 66% for IL-1β) and Mac-1 α (>95% in both cases) but not by control mAb. Unstimulated HUVECs, which express lower amounts of ICAM-1 (18), do not attach significantly to purified Mac-1 at this stringency of wash. However, if the assay is performed under gentler wash conditions, unstimulated HUVECs adhere to purified Mac-1 in a dose-dependent manner (Fig. 9). There is complete inhibition with mAb to Mac-1 but only a small inhibition of unstimulated HUVEC adhesion with mAb to ICAM-1, suggesting the presence of an additional ligand(s) for Mac-1 on HUVEC. mAbs to ICAM-2 that block its interaction with LFA-1, alone or in combination with ICAM-1 mAbs, have little effect on HUVEC adhesion to Mac-1 (Fig. 9 and data not shown).

Difference among ICAM-1 mAbs in Blocking Adhesion to Mac-1 and LFA-1

Our reciprocal adhesion assays show that both Mac-1 and LFA-1 bind to ICAM-1. This result prompts the question of which epitopes on ICAM-1 are involved in interactions with LFA-1 and Mac-1. Studies above were performed with a mixture of RR1/1 and R6.5 mAb. We examined ICAM-1+ l. cell adhesion to both Mac-1 and LFA-1 in the presence of a panel of individual ICAM-1 mAb. In addition to previously characterized ICAM-1 mAbs (RR1/1, R6.5, LB-2, CL203, 84H10), four new ICAM-1 mAbs were generated against HUVEC stimulated with IL-1β (S. Stacke and T. Springer, manuscript in preparation). There is a striking difference among mAbs to ICAM-1 in their ability to block adhesion of ICAM-1+ l. cells to Mac-1 and LFA-1 (Fig. 10). As previously reported (34, 38, 41, 50, 55), RR1/1, R6.5, LB-2, and 84H10 all inhibit LFA-1–ICAM-1 interaction to varying degrees; in addition, one of our new mAbs, CBRIIC1/1, inhibits LFA-1–ICAM-1 binding. Only R6.5 strongly inhibits ICAM-1 adhesion to Mac-1 and LFA-1, whereas CBRIIC1/1 blocks roughly 35-40% of Mac-1–ICAM-1 binding but does not inhibit LFA-1–ICAM-1 adhesion. RR1/1 does not appear to block ICAM-1–Mac-1 interactions.

Neutrophil-HUVEC Conjugate Formation

While the reciprocal assays of cell binding to purified protein strongly suggest that Mac-1 interacts with ICAM-1, we wanted to confirm that this interaction is physiologic, i.e., that it could occur between cells that both normally express Mac-1 and ICAM-1 and adhere to each other in vivo. We used fMLP-activated neutrophils which strongly express Mac-1 (45) and 24-h LPS-treated HUVECs which express high levels of ICAM-1 (16) but low amounts of ELAM-1 (10, 49). To characterize adhesion quantitatively, a two color fluorescence conjugate assay was developed in which neutrophils and HUVECs are labeled with red and green fluorescent compounds, respectively. This system differs from the binding of neutrophils to HUVEC monolayers under static conditions and removal of nonadherent cells by 1 g sedimentation, an assay that was used in a previous report (56) that described ICAM-1 as a ligand for Mac-1. In our system, neutrophil–endothelial cell heterotypic conjugates form in suspension while the cells are gently agitated.

Chemotactic stimulation of neutrophils resulted in a...
significant increase in conjugation with HUVEC over the baseline conjugate formation of unstimulated neutrophils (Table I). There was a three- to fourfold increase in the number of HUVEC's conjugated with neutrophils after stimulation. When TS1/22, an anti-LFA-1 α mAb was added, there was a slight but not statistically significant inhibition of conjugate formation. If an mAb to the Mac-1 α subunit was added, there was a significant inhibition (73%) of stimulated cell conjugates. A combination of anti-LFA-1 and anti-Mac-1 mAbs or an antibody against the common β chain showed the greatest inhibition (88-98%). A mixture of R6.5 Fab and RR1/1 Fab2 fragments decreased conjugate formation by 48%. Since this inhibition is greater than that of LFA-1 antibodies alone, the results suggest that both LFA-1 and Mac-1 interact with ICAM-1. Because the CD18-dependent adhesion cannot be inhibited solely with mAbs to ICAM-1, LFA-1 and/or Mac-1 may be interacting with additional ligands. For LFA-1, ICAM-2 is a strong possibility, yet it does not appear that Mac-1 binds to ICAM-2 (Fig. 9) and, thus, other endothelial cell surface counter receptors may be involved.

Discussion

We prove that Mac-1, like LFA-1 (41), is a counter-receptor for ICAM-1 by demonstrating that purified Mac-1 mediates cell adhesion dependent on ICAM-1, and reciprocally that purified ICAM-1 mediates cell adhesion dependent on Mac-1. Cells transfected with specific cDNAs of putative counter receptors were used to eliminate complications associated with additional receptor-ligand interactions. We show that ICAM-1+ transfectants and Mac-1+ transfectants bind purified Mac-1 and ICAM-1, respectively. To rule out the possibility that this result is an effect of the experimental system, we show a Mac-1, ICAM-1-dependent adhesion between stimulated endothelial cells and purified Mac-1. We also demonstrate a Mac-1–ICAM-1 interaction in a cell–cell context as stimulated Mac-1+ peripheral blood neutrophils form cell conjugates with ICAM-1+ HUVEC in a manner that is inhibited with Mac-1 and ICAM-1 mAbs.

The binding of ICAM-1 to both Mac-1 and LFA-1 shows that an immunoglobulin-like molecule can adhere to more
than one integrin. However, the interaction between the two sister leukocyte integrins and ICAM-1 is not identical. mAb blocking data (Fig. 10) suggests differences with respect to the site of interaction on ICAM-1 for LFA-1 and Mac-1. Only one mAb (R6.5) is able to significantly inhibit binding of ICAM-1-transfected L cells to substrates coated with Mac-1 and LFA-1, whereas others (RR1/1, LB-2, 84H10, CBRIC1/4) that block LFA-1-ICAM-1 function (34, 38, 41, 50, 55) do not decrease binding to Mac-1. Furthermore, one new mAb (CBRIC1/I) partially blocks Mac-I-ICAM-1 binding but has little or no effect on LFA-1-ICAM-1 adhesion. Additional evi-
Table 1. Conjugate Formation between fMLP-stimulated Neutrophils and 24-h LPS-cultured HUVEC

<table>
<thead>
<tr>
<th>mAb</th>
<th>fMLP in conjugates</th>
<th>HUVEC in conjugates</th>
<th>n</th>
<th>Inhibition of adherence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>CD18</td>
<td>-</td>
<td>12 ± 3</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>42 ± 6</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>41 ± 5</td>
<td>7</td>
<td>0.3 NS</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>+</td>
<td>44 ± 6</td>
<td>5</td>
<td>-0.6 NS</td>
</tr>
<tr>
<td>LFA-1</td>
<td>+</td>
<td>38 ± 6</td>
<td>5</td>
<td>14.4 NS</td>
</tr>
<tr>
<td>Mac-1</td>
<td>+</td>
<td>21 ± 6</td>
<td>7</td>
<td>73.0 (p &lt; 0.0005)</td>
</tr>
<tr>
<td>CD18</td>
<td>+</td>
<td>16 ± 5</td>
<td>5</td>
<td>88.0 (p &lt; 0.0005)</td>
</tr>
<tr>
<td>Mac-1 + LFA-1</td>
<td>+</td>
<td>13 ± 5</td>
<td>8</td>
<td>98.3 (p &lt; 0.0005)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>+</td>
<td>27 ± 3</td>
<td>8</td>
<td>48.3 (p &lt; 0.0005)</td>
</tr>
</tbody>
</table>

Neutrophils (1.5 x 10⁶) stained red with HE and HUVECs (6 x 10⁵) stained green with SFDA are resuspended and pretreated separately with the following mAbs: Control (W6/32), ICAM-2 (C8B12/C1), LFA-1 (TS1/22), Mac-1 (LPMI90), CD18 (YFC51.1), ICAM-1 (a mixture of R6.5 Fab and RR1/Fab', fragments). The cells are mixed in 24-well plates and preincubated at 37°C. fMLP (10⁻⁹ M) is added and the cells are agitated at 75 rpm for 10 min at 37°C. Resultant suspensions are analyzed immediately by flow cytometry. The data is expressed as the percentage of HUVEC found in heterotypic conjugates with neutrophils, and the inhibition of conjugation of stimulated neutrophils is expressed relative to the baseline level of conjugates without stimulation with fMLP. Significance values were determined using a pooled t test. NS means that values were not statistically different (p < 0.05) from control values. Values after the ± sign show standard deviations.

The binding of Mac-1 to ICAM-1 demonstrates directly that Mac-1 interacts with an endothelial cell surface counter receptor. Our results agree with previous studies that show neutrophil adhesion to endothelial cells is inhibited by mAb to Mac-1 (3), and that Mac-1- and LFA-1-dependent adhesion of fMLP-stimulated neutrophils is additive to unstimulated endothelial cells (55) or ICAM-1 containing planar membranes (56). ICAM-1 is blocked completely by a mAb to ICAM-1 (R6.5). In contrast, ICAM-1 has been reported not to be a ligand for Mac-1 (34). In that study, neutrophils stimulated with phorbol esters adhere to unstimulated endothelial cells in a manner that is ICAM-1-, Mac-1-, and ICAM-1-dependent, results that agree with our findings. However, this group concluded that only LFA-1 interacts with ICAM-1 because the inhibition with LFA-1 and ICAM-1 mAbs (LB-2, 84H10) is not additive whereas the inhibition with Mac-1 and ICAM-1 mAbs is additive. The differences in the previous reports may be explained partially by the disparity in mAb selection; here we show that R6.5 mAb blocks both Mac-1 and LFA-1-ICAM-1 interactions, whereas LB2 and 84H10 mAbs only inhibit LFA-1-ICAM-1-binding (Fig. 10). Our results do not explain, however, a previous observation that when macrophages are plated on an ICAM-1 substrate, LFA-1 but not Mac-1 is down-modulated from the apical surface (34). Our mAb blocking data is consistent with mutagenesis studies that map mAb epitopes to distinct regions of the ICAM-1 molecule (60). RR1 and LB-2 map to the first NH₂-terminal immunoglobulin domain whereas R6.5 maps to the second domain. Our data is also consistent with in vivo experiments (8) that show a reduction in the granulocyte infiltration into rabbit lungs inflamed with phorbol esters after pretreatment with mAbs to CD18 (R3.3) or ICAM-1 (R6.5), but not with mAb to LFA-1 (R3.1). These findings suggest that stimulated neutrophils may utilize a Mac-1-ICAM-1-dependent pathway of adhesion to mediate attachment in vivo to inflamed endothelium.

Experiments presented here are consistent with the possibility of counter-receptors for Mac-1 distinct from ICAM-1 on the surface of unstimulated (34) and stimulated endothelial cells. In our assays, endothelial cell ICAM-1 cannot by itself account for all of the Mac-1-dependent adhesion of neutrophils. Adhesion of stimulated HUVEC to purified Mac-1 under high stringency wash conditions is only partially (68-82%) blocked by mAb to ICAM-1 (Fig. 8). Furthermore, there is little adhesion of unstimulated HUVEC to Mac-1 at this stringency, but when washed at a lower stringency (Fig. 9), there is significant non-ICAM-1-dependent...
adhesion to purified Mac-1. This result conflicts with a report that showed that FMLP-stimulated neutrophil adhesion to unstimulated endothelial cells was blocked 84% by a mAb to ICAM-1 (55). We do not understand the discrepancy, but it may be explained by differences in tissue culture conditions of untreated HUVEC which may induce a second ligand for Mac-1. At present, we do not understand fully the role of ICAM-2 in neutrophil adhesion to unstimulated or stimulated endothelial cells, although ICAM-1 and ICAM-2 inhibit the binding of purified Mac-1 to ICAM-1 (60). Similar experiments must now be done to map the binding site for LFA-1 is localized in the most NH2-terminal of the five Ig-like domains of ICAM-1 (60). Similar experiments must now be done to map the binding site for LFA-1 is localized in the most NH2-terminal of the five Ig-like domains of ICAM-1 (60). Simple experiments must now be done to map the region of ICAM-1 that contacts Mac-1.

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