Heparin Is an Adhesive Ligand for the Leukocyte Integrin Mac-1 (CD11b/CD18)

Michael S. Diamond,* Ronen Alon,* Charles A. Parkos,‡ Mark T. Quinn,§ and Timothy A. Springer*

*Center for Blood Research, Harvard Medical School, Boston, Massachusetts 02115; ‡Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115; and §Department of Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717

Abstract. Previous studies have demonstrated that the leukocyte integrin Mac-1 adheres to several cell surface and soluble ligands including intercellular adhesion molecule-1, fibrinogen, iC3b, and factor X. However, experiments with Mac-1–expressing transfectants, purified Mac-1, and mAbs to Mac-1 indicate the existence of additional ligands. In this paper, we demonstrate a direct interaction between Mac-1 and heparan sulfate glycans. Heparin affinity resins immunoprecipitate Mac-1, and neutrophils and transfectant cells that express Mac-1 bind to heparin and heparan sulfate, but not to other sulfated glycosaminoglycans. Inhibition studies with mAbs and chemically modified forms of heparin suggest the I domain as a recognition site on Mac-1 for heparin, and suggest that either N- or O-sulfation is sufficient for heparin to bind efficiently to Mac-1. Under conditions of continuous flow in which heparins and E-selectin are cosubstrates, neutrophils tether to E-selectin and form firm adhesions through a Mac-1–heparin interaction.

In many immune responses, neutrophils are among the first cells to exit from the circulatory system and traffic to an inflammatory site. For this to occur, neutrophils must attach to endothelial cells, change shape, diapedese, and migrate. Over the past few years, several families of surface receptors on the neutrophil and endothelial cell surface that facilitate movement from the bloodstream have been identified. Selectins form transient attachments that allow neutrophil rolling on inflamed endothelium (11, 66). G-protein–coupled receptors bind soluble inflammatory molecules (25, 66), and signal integrins to strengthen attachment to endothelial cells, and initiate movement to the underlying tissue (11, 66). The details of neutrophil migration to the target site are less clear. A chemotactic gradient of soluble or tethered inflammatory peptides or lipids augments the adhesiveness of surface receptors and reorganizes the cytoskeleton. To move directionally, neutrophils adhere transiently to molecules in the extracellular environment; although the matrix contains an abundant number of adhesive proteins, proteoglycans, and carbohydrates, it is unknown which participate in chemotaxis.

The leukocyte integrins comprise a subfamily of related cell-surface glycoproteins that coordinate adhesive functions including leukocyte migration (12, 65). The members of this family include lymphocyte function–associated antigen-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18). They share a common β subunit that is noncovalently associated with unique, but closely related, α subunits (32). The α subunits share two features in their extracellular region, a 200–amino acid inserted I domain and three tandem EF handlike putative divalent cation-binding repeats (32).

Mac-1 is expressed primarily on myeloid cells. Experiments with blocking mAbs and with neutrophils from patients with a genetic deficiency of leukocyte integrins demonstrate a role for Mac-1 in an array of adhesive interactions that include myeloid cell adhesion to, and transmigration across, endothelium or epithelium, neutrophil homotypic adhesion and chemotaxis, myeloid cell adhesion to serum-coated substrates, and the binding and phagocytosis of opsonized particles (4, 8, 52, 54, 63, 72). Mac-1 sustains these interactions by binding to several cell surface and soluble ligands including intercellular adhesion molecule (ICAM)-1[1], fibrinogen, iC3b, and factor X (1, 2, 8, 19, 63, 72, 73). Recent experiments with mAbs, integrin chimeras, and soluble protein fragments define the 200–amino acid I domain on Mac-1 as a recognition site for at least three of its ligands (21, 70, 75).

Although these four ligands subserve many Mac-1–dependent adhesive functions, they do not account for the entirety of its adhesive interactions. For example, a recep-

Address all correspondence to Timothy A. Springer, Center for Blood Research, 200 Longwood Avenue, Boston, MA 02115. Tel.: (617) 278-3200. Fax: (617) 278-3232.

M. S. Diamond's present address is Department of Medicine, Box 0120, 505 Parnassus, University of California, San Francisco, CA 94143.

1. *Abbreviations used in this paper*: fMLP, formylmethionylleucylphenylalanine; ICAM, intercellular adhesion molecule; LAD, leukocyte adhesion deficiency.

tor on resting endothelial cells and epithelial cells that lack ICAM-1 has been suggested, but not yet identified (19, 39, 52). A counterreceptor on neutrophils that participates in Mac-1–dependent homotypic adhesion remains postulated, but uncharacterized (4, 54). Finally, the chemotaxis of neutrophils requires Mac-1, but no extracellular matrix ligand has been documented. Several groups, including our own, have attempted unsuccessfully to raise blocking mAbs or to identify novel cDNAs that confer adhesion to purified or cellular Mac-1. Moreover, a recent study suggests that the adhesion of bone marrow cells to stromal fibroblasts may occur, in part through a heparan sulfate interaction with Mac-1 (13). In this report, we document heparin and heparan sulfate as glycosaminoglycan, adhesive ligands for the leukocyte integrin Mac-1. Using data from static and shear flow adhesion assays we suggest novel adhesive pathways through which Mac-1–heparin interactions may mediate neutrophil trafficking from the circulatory system to an inflammatory site.

Materials and Methods

Glycosaminoglycans

High molecular weight (mol wt = 13,000–15,000) porcine intestine mucosal heparin and low molecular weight (mol wt = 5,000) bovine intestine mucosal heparin were purchased from Calbiochem Corp. (La Jolla, CA). Chemically modified forms of heparin were obtained from Seikagaku Koygo Co. (Tokyo, Japan). Heparan sulfate (mol wt = 7,500) from bovine intestinal mucosa, keratan sulfate from bovine cornea, and all forms of shark cartilage chondroitin sulfate (A, B, C) were purchased from Sigma Chemical Co. (St. Louis, MO).

mAbs

The following mAbs were used from ascites: TS1/22 (anti-CD11a) (60), Mn41 (anti-CD11b) (22), LPM19c (anti-CD11b) (69), 44a (anti-CD11b) (5), OKM9 (anti-CD11b) (72), TMG-65 (anti-CD11b) (69), 14B6E.2 (anti-CD11b) (69), the CBRM1 series (anti-CD11b: M1/1, M1/2, M1/4, M1/9, M1/10, M1/13, M1/16, M1/17, M1/18, M1/20, M1/21, M1/22, M1/23, M1/24, M1/25, M1/26, M1/27, M1/28, M1/29, M1/30, M1/31, M1/32, M1/33, M1/34) (21), CBRp150,95/4G1 (anti-CD11c) (67), and TS1/18 (anti-CD18) (60). The following mAbs were used as purified IgG: W6/32 (anti-HLA A, B, C) (7), OKM1 (anti-CD11b) (72), LM2/1 (anti-CD11b) (45), R15.7 (anti-CD18, a gift of Dr. R. Rothlein, Boehringer Ingelheim Ltd., Ridgefield, CT) (23), and DREG-56 (anti–L-selectin, a gift of Dr. T. Kishimoto, Boehringer Ingelheim Ltd.) (33).

Tissue Culture, Transfection, and Cell Preparation

Peripheral blood neutrophils from healthy volunteers or patients with leukocyte adhesion deficiency (LAD) were isolated from whole venous blood by dextran sedimentation, Ficoll gradient centrifugation, and hypotonic lysis (46). Before experimentation, cells were maintained at room temperature in HBSS, 10 mM Hepes, pH 7.3, and 1 mM $MgCl_2$ in polypropylene tubes (2097; Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ). In some experiments, neutrophils were maintained in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

CHO cell stable transfectants that express Mac-1, p150,95 or ICAM-1 have been described (21). These cells were maintained in α-MEM, 10% dialyzed FCS, 16 μM thymidine, 0.05 μM methotrexate, 2 mM glutamine, and 50 μg/ml gentamicin.

Protein Purification and SDS Gel Electrophoresis

As described (51), the procedure for isolating cytochrome b559 and Mac-1 includes solubilizing stimulated granulocyte membranes in 2% *n*-octyl-β-D-glucopyranoside followed by passage of the detergent extract over a column of wheat germ agglutinin–Sepharose 4B. After washing, the bound proteins were eluted from the wheat germ agglutinin–Sepharose 4B with a membrane resuspension buffer (100 mM KCl, 10 mM NaCl, 10 mM Hepes, pH 7.3, 1 mM EDTA, 1 mM PMSF, 10 μg/ml chymostatin, 200 mM *N*-acetyl glucosamine, 200 μg/ml chitobiose, 0.4 M NaCl, and 0.2% Triton X-100). The eluate was concentrated and diluted 10-fold to reduce the salt concentration to 50 mM, and passed over a 5-ml column of heparin-Ultrogel (LKB Instruments Inc., Bromma, Sweden). Cytochrome b559 and Mac-1 were eluted from the heparin column with a gradient of increasing NaCl containing 0.1% Triton X-100. Peak heparin eluate fractions were subjected to SDS-PAGE on linear polyacrylamide gradient gels (8–16%) followed by either staining or electrophoretic transfer onto nitrocellulose as described (51).

The purification of Mac-1 by LM2/1 immunoaffinity chromatography has been described (19). Approximately 2–5 μg of purified Mac-1 in 300 mM NaCl, 50 mM triethanolamine, 0.1 M Tris, 1% *n*-octyl-β-D-glucopyranoside, pH 8.0, was diluted 20-fold with 10 mM Hepes, pH 7.4, 0.1% Triton X-100, 35 mM NaCl, and incubated for 3 h at 4°C with either 50 μl of heparin–Ultrogel or, as a control, Ultrogel alone. After washing with 40 mM NaCl, 10 mM KCl, 10 mM Hepes, pH 7.4, 0.1% Triton X-100, the beads were eluted for 1 h at 4°C with 0.5 ml of 400 mM NaCl, 100 mM KCl, 10 mM Hepes, pH 7.4, and 0.1% Triton X-100. The eluates were concentrated 10-fold using a 30-kD cutoff microconcentrating device (Amicon Corp., Danvers, MA) and subjected to SDS-PAGE followed by silver staining as described above.

Static Adhesion Assays

The neutrophil binding assay was based on a previously published protocol (20). Purified heparins (5 mg/ml in PBS), chondroitin sulfates (A, B, or C, 5 mg/ml in PBS), or fibrinogen (2 mg/ml in PBS; Sigma Chemical Co.) were spotted (25 μl) onto 6-cm bacterial petri dishes (1007; Fisher Scientific Co., Pittsburgh, PA) for 90 min at room temperature. Protein or glycosaminoglycan was removed, and the plates were blocked with the detergent Tween 20. Neutrophils (4×10^6 cells in 1 ml) were resuspended in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, and for some experiments, preincubated with mAbs (1/200 dilution of ascites or 20 μg/ml purified mAb) or with soluble glycosaminoglycan for 15 min at room temperature. In some experiments, 1 mM $CaCl_2$ was added to the HBSS binding buffer. Subsequently, cells were added to the petri dishes in the presence of formylmethionylleucylphenylalanine (fMLP) (10^{-7} M, final volume of 3 ml) and allowed to adhere for 3.5 min. Nonadherent cells were removed, and binding was quantitated as described (20, 21).

The binding of CHO cell transfectants is a modification of a previously described protocol (21). Heparins and chondroitin sulfates were adsorbed to 6-cm petri dishes. After a 90-min incubation at room temperature, nonspecific binding sites were blocked with a 0.5% heat-treated BSA solution (PBS, 1 mM $MgCl_2$, 0.025% NaN_3). CHO cell transfectants, after detachment from tissue culture plates with HBSS, 10 mM Hepes, pH 7.3, 5 mM EDTA, were washed twice, resuspended (8×10^5 cells/ml) in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, 0.5% heat-treated BSA, and bound to glycosaminoglycan-coated petri dishes for 20 min at room temperature. Nonadherent cells were removed, and binding was quantitated as described (21). In some experiments, the number of nonviable, adherent transfectants was determined by staining with PBS, 0.4% trypan blue, and this value was subtracted.

Laminar Flow Adhesion Assays

Purified heparin (5 mg/ml) or heparan sulfate (10 mg/ml) was spotted on bacterial petri dishes (Nunc Inc., Naperville, IL) and blocked with Tween 20 as described above. Control substrates were prepared by blocking dishes solely with Tween 20. Soluble recombinant E-selectin (36) (a generous gift of Dr. R. Lobb, Biogen, Cambridge, MA) was diluted (0.4 μg/ml in 50 mM $NaHCO_3$, pH 9.1) and absorbed on bacterial petri dishes for 2 h at room temperature. The substrate was washed twice with PBS, and heparin (5 mg/ml) or control PBS solution was adsorbed onto the E-selectin–coated substrate for 10 h at room temperature. The substrate was then blocked with 0.2% Tween 20, PBS for 60 min at room temperature.

The laminar flow assays were performed in a parallel wall flow chamber as described previously (35, 36). Briefly, a petri dish slide on which purified heparin, heparan sulfate, and/or recombinant E-selectin had been absorbed was assembled as the lower wall of the parallel flow chamber and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon Inc., Instrument Division, Garden City, NY). Cells that tethered or arrested were quantitated by analysis of images videotaped with a video camera (TEC-470 CCD; Optronics Engineering, Goleta, CA) and Hi 8 recorder (CVD-1000; Sony Corp., Park Ridge, NJ). Peripheral blood neutrophils were resuspended (5×10^5 cells/ml) in binding media

(HBSS, 10 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2 mM $CaCl_2$), in the absence or presence of IL-8 (50 ng/ml, Genzyme Corp., Cambridge, MA) and immediately perfused through the chamber at different flow rates to obtain the indicated shear stresses at the chamber wall. In experiments with a single divalent cation, neutrophils were washed in HBSS, 10 mM Hepes, pH 7.4, 10 mM EDTA and resuspended in HBSS, 10 mM Hepes, pH 7.4, 2 mM $CaCl_2$.

For mAb inhibition studies, neutrophils (10^7 cells/ml) were incubated in HBSS, 10 mM Hepes, pH 7.4 for 5 min at room temperature with 30 μg/ml of purified mAbs (CBRM1/29, DREG-56, W6/32) or a 1:30 dilution of ascites (CBRM1/34). The cell suspension was diluted in a 20-fold volume of binding media and immediately perfused into the flow chamber. In some experiments, to assess the requirement of active metabolism on integrin-mediated adhesion, cells were preincubated for 5 min in binding media supplemented with 0.5% NaN_3.

Neutrophil binding to heparin or control substrates at subphysiologic shear stress was determined by counting the number of cells that attached over a 3-min period of continuous shear flow for a given field of view (0.43 mm^2). Detachment assays were performed on cells tethered at subphysiologic shear flow to purified heparin. The shear flow was increased every 10 s to a maximum of 14.6 dyn/cm^2, in 2–2.5-fold increments and the number of cells remaining bound at the end of each 10-s interval was determined.

Neutrophil tethering to E-selectin substrate coimmobilized with heparin was performed under physiologic shear flow (1.05 dyn/cm^2), and the rate of cell tethering (number of events lasting at least 2 s/min per 0.43 mm^2 field) was determined. No tethering was observed under these conditions to substrates coated with heparin alone. Rolling velocities of neutrophils (30–35 per field) that tethered under flow were determined at a shear stress of 1.5 dyn/cm^2 by cell displacements measured over 5-s intervals. For comparison of effects of mAbs or chemical treatments, identical fields were used for binding experiments to ensure that results reflected uniform site density of immobilized adhesive molecules.

Results

Previously, we identified ICAM-1 on endothelial cells as a counterreceptor for Mac-1 (19). In the course of these experiments, we observed that unstimulated endothelial cells bind to purified Mac-1 even in the presence of blocking mAbs to ICAM-1. Since Mac-1 does not bind to ICAM-2 (15, 19), we postulated the existence of an additional adhesive ligand on endothelium for Mac-1. Subsequently, we found several mesenchymally derived tumor cell lines (e.g., RD 3/5 and FS 12/3) that lack or had low expression of ICAM-1, ICAM-2, and ICAM-3 (15, 16), but adhere strongly to purified Mac-1 (Diamond, M. S., and T. A. Springer, unpublished observations). Because we were unable to clone a cDNA or make function blocking antibodies against this putative ligand, we hypothesized a highly conserved molecule. Two candidates were proteoglycans and sialylated carbohydrates, molecules that function broadly in cell adhesion in the extracellular matrix and on the cell surface.

While purifying cytochrome b559 from solubilized granulocytes, a "contaminating" 150-kD protein was observed that eluted from a heparin affinity resin under conditions of moderately high ionic strength (250 mM NaCl; Fig. 1, lane *4*) (51). Because of the similarity of molecular weight to Mac-1, this eluate was immunoblotted. Both mAbs (Fig. 1, lane *3*) and polyclonal antisera (data not shown) against CD11b identified the 150-kD protein as a subunit of Mac-1. Additionally, functionally active Mac-1 binds to immobilized heparin directly; Mac-1 purified by immunoaffinity chromatography (19) is reprecipitated by heparin-Ultrogel (Fig. 1, lane 5), but not by Ultrogel alone (Fig. 1, lane 7). Scanning densitometry showed that 73% of the purified Mac-1 bound to Heparin-Ultrogel (data not shown).

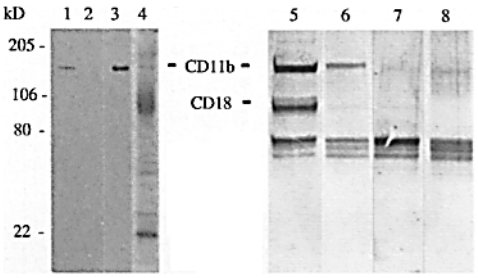

Figure 1. Immunoblotting and SDS 8–16% gradient PAGE of Mac-1 purified from detergent-solubilized granulocyte lysates by immunoaffinity or heparin affinity chromatography. Lane *1*, Mac-1 purified by mAb (LM2/1) affinity chromatography, immunoblotted with anti–Mac-1 mAb (44a), and visualized with peroxidase-conjugated goat anti–mouse IgG; lane *2*, Mac-1 purified by mAb (LM2/1) affinity chromatography, immunoblotted with negative control mAb, and visualized with peroxidase-conjugated goat anti–mouse; lane *3*, immunoblot with anti–Mac-1 mAb (44a) of the peak heparin eluate fraction from the purification of granulocyte cytochrome b559 (see Materials and Methods), and visualized with peroxidase-conjugated goat anti–mouse IgG; lane *4*, Coomassie stain of SDS-PAGE of peak heparin eluate fraction shown as an immunoblot in lane *3*; lanes *5–8*; silver staining of reduced SDS-PAGE of purified Mac-1 after binding and elution from heparin-Ultrogel (see Materials and Methods); lane *5*, Mac-1 eluted (400 mM NaCl) from heparin-Ultrogel showing the characteristic 150 and 95 kD subunits; lane *6*, SDS denaturation of heparin-Ultrogel matrix after high salt elution of Mac-1 demonstrating minimal residual integrin on the matrix; lane *7*, Control high salt (400 mM NaCl) eluate from Ultrogel after incubation with purified Mac-1 demonstrating a lack of nonspecific binding of the two subunits of Mac-1; lane *8*, SDS denaturation of the Ultrogel matrix after high salt elution demonstrating no residual integrin on the matrix.

To assess the significance of this biochemical interaction between Mac-1 and heparin, we assayed neutrophil adhesion to different purified glycosaminoglycans. Resting neutrophils bind weakly to both low (mol wt = 5,000) and high (mol wt = 13,000–15,000) molecular weight forms of heparin, but not to chondroitin sulfate A, B, or C (Fig. 2, and data not shown). Stimulation with the chemotactic peptide fMLP enhances adhesion of neutrophils to heparin, but not to chondroitin sulfate; in parallel experiments, an equivalent number of activated neutrophils bind to ICAM-1 and fibrinogen (data not shown). To confirm that Mac-1 on neutrophils mediates the adhesion to heparin, we tested neutrophils from patients who have the genetic disease LAD which is characterized by an absence of Mac-1 on leukocytes (3). LAD patient neutrophils do not adhere to any form of heparin or chondroitin sulfate in the absence or presence of fMLP (Fig. 2) or in buffers that contain only Mg^{2+} or both Mg^{2+} and Ca^{2+} (data not shown).

Inhibition experiments with mAbs sustain the role of Mac-1 in neutrophil adhesion to heparin. Blocking mAbs to Mac-1 (LPM19c) but not to LFA-1 (TS1/22), p150,95 (CBRp150,95/4G1), or L-selectin (DREG-56) abolish adhesion to heparin (Fig. 3; see Fig. 7). A panel of mAbs to Mac-1 for which epitopes have been mapped on the α subunit, and which have been tested for inhibition of binding to other ligands (21), was used to localize the recognition site for heparin (Table I). mAbs to the I domain inhibited

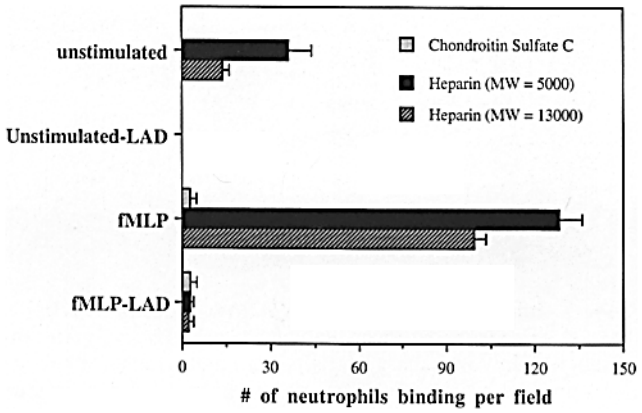

Figure 2. Neutrophil adhesion to different glycosaminoglycans. Peripheral blood neutrophils from healthy volunteers or LAD patients were resuspended (4×10^6 cells/ml) and added to 60-mm petri dishes coated with spots of chondroitin sulfate C, or low or high molecular weight heparin. Binding in the absence or presence of 10^{-7} M fMLP was for 3.5 min at room temperature, and unbound cells were removed by 10 washes with a Pasteur pipette. Bound cells were quantitated by visually scoring the number of cells in five microscopic fields (×100). One representative experiment of four is shown, and bars indicate standard deviations.

binding with a mean of 57.3 ± 24.4%, whereas mAbs to the COOH-terminal region blocked with a mean of 11.0 ± 9.8%. 5 of 18 mAbs that mapped to the I domain blocked strongly (>80%), whereas no mAb that localized to the COOH-terminal region showed >30% inhibition. The one mAb (CBRM1/20) that mapped directly to the divalent cation-binding region had little inhibitory effect. The one mAb that mapped to sites in both the NH_2-terminal and divalent cation-binding regions blocked 63% of the binding. Collectively, these data suggest a recognition site in the I domain on Mac-1 for heparin.

To eliminate the possibility that mAbs to Mac-1 inhibit neutrophil adhesion by an indirect effect, we examined

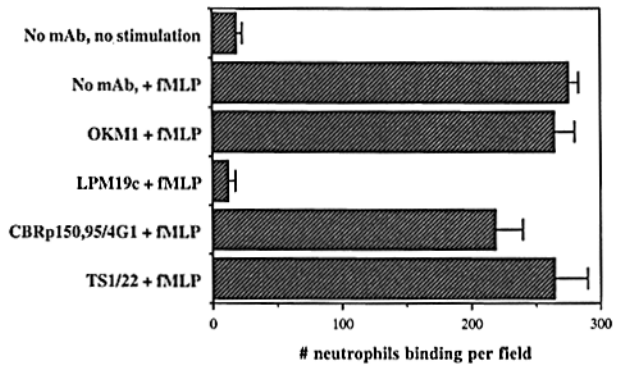

Figure 3. The effect of mAb on neutrophil adhesion to high molecular weight heparin. Peripheral blood neutrophils were resuspended (4×10^6 cells/ml), preincubated at room temperature for 15 min with mAbs, and added to 60-mm petri dishes coated with heparin in the absence or presence of 10^{-7} M fMLP. The cell-binding assay, washing, and quantitation were performed as described in Fig. 2. mAbs: Media (*no mAb*), anti–Mac-1 (*OKM1*, *LPM19c*), anti-p150,95 (*CBRp150,95/4G1*), anti-LFA-1 (*TS1/22*). One representative experiment of three is shown, and bars indicate standard deviations.

Table I. Summary of Inhibition of Neutrophil Adhesion to Heparin by mAbs and Comparison with Structural Epitope

mAb	Epitope	Percent inhibition ± SEM
LPM19c	I domain	94.1 ± 1
OKM9	I domain	45.4 ± 2
LM2/1	I domain	29.1 ± 9
TMG-65	I domain	82.0 ± 3
Mn41	I domain	84.5 ± 9
14B6E.2	I domain	28.8 ± 8
CBRM1/1	I domain	57.4 ± 8
CBRM1/2	I domain	41.6 ± 7
CBRM1/4	I domain	27.8 ± 11
CBRM1/13	I domain	8.5 ± 13
CBRM1/21	I domain	73.3 ± 15
CBRM1/22	I domain	64.2 ± 13
CBRM1/24	I domain	34.4 ± 14
CBRM1/27	I domain	86.3 ± 9
CBRM1/29	I domain	69.6 ± 8
CBRM1/31	I domain	60.5 ± 8
CBRM1/33	I domain	55.7 ± 9
CBRM1/34	I domain	88.0 ± 5
OKM1	COOH-terminal	2.8 ± 14
CBRM1/9	COOH-terminal	−6.2 ± 13
CBRM1/10	COOH-terminal	24.0 ± 5
CBRM1/16	COOH-terminal	10.8 ± 10
CBRM1/17	COOH-terminal	10.8 ± 15
CBRM1/18	COOH-terminal	9.8 ± 17
CBRM1/23	COOH-terminal	6.1 ± 1
CBRM1/25	COOH-terminal	3.7 ± 5
CBRM1/26	COOH-terminal	22.2 ± 17
CBRM1/30	COOH-terminal	26.4 ± 6
CBRM1/32	NH_2-cation	62.8 ± 13
CBRM1/20	cation	13.1 ± 2
CBRM1/28	?	12.3 ± 6
CBRp150,95/4G1	p150,95	−3.3 ± 10
TS1/18	CD18	16.8 ± 0.5
R15.7	CD18	60.2 ± 7

Neutrophils were prepared as described in Fig. 3. Results are the average of three to five independent experiments. Epitopes are assigned according to their binding to a series of Mac-1/p150,95 chimeras as described (21).

transfectant cell binding to heparin. CHO cells that express Mac-1 adhere strongly to heparin but not to chondroitin sulfate (Fig. 4 *A*); adhesion is specific as mAbs to Mac-1, but not to LFA-1 block (Fig. 4 *B*). In contrast, CHO cells that express ICAM-1 do not bind to heparin. Thus, Mac-1 is both necessary and sufficient for attachment to heparin. Interestingly, CHO cells that express p150,95 bind to heparin, although at lower efficiency.

To evaluate the interaction more quantitatively, we tested soluble heparin for its ability to inhibit Mac-1–dependent neutrophil adhesion to immobilized heparin (Fig. 5). Preincubation of neutrophils with soluble heparin dose dependently inhibited adhesion to heparin (inhibition constant $[K_i] = 9$ μM) but not to fibrinogen or iC3b-coated erythrocytes (data not shown). In contrast, soluble chondroitin sulfate C, which also bears a strong negative charge secondary to sulfation, had no effect on Mac-1–dependent neutrophil adhesion to heparin or fibrinogen over a wide range of concentrations.

Because heparins are a heterogeneous group of molecules with differences in carbohydrate backbone and extent of sulfation (38), we tested how neutrophils adhere to a series of chemically modified heparins (Fig. 6). Most hep-

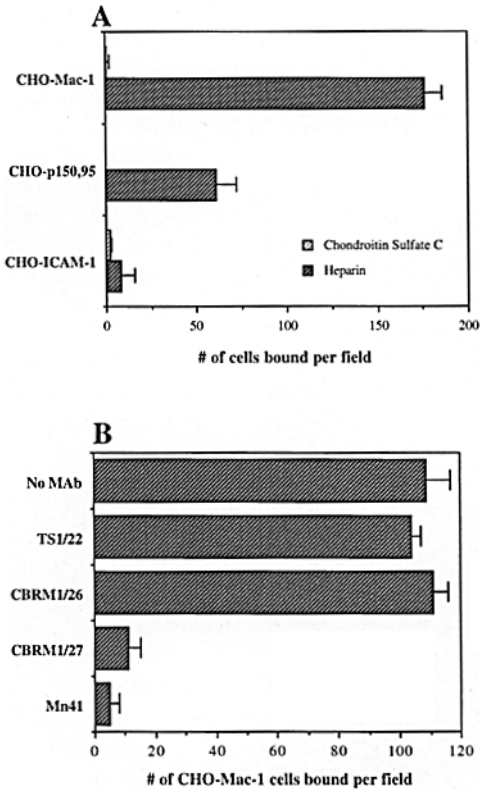

Figure 4. Binding of CHO cell transfectants to high molecular weight heparin. (*A*) Mac-1, p150,95 and ICAM-1–expressing CHO cells were detached, resuspended (8×10^5 cells) in 1 ml, and bound to 60-mm petri dishes coated with spots of heparin and chondroitin sulfate C for 20 min at room temperature. Unbound cells were removed after five washes with a Pasteur pipette, and binding was quantitated as described in Fig. 2. One representative experiment of three is shown, and bars indicate standard deviations. (*B*) Mac-expressing CHO cells were prepared and assayed as in *A* for binding to heparin in the presence of no mAb, mAb to LFA-1 (*TS1/22*), or mAb to Mac-1 (*CBRM1/26*, *CBRM1/27*, *Mn41*). One representative experiment of three is shown, and bars indicate standard deviations.

arins are N-sulfated at the free amino group of GlcNAc, and O-sulfated at C-6 of GlcNAc and at C-2 of IdoA. However, some heparins show additional O-sulfation at the C-2 and C-3 of GlcA or the C3 of GlcNAc. Activated neutrophils adhere poorly to heparins that are completely desulfated (CDSNAc). Sulfation at the N-position of the glucosamine residue of a completely desulfated heparin (CDSNS) restores adhesion. Although this suggests an important role for the N-sulfate group, additional motifs must be critical because N-desulfated forms of heparin (NDSNAc) sustain neutrophil adhesion.

Thus far, static adhesion assays have been used to define an interaction between Mac-1 and heparin. Because closely related moieties (e.g., heparan sulfate containing proteoglycans) are expressed on endothelial cell surfaces in vivo, we examined how neutrophils interact with immobilized heparin under conditions of continuous laminar flow. At low, subphysiologic shear stress (0.36 dyn/cm^2), neutrophils activated with the inflammatory cytokine IL-8 bind to heparin. The adhesion is blocked completely by mAbs to Mac-1, but not L-selectin (Fig. 7 *A*), even when 20–40% of the L-selectin is retained on the cell surface (data not shown). At physiologic shear stress (0.7–0.8 dyn/cm^2), neutrophil attachment to heparin is diminished. Resting or activated neutrophils, once adhered, do not roll on heparin at any shear stress examined. In contrast, neutrophil rolling is supported by immobilized E- or P-selectins (35, 36), and by the L-selectin ligand, peripheral node in (37). Much like its interaction with ICAM-1 (35), Mac-1 binds to heparin at subphysiologic shear flows, and attachment results in spreading and resistance to detachment at high physiological shear stresses (e.g., 15 dyn/cm^2, Fig. 7 *B*). Similarly, heparan sulfate supports Mac-1–dependent firm adhesions that are highly resistant to detaching shear forces (Fig. 7 *C*). In the presence of Ca^{2+} alone, no neutrophil tethering or rolling on heparan sulfate under physiologic or subphysiologic shear flow was observed.

Because Mac-1 on neutrophils does not attach to heparin under physiologically relevant flow conditions, we questioned whether an initial tethering interaction via E-selectin could deaccelerate a neutrophil enough to facilitate a Mac-1–heparin interaction. To dissect the different modes of adhesion, tethering rates and the shear resistance of adhesion were measured for a substrate adsorbed with E-selectin alone or coadsorbed with E-selectin and heparin (Fig. 8). Neutrophils bind to E-selectin via counterreceptors that are decorated with sialyl Le^x (34). E-selectin sustains neutrophil tethering, but does not support arrest at physiologic shear stresses (36). Experiments were performed in buffers containing Ca^{2+} only, Ca^{2+} and Mg^{2+}, and in the presence or absence of blocking mAb to Mac-1;

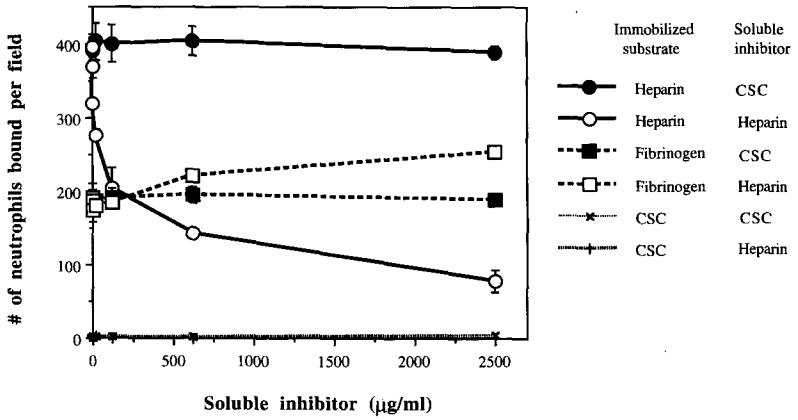

Figure 5. Neutrophil adhesion to fibrinogen, high molecular weight heparin, and chondroitin sulfate C in the presence of soluble high molecular weight heparin or chondroitin sulfate C. Peripheral blood neutrophils were resuspended (4×10^6 cells/ml), preincubated at room temperature for 15 min with varying concentrations of soluble heparin or chondroitin sulfate C, and added to 60-mm petri dishes coated with fibrinogen, heparin, and chondroitin sulfate C in the presence of 10^{-7} M fMLP. The cell-binding assay, washing, and quantitation were performed as described in Fig. 2. One representative experiment of three is shown, and bars indicate standard deviations.

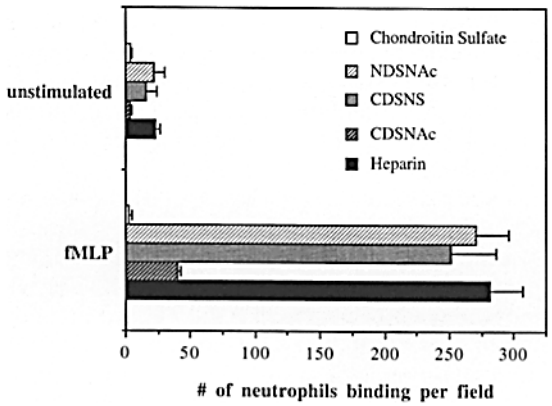

Figure 6. Neutrophil adhesion to chemically modified derivatives of heparin and chondroitin sulfate C. Peripheral blood neutrophils were resuspended (4×10^6 cells/ml), preincubated at room temperature for 15 min with mAbs, and added to 60-mm petri dishes coated with chemically modified forms of heparin (*NDSNAc*, N-desulfated, N-acetylated; *CDSNS*, completely desulfated, N-sulfated; CDSNAc, completely desulfated, N-acetylated) and chondroitin sulfate C in the absence or presence of 10^{-7} M fMLP. The cell-binding assay, washing, and quantitation were performed as described in Fig. 2. One representative experiment of three is shown, and bars indicate standard deviations.

selectins require Ca^{2+}, and leukocyte integrins require Mg^{2+} for adhesion to their respective ligands (28, 34). Under conditions of physiological shear stress (1.0 dyn/cm^2), neutrophils do not tether on heparin substrates or on E-selectin–heparin substrates in the presence of mAbs to E-selectin (data not shown). Ca^{2+} is required for neutrophil tethering on E-selectin (Fig. 8 *A*), but no firm adhesion or neutrophil arrest develops on selectins even in the presence of Mg^{2+} (data not shown and 36). The addition of Mg^{2+} does not affect the rate of tethering to E-selectin. When heparin is coadsorbed with E-selectin, Ca^{2+} enables optimal tethering and rolling as the addition of Mg^{2+} does not augment the tethering rate (Fig. 8 *A*). Mg^{2+}, however, increases the efficiency of events that occur after tethering and rolling. In the absence of mAbs to Mac-1, the arrest of tethered and rolling neutrophils, and hence accumulation of cells, is increased by the addition of Mg^{2+} on an E-selectin–heparin substrate, but not on E-selectin alone (data not shown); arrest occurred immediately upon tethering or after a brief period of rolling. Nonarrested neutrophils roll at identical velocities on E-selectin or E-selectin–heparin substrates in the presence of Ca^{2+} or Ca^{2+}/Mg^{2+}, with or without blocking mAbs to Mac-1. Finally, if neutrophils attach to an E-selectin–heparin substrate in the presence of both divalent cations at physiologic shear stresses, they form firm adhesions and do not detach at elevated shear stresses unless the Mac-1 component is blocked (Fig. 8 *B*). Collectively, these experiments distinguish a Mac-1–heparin interaction from neutrophil rolling on selectins, and suggest that neutrophil rolling on E-selectin at physiological shear is required so that a second, firm attachment between Mac-1 and a heparin ligand will occur, resulting in cell arrest.

Discussion

In this paper, we describe a receptor–ligand interaction between the leukocyte integrin Mac-1 and heparins. The following evidence was obtained for this interaction: (*a*) Mac-1 that is isolated from human granulocytes directly binds to heparin affinity resins; (*b*) neutrophils that are activated with fMLP or IL-8 bind to heparins, but not to other sulfated glycosaminoglycans; (*c*) LAD patient neutrophils that lack surface expression of Mac-1 do not bind heparin; (*d*) mAbs to Mac-1, but neither lymphocyte function–associated antigen-1 nor L-selectin inhibit neutrophil attachment to heparin or heparan sulfate; and (*e*) CHO cells that express Mac-1 bind strongly to heparin, and this adhesion is inhibited specifically by mAbs.

Although interactions with carbohydrates have not been characterized widely for integrins, previous reports indicated that Mac-1 might recognize polysaccharide determinants. Mac-1 appears to bind carbohydrate moieties on yeast β glucan and bacterial lipopolysaccharide (56, 57, 74), and more recently, heparin or heparan sulfate has been suggested to interact with Mac-1 (13). This latter study showed that soluble heparins and enzymatic treatment with heparinase inhibited the binding of CD45 and Mac-1 in A4 bone marrow–derived cell lysates to Swiss 3T3 fibroblasts (13); however, no direct adhesion to heparin by cells that express Mac-1 was documented. In contrast, we demonstrate specific adhesion to heparin of neutrophils and transfectants that express Mac-1, and no adhesion to heparin with LAD neutrophils that lack Mac-1 but express CD45 (62, and data not shown).

To localize the recognition site of heparin on Mac-1, mAbs to defined structural epitopes were assayed for their capacity to inhibit adhesion. This technique previously predicted the 200–amino acid I domain as a recognition site for ICAM-1, fibrinogen, and iC3b (21). Subsequent investigations with recombinant I domain polypeptides confirm that this region participates in ligand recognition (44, 70, 75). Inhibition experiments with mAbs suggest that heparin interacts with Mac-1 through at least one site in the I domain. Because fewer I domain mAbs (27% compared to 67% for iC3b, ICAM-1, and fibrinogen) strongly block adhesion to heparin, the binding site may be smaller than that for other characterized Mac-1 ligands. It appears that the recognition site for heparin may be distinct or partially overlapping with respect to other Mac-1 ligands. Two mAbs (14B6E.2, CBRM1/4), that abolish binding to iC3b, ICAM-1, and fibrinogen, do not affect binding to heparin. One mAb (CBRM1/27) that poorly inhibits adhesion to ICAM-1 and iC3b blocks adhesion to heparin, and soluble heparin does not reduce Mac-1–dependent neutrophil adhesion to fibrinogen.

Transfectant studies in CHO cells suggest a heparin interaction with p150,95 that is lower affinity than with Mac-1. The p150,95 molecule is expressed at substantially lower levels than Mac-1 on neutrophils, and this lower expression, or greater resistance to activation by fMLP and IL-8, may explain the dominant role of Mac-1 on neutrophils in interacting with heparin. Mac-1 and p150,95 already share several ligands including fibrinogen (40), iC3b (10, 47, 55), and ICAM-1 (21). Some mesenchymal cell lines that lack ICAM expression bind to purified Mac-1 and p150,95 but not to lymphocyte function–associated antigen-1 (Diamond, M. A., and T. A. Springer, unpublished observations).

Previous studies have suggested that L-selectin binds

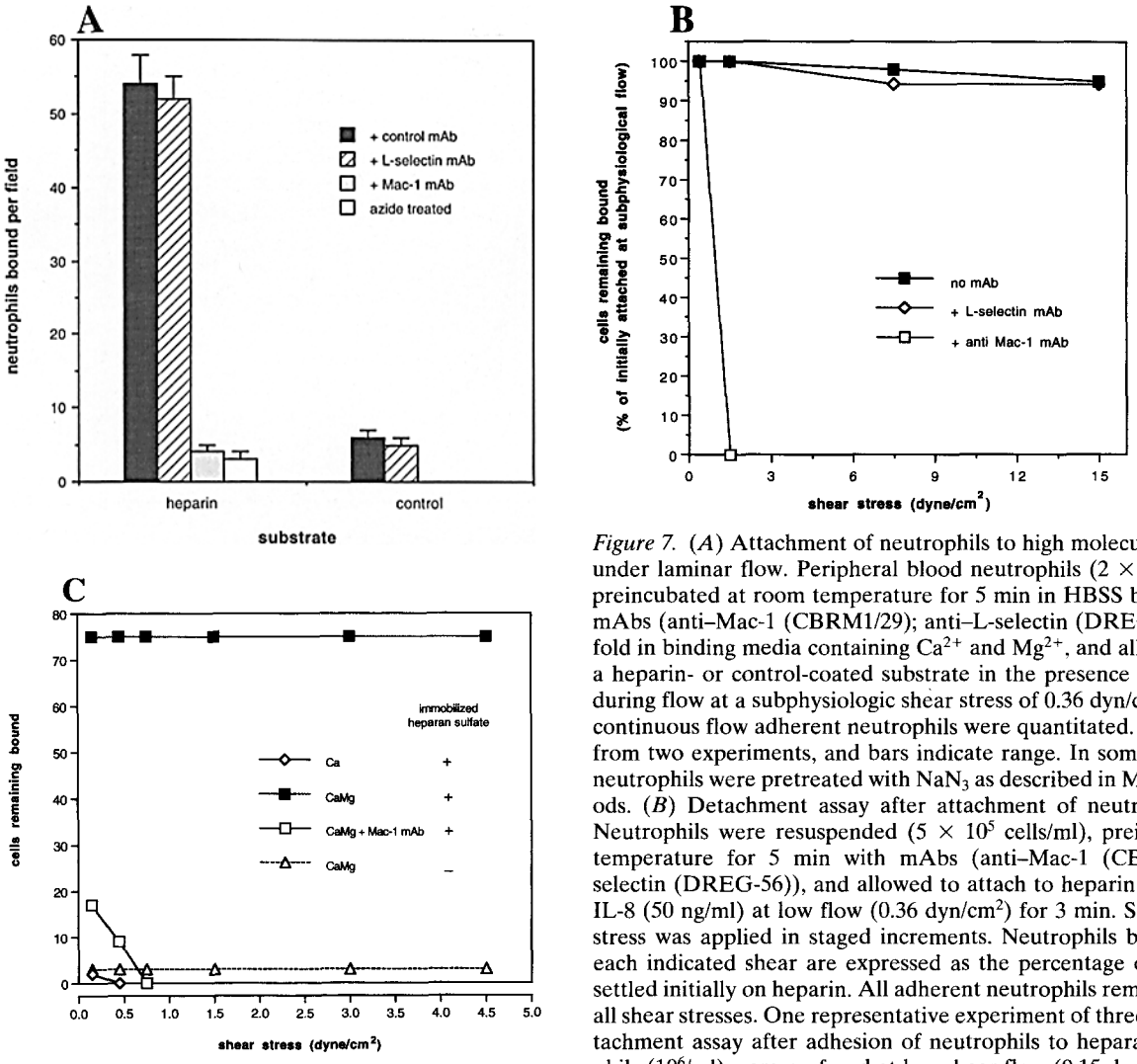

Figure 7. (*A*) Attachment of neutrophils to high molecular weight heparin under laminar flow. Peripheral blood neutrophils (2×10^7 cells/ml) were preincubated at room temperature for 5 min in HBSS binding media with mAbs (anti–Mac-1 (CBRM1/29); anti–L-selectin (DREG-56)), diluted 20-fold in binding media containing Ca^{2+} and Mg^{2+}, and allowed to adhere to a heparin- or control-coated substrate in the presence of IL-8 (50 ng/ml) during flow at a subphysiologic shear stress of 0.36 dyn/cm^2. After 3 min of continuous flow adherent neutrophils were quantitated. Data are averaged from two experiments, and bars indicate range. In some experiments, the neutrophils were pretreated with NaN_3 as described in Materials and Methods. (*B*) Detachment assay after attachment of neutrophils to heparin. Neutrophils were resuspended (5×10^5 cells/ml), preincubated at room temperature for 5 min with mAbs (anti–Mac-1 (CBRM1/29); anti–L-selectin (DREG-56)), and allowed to attach to heparin in the presence of IL-8 (50 ng/ml) at low flow (0.36 dyn/cm^2) for 3 min. Subsequently, shear stress was applied in staged increments. Neutrophils bound after 10 s at each indicated shear are expressed as the percentage of neutrophils that settled initially on heparin. All adherent neutrophils remained stationary at all shear stresses. One representative experiment of three is shown. (*C*) Detachment assay after adhesion of neutrophils to heparan sulfate. Neutrophils (10^6/ml) were perfused at low shear flow (0.15 dyn/cm^2) and allowed to adhere to heparan sulfate coated substrates for 2 min in medium containing the indicated divalent cations. In some experiments, neutrophils were preincubated at room temperature for 5 min with a mAb to Mac-1 (CBRM1/34). Shear stress was increased in staged increments and cells remaining bound were quantitated as described in *B*. Number of cells bound to a field at each indicated shear stress is shown. One experiment of three is shown.

heparin-like molecules (48, 50), yet we do not observe a significant contribution of this interaction in either of our assays. In the presence of Ca^{2+} but not Mg^{2+}, resting neutrophils which express high levels of functional L-selectin do not interact with heparin or heparan sulfate under static or flow conditions (Fig. 7 *C*, and data not shown). mAbs to L-selectin do not inhibit neutrophil adhesion to heparin or heparan sulfate (data not shown) and LAD patient neutrophils that lack Mac-1 but express L-selectin do not bind heparin. Furthermore, neutrophil attachment to heparin improves after stimulation with fMLP, a condition that prompts rapid shedding of L-selectin (31). Even when L-selectin is retained on the neutrophil surface, mAbs to Mac-1 fully abrogate the formation of firm adhesions on heparin and heparan sulfate. Finally, the adhesion to heparin is energy and temperature dependent (data not shown), requires Mg^{2+} and low shear stress flow conditions for attachment, and is not rolling in nature.

The interaction between Mac-1 and heparin is equivalent in strength (K_i of adhesion = 9 μM) to many other integrin–ligand pairs (18), and the level of binding is similar to that of two other described Mac-1 ligands, ICAM-1 and fibrinogen (19, 21). Complete chemical desulfation of heparin significantly reduces binding to Mac-1, whereas N-sulfation of the amino group of GlcNAc restores adhesion. Thus, the presence of at least one type of sulfate group on heparin appears crucial for its interaction with Mac-1. Because forms of heparin that lack N-sulfation (NDSNAc) retain the ability to bind Mac-1, secondary O-sulfated moieties, and carbohydrate structures probably contribute to the recognition site. Furthermore, heparan sulfate sustains Mac-1–dependent adhesion of neutrophils; heparan sulfate shows a lower proportion of N-sulfated GlcNAc residues and a lower overall degree of O-sulfation (38). Since Mac-1 does not interact with chondroitin sulfates, keratan sulfate, or hyaluronic acid (data not shown), the interaction with heparan sulfate glycosaminoglycans is specific, and does not reflect nonspecific binding to sulfate groups or other highly negatively charged structures.

Although we demonstrate an in vitro adhesive interac-

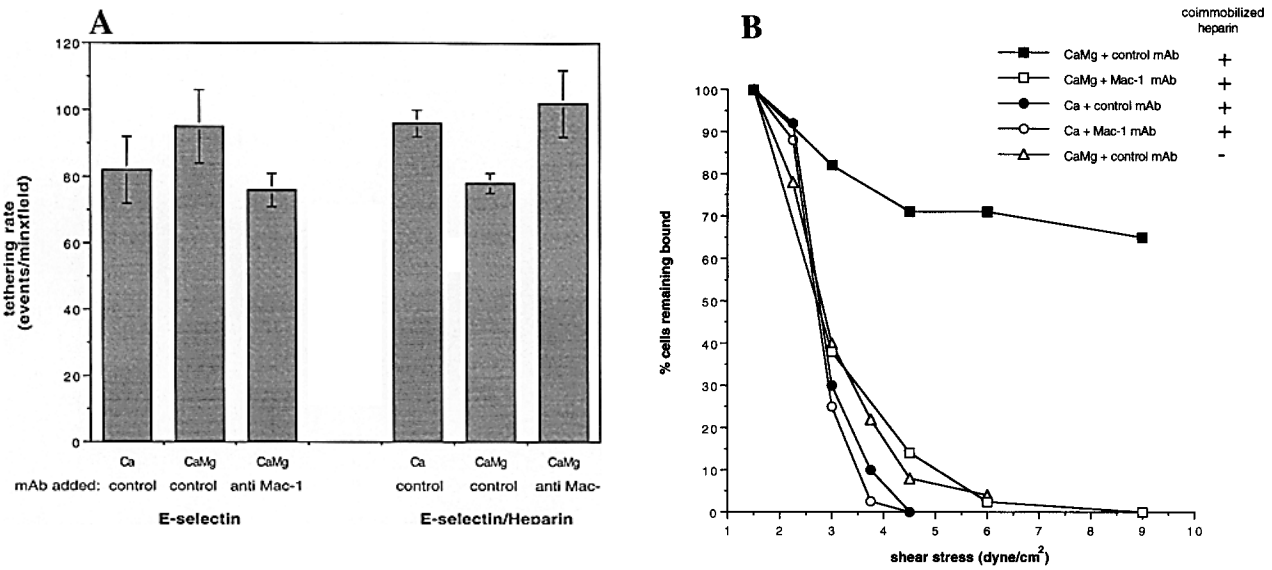

Figure 8. The effect of divalent cations on rolling and firm adhesions of neutrophils tethered to E-selectin–heparin substrate under physiological shear flow. Neutrophils were suspended in a HBSS buffer containing 2 mM Ca^{2+} alone, or with 1 mM Mg^{2+} and perfused through a flow chamber at a shear of 1.05 dyn/cm^2. (*A*) Neutrophil tethering events (cell attachments of at least 2 s duration) were determined on substrates adsorbed with recombinant E-selectin alone or with heparin. Tethering is expressed as the number of tethering events per minute per field (0.43 mm^2 in area). Cells were preincubated with anti–Mac-1 mAb (CBRM1/34) or control anti-HLA class I mAb (W6/32) for 5 min before perfusion into the chamber. Results are expressed as the mean ± range. (*B*) After 1 min of cell perfusion at a shear of 1.05 dyn/cm^2 that allowed accumulation of tethered cells, the flow rate was increased, and the number of cells that remained bound to the substrate was measured. The data is expressed as the percentage of the initially bound neutrophils. For blocking experiments, cells were preincubated with mAbs to Mac-1 and HLA class I as described above. The control mAb had no effect on neutrophil tethering, rolling, or arrest. Results in *A* and *B* are representative of three separate experiments.

tion between purified heparins and Mac-1, the form to which Mac-1 binds in vivo remains unknown. Physiologically, heparins decorate proteoglycans, molecules that function broadly in cell adhesion and communication (9, 58). Many cell surface and matrix-associated proteoglycans contain heparan sulfate, a less sulfated form of heparin (38, 58), whereas those found in intracellular granules of some leukocytes contain the more highly sulfated heparin molecule (68). Candidate proteoglycan ligands that display heparan sulfate include syndecans, perlecan, and glypican (14, 42, 49, 59). Syndecans are an evolutionarily related gene family of four transmembrane proteoglycans, and are expressed broadly in epithelial, endothelial, connective, and neural tissues (30). A common ancestral gene family member in *Drosophila* colocalizes with integrins at sites of muscle attachment during wing morphogenesis (26, 64). Antimicrobial peptides generated during wound repair induce expression of syndecans on endothelial cells, fibroblasts, and in the surrounding extracellular matrix of granulation tissue (24). Perlecan is a basal lamina transmembrane proteoglycan involved in neovascularization (6), and has been suggested to interact with both β_1 and β_3 integrins in a heparin-dependent manner (27). Glypican is a heparan sulfate proteoglycan that is membrane linked through a glycosyl phosphatidylinositol anchor (14), and is expressed on aortic and umbilical vein endothelial cells (43). Future studies will assess which of the described heparan sulfate proteoglycans serve as adhesive ligands for Mac-1. Preliminary studies indicate a high level of specificity of Mac-1 and heparan sulfate proteoglycan interactions; the purified extracellular domain of syndecan-1 (29), which is expressed primarily on epithelial cells (30), does not support neutrophil adhesion (Alon, R., and T. A. Springer, unpublished observations).

Where could a Mac-1–heparin interaction be important? It may contribute to neutrophil migration through the endothelium during inflammation. The laminar flow experiments suggest that Mac-1 does not make transient interactions with heparin or heparan sulfate that characterize rolling; at physiological shear stresses, activated or resting neutrophils do not attach to heparin substrates. In contrast, at conditions of low shear stress, activated neutrophils attach to and spread on heparins in a Mac-1–dependent manner, similar to that observed on ICAM-1 (35). At high shear flows, when E-selectin is coadsorbed with heparin, neutrophils make transient rolling attachments via selectins, and then arrest and develop high shear resistance through a heparin–Mac-1 interaction. We have no evidence for an overlap between selectin- and Mac-1–mediated adhesion to heparin; Mac-1 binding to heparin does not support neutrophil rolling. Instead, it enables the firm adhesion of cells that have been slowed physiologically by selectin-mediated rolling, or allowed sufficient contact time experimentally by binding at subphysiologic flow. Because they bind inflammatory cytokines and chemokines such as IL-8 (66, 71), heparins can both present molecules that activate Mac-1 and serve as ligands for adhesion and spreading. Thus, heparin moieties may complement other receptors such as ICAM-1 in the Mac-1–mediated neutrophil extravasation process.

Another role for a Mac-1–heparin interaction may be in neutrophil migration after extravasation. Heparan sulfates

are present in the extracellular matrix as components of secreted proteoglycans, and may serve as adhesive tracks for migration and haptotaxis of leukocytes that express Mac-1. In addition, heparin-containing proteoglycans reside within primary granules of resting neutrophils (53). After activation, these molecules translocate to the surface and into the surrounding environment. A chemotactic stimulus in the periphery may induce neutrophils to activate Mac-1 and directionally secrete an adhesive ligand to create a path for locomotion.

A Mac-1–heparin interaction also may explain the phenomenon of neutrophil homotypic aggregation. Activation by high concentrations of certain chemotactic factors (e.g., fMLP) induces neutrophil degranulation, activation of surface Mac-1 (17), and a Mac-1–dependent homotypic adhesion (54). Because neutrophil granules contain proteoglycans, such as serglycin, that display polyvalent heparin-like moieties (41, 53), degranulation may promote homotypic aggregation by bridging Mac-1 on adjacent cells. Interestingly, neutrophil aggregation does not occur at 16°C, a temperature that prevents degranulation, but is permissive for the activation of neutrophil Mac-1 (61, and Diamond, M. S., and T. A. Springer, unpublished observations).

In this report we have described a specific interaction between heparin, heparan sulfate, and the leukocyte integrin Mac-1. Future studies must aim at identifying the precise functional side chain on heparan sulfate glycans that binds to Mac-1, the proteoglycans that are decorated with this moiety, and the biological context for Mac-1–heparin adhesion. Such information may enable the generation of heparin or heparan sulfate analogues that serve selectively as antiinflammatory agents in vivo.

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