

# GMP-140 Binds to a Glycoprotein Receptor on Human Neutrophils: Evidence for a Lectin-like Interaction

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**Abstract.** GMP-140 is a rapidly inducible receptor for neutrophils and monocytes expressed on activated platelets and endothelial cells. It is a member of the selectin family of lectin-like cell surface molecules that mediate leukocyte adhesion. We used a radioligand binding assay to characterize the interaction of purified GMP-140 with human neutrophils. Unstimulated neutrophils rapidly bound [ $^{125}$ I]GMP-140 at 4°C, reaching equilibrium in 10–15 min. Binding was  $\text{Ca}^{2+}$  dependent, reversible, and saturable at 3–6 nM free GMP-140 with half-maximal binding at  $\approx 1.5$  nM. Receptor density and apparent affinity were not altered when neutrophils were stimulated with 4 $\beta$ -phorbol 12-myristate 13-acetate. Treatment of neutrophils with

proteases abolished specific binding of [ $^{125}$ I]GMP-140. Binding was also diminished when neutrophils were treated with neuraminidase from *Vibrio cholerae*, which cleaves  $\alpha 2$ -3-,  $\alpha 2$ -6-, and  $\alpha 2$ -8-linked sialic acids, or from Newcastle disease virus, which cleaves only  $\alpha 2$ -3- and  $\alpha 2$ -8-linked sialic acids. Binding was not inhibited by an mAb to the abundant myeloid oligosaccharide, Le $^x$  (CD15), or by the neoglycoproteins Le $^x$ -BSA and sialyl-Le $^x$ -BSA. We conclude that neutrophils constitutively express a glycoprotein receptor for GMP-140, which contains sialic acid residues that are essential for function. These findings support the concept that GMP-140 interacts with leukocytes by a lectin-like mechanism.

**A**DHESIVE interactions of leukocytes with other blood cells or vascular endothelium are fundamental to many aspects of immune and inflammatory responses (15, 25, 60, 61). The selectins are a new family of structurally related molecules that participate in these events (5, 20, 28, 46). The three known selectins are (a) endothelial leukocyte adhesion molecule-1 (ELAM-1),<sup>1</sup> a cytokine-inducible endothelial cell receptor for neutrophils (4) and monocytes (17); (b) the peripheral lymph node lymphocyte homing receptor (murine Mel 14 antigen or human Leu 8 antigen/LAM-1), a molecule also expressed on neutrophils and monocytes which may participate in leukocyte extravasation at sites of inflammation (22, 24); and (c) granule membrane protein-140 (GMP-140, PADGEM protein, CD62), a receptor for neutrophils and monocytes that is rapidly mobilized from secretory storage granules to the surface of activated platelets (14, 27) and endothelium (13, 16, 32). Each selectin contains an  $\text{NH}_2$ -terminal domain homologous to  $\text{Ca}^{2+}$ -dependent lectins (9), followed by an EGF-like domain, a series of consensus repeats similar to those in complement-regulatory proteins, a transmembrane domain, and a short cytoplasmic tail (5–7, 20, 28, 46, 55).

1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; ELAM-1, endothelial leukocyte adhesion molecule 1; GMP-140, granule membrane protein-140; HSA, human serum albumin; PPME, phosphomannan monoester core from *Hansenula hostii*.

The binding properties of selectins have not been well studied nor have any of their receptors been identified. However, the conservation of domains related to  $\text{Ca}^{2+}$ -dependent lectins and the common requirement for  $\text{Ca}^{2+}$  to support leukocyte adhesion (13, 14, 17, 27, 64) suggest that the selectins may bind to oligosaccharide targets. This hypothesis is strengthened by observations that lymphocyte adhesion to peripheral lymph node HEVs mediated by the peripheral lymph node homing receptor is inhibited by certain carbohydrate-rich structures such as fucoidin and the mannose-6-phosphate-rich polysaccharide PPME (64) and by pretreatment of HEVs with neuraminidase (40).

In this study, we used a radioligand binding assay to examine the interaction of purified GMP-140 with blood cells. The time course, reversibility, and saturability of GMP-140 binding to human neutrophils were studied. We also examined whether binding was affected by neutrophil activation or fixation, by soluble carbohydrates, or by pretreatment of neutrophils with proteases or glycosidases.

## Materials and Methods

### Materials

Leupeptin, antipain, aprotinin, benzamidin, 4 $\beta$ -phorbol 12-myristate 13-acetate, 0.4% trypan blue stain, Histopaque, Sephadex G-75 and phos-

phorylated monosaccharides were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin; 222 U/mg) and porcine pancreatic elastase (7.8 U/mg) were purchased from Worthington Biochemical Corp. (Freehold, NJ). Endo- $\beta$ -galactosidase from *Escherichia freundii* was a gift from Michiko Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). Endo- $\beta$ -galactosidase from *Bacteroides fragilis*, neuraminidase from *Vibrio cholerae*, and 2,3-dehydro 2,6-anhydro *N*-acetyl neuraminic acid (Neu2en5Ac) were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Newcastle disease virus (NDV) was isolated as previously described (37). Enzymobeads and Affigel-10 were purchased from Bio-Rad Laboratories (Richmond, CA), carrier-free Na<sup>125</sup>I from Amersham Corp. (Arlington Heights, IL), and HBSS from Gibco Laboratories (Grand Island, NY). Diisopropylfluorophosphate (DFP) and Triton X-100 (Protein Grade) were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Human serum albumin (HSA) was from Cutter Biologicals (Elkhart, IN). The Micro BCA protein assay kit and Lubrol-PX (Surfact Amps PX) were purchased from Pierce Chemical Co. (Rockford, IL). PPME, the phosphomannan monomer core from *Hansenula hostii* (48), was a gift from Steven Rosen (University of California, San Francisco). FITC-anti-Leu 8, FITC-anti-Leu-M1 (Le<sup>x</sup>, CD15) and PE-anti-Leu 15 (CD11b/CD18) mAbs were purchased from Becton-Dickinson Co. (Mountain View, CA). FITC-conjugated goat anti-mouse IgG/IgM F(ab)<sup>2</sup> was purchased from Caltag Laboratories (South San Francisco, CA). The murine anti-Lewis<sup>x</sup> (Le<sup>x</sup>, CD15) IgM mAb, 82H5 (19), was isolated by affinity chromatography on a silica column to which Le<sup>x</sup> (Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc) was immobilized (0.5  $\mu$ mol oligosaccharide/g silica; Synsorb-X). Le<sup>x</sup> and sialyl-Le<sup>x</sup> were synthesized (36) and coupled to BSA ( $\approx$ 10 oligosaccharides/BSA molecule) as previously described (29). The anti-Le<sup>x</sup> monoclonal antibody, sialyl-Le<sup>x</sup>-BSA, and Le<sup>x</sup>-BSA were gifts from Murray Ratcliffe (Chembion Ltd., Edmonton, Alberta).

### Neutrophil Isolation

Human neutrophils, isolated as previously described (14), were suspended to  $4 \times 10^6$ /ml in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS, supplemented with 1 mg/ml HSA and 1 mM Ca<sup>2+</sup> (HBSS/HSA/Ca), and kept at 4°C until use. Neutrophil suspensions were routinely >95% pure by examination of Wright-Giemsa-stained smears and >95% viable by trypan blue exclusion. For certain experiments, neutrophils were incubated with HBSS/HSA/Ca containing either 100 nM PMA or diluent at 22°C for 30 min. Quiescent and activated neutrophils were either washed once with HBSS/HSA/Ca and chilled on an ice bath, or fixed with one-eighth volume of 8% paraformaldehyde for 30 min at 22°C, followed by addition of one-eighth volume of 0.5 M glycine/0.25 M Tris, pH 7.5. Fixed cells were washed three times with HBSS/HSA/Ca and chilled in an ice bath before use.

### Erythrocyte Isolation

9 ml of whole blood anticoagulated with 1 ml ACD Solution A (Becton-Dickinson Co., Rutherford, NJ) was centrifuged for 10 min at 1,100 g at 22°C. After removing the plasma and buffy coat, 1 ml of packed erythrocytes was diluted with 10 ml of HBSS/HSA/Ca, and centrifuged for 5 min at 1,100 g. This wash step was repeated three times. The erythrocyte suspensions contained <0.1% leukocytes and platelets as assessed by phase contrast microscopy.

### Lymphocyte and Monocyte Isolation

Whole blood anticoagulated with 5 mM EDTA was diluted 1:1 with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS supplemented with 1 mg/ml HSA and 5 mM EDTA. 25 ml of diluted blood were layered on 10 ml of Histopaque and centrifuged at 400 g at 22°C for 40 min. The mononuclear cell layer was collected, diluted with 10 volumes of Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS supplemented with 1 mg/ml human serum albumin and 1 mM EDTA (HBSS/HSA/EDTA), and centrifuged at 400 g for 5 min. The cell pellet was resuspended with 40 ml of HBSS/HSA/EDTA and centrifuged at 100 g for 10 min at 22°C. After removing the platelet-rich supernatant, the mononuclear cell pellet was resuspended with 5 ml of HBSS/HSA/EDTA. Lymphocytes and monocytes were separated by countercurrent centrifugal elutriation using a centrifuge (model JA-21; Beckman Instruments, Fullerton, CA) equipped with an Beckman JE-6 elutriation rotor using minor modifications of previously described methods (63). Before use, the elutriation system was flushed with 500 ml sterile water followed by 500 ml 6% hydrogen peroxide (Mallinckrodt, Paris, KY), 1,000 ml pyrogen-free sterile water, USP (Abbott Laboratories, North Chicago, IL), and 500 ml HBSS/HSA/EDTA. After elutriation

was completed, the fractions were centrifuged at 400 g for 10 min at 4°C and each pellet was resuspended in 0.5 ml HBSS/HSA/EDTA. Before use, the fractions were washed with HBSS/HSA/Ca. The cellular compositions of the pooled lymphocyte and monocyte fractions were assessed by differential counting of Wright-Giemsa-stained smears. Lymphocyte fractions were >99% pure and monocyte fractions were 60–95% pure. The contaminating cells in the monocyte fractions were predominantly lymphocytes. In three separate experiments, [<sup>125</sup>I]GMP-140 binding to pure lymphocytes and to monocyte-enriched populations was determined as described below. As these studies revealed minimal binding of GMP-140 to lymphocytes, GMP-140 binding per 10<sup>6</sup> monocytes was calculated by subtracting the low level binding due to contaminating lymphocytes from total GMP-140 bound to the monocyte-enriched fraction.

### Platelet Isolation and Activation

Quiescent human platelets were isolated as previously described (14). Platelets ( $5 \times 10^8$ /ml) in HBSS/HSA/Ca were incubated with 1 U/ml bovine  $\alpha$ -thrombin or buffer for 10 min at 22°C without stirring, in the presence of 1 mM Arg-Gly-Asp-Ser (RGDS). Aliquots (250  $\mu$ l) were then added directly to mixtures containing 2.3 nM [<sup>125</sup>I]GMP-140 with or without 100-fold excess unlabeled GMP-140 as described below. Control experiments showed that 1 mM RGDS had no effect on binding of GMP-140 to neutrophils.

### GMP-140 Purification

Platelet GMP-140 was purified from outdated human platelets obtained from the Oklahoma Blood Institute and the American Red Cross of Tulsa using a modification of a previously reported procedure (33). Briefly, platelets were extensively washed with 0.1 M NaCl, 20 mM Tris, pH 7.5, 5 mM benzamidine, 5 mM EDTA, and frozen at  $-70^\circ\text{C}$  until use. Frozen platelets (100 units) were thawed, and leupeptin (100  $\mu$ M) and DFP (1 mM) were added. The platelet suspension was freeze-thawed three times in a dry ice-acetone bath and then homogenized. After centrifuging the homogenate for 45 min at 4°C at 46,000 g, the membrane pellet was solubilized in 200 ml of 0.1 M NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, 5 mM benzamidine, 100  $\mu$ M leupeptin, 2% Triton X-100. This mixture was stirred for 30 min at 4°C and then centrifuged for 45 min at 46,000 g. The supernatant was made 0.5 M in NaCl and passed through a  $2.5 \times 10$  cm Sephadex G-75 column hooked in series to a  $1.5 \times 20$  cm Affigel-10 column to which the anti-GMP-140 mAb S12 (33) was coupled at a density of 5 mg of antibody per ml of resin. Both columns were equilibrated at room temperature with 0.5 M NaCl, 20 mM Tris, pH 7.5, 0.02% sodium azide. The S12-Affigel-10 column was extensively washed with equilibration buffer containing 0.01% Lubrol PX at 4°C and bound protein was eluted with 80% ethylene glycol, 1 mM MES, pH 6.0, 0.02% sodium azide, 0.01% Lubrol PX. Protein-containing fractions were pooled, dialyzed against 0.1 M NaCl, 20 mM Tris, pH 7.5, 0.01% Lubrol PX, and applied to a  $5 \times 50$  mm Mono Q column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in the same buffer. The column was washed extensively and then step eluted with 2 M NaCl, 20 mM Tris, pH 7.5, 0.01% Lubrol PX. The protein-containing fractions were pooled and dialyzed against 0.1 M NaCl, 20 mM Tris, pH 7.5, 0.01% Lubrol PX, and stored at 4°C. This material was homogeneous as judged by SDS-PAGE (26) and NH<sub>2</sub>-terminal amino acid sequencing.

### Protein Iodination

GMP-140 was iodinated by the lactoperoxidase method using Enzymobeads according to the manufacturer's instructions. 40  $\mu$ g of GMP-140 was labeled with 0.1–1.0 mCi carrier-free Na<sup>125</sup>I and free [<sup>125</sup>I] was removed by gel filtration through a prepacked Sephadex G-25 column (PD-10, Pharmacia Fine Chemicals) equilibrated in 0.1 M NaCl, 20 mM Tris, pH 7.5, 0.01% Lubrol PX. The void volume fractions were pooled and the protein concentration was determined with the Micro BCA protein assay reagent using BSA as a standard. The labeled protein was made 0.1% gelatin and stored at 4°C until use. The specific activity of the radiolabeled protein ranged from 0.81–15.2  $\mu$ Ci/ $\mu$ g protein and was routinely >95% TCA precipitable.

### Radioligand Binding Assay

Binding assays were performed by combining 10  $\mu$ l [<sup>125</sup>I]GMP-140 and 250  $\mu$ l HBSS/HSA/Ca containing varying concentrations of unlabeled GMP-140 in a 1.5-ml siliconized polypropylene microfuge tube. The assay was initiated by adding 250  $\mu$ l of neutrophils suspended in HBSS/HSA/Ca at  $4 \times 10^6$ /ml. The tubes were incubated at 4°C on an oscillating platform. At various times, the tubes were vortexed briefly, and 450  $\mu$ l of the mixture

was layered over 0.5 ml of dibutyl phthalate (J. T. Baker Inc., Phillipsburg, NJ) in another 1.5-ml microfuge tube. To separate free from cell-bound radiolabeled GMP-140, the tubes were centrifuged for 5 min at 16,000 g in an Eppendorf model 5415 centrifuge equipped with a fixed angle rotor. The tips were amputated into 12 × 75 mm test tubes and the radioactivity in each cell pellet was measured in a gamma counter (series 500; Iso-Data Inc., Rolling Meadows, IL). All components were chilled on an ice bath before the assay and unless indicated, all assays were performed in duplicate.

For studies of time course, antibody inhibition, enzyme susceptibility, and reversibility of binding, [ $^{125}$ I]GMP-140 was present at a final concentration of 0.1–0.3 nM, whereas for experiments measuring maximal GMP-140 binding to various blood cells, [ $^{125}$ I]GMP-140 was present at 2–3 nM. In these experiments, specific binding was calculated as total binding minus nonspecific binding, defined as [ $^{125}$ I]GMP-140 bound in the presence of 100-fold molar excess unlabeled GMP-140. Nonspecific binding ranged from 10–30% of total binding.

Saturability of [ $^{125}$ I]GMP-140 binding to neutrophils was analyzed by incubating cells with 0.1–0.2 nM [ $^{125}$ I]GMP-140 in the presence of increasing concentrations of unlabeled GMP-140 (0–6.3 nM). Disintegrations per minute bound in the presence of 50 nM unlabeled GMP-140 (>250-fold molar excess over added [ $^{125}$ I]GMP-140) was defined as non-specific binding. Nonspecific binding, which was assumed to be constant, was subtracted from total binding at each input of unlabeled GMP-140. GMP-140 bound (femtomoles) was then calculated by dividing specific binding (disintegrations per minute) by specific activity (disintegrations per minute/femtomoles), which decreases as a function of increasing input of unlabeled GMP-140.

### Elution of Bound [ $^{125}$ I]GMP-140

Neutrophils ( $10^6$ ) were incubated with 2.6 nM [ $^{125}$ I]GMP-140 in HBSS/HSA/Ca as described above for 30 min, then rapidly washed three times with cold HBSS/HSA/Ca. The washed neutrophils were resuspended in 250  $\mu$ l of HBSS/HSA/Ca containing 260 nM unlabeled GMP-140 and incubated an additional 10 min at 4°C. After centrifugation, the radioactivity was measured in both the supernatant and cell pellet;  $\approx$ 62% of the total [ $^{125}$ I]GMP-140 was recovered in the supernatant. An aliquot of the supernatant and an equimolar quantity of [ $^{125}$ I]GMP-140 from the same iodination were electrophoresed on a 7.5% SDS polyacrylamide gel under reducing conditions and analyzed by autoradiography using an AMBIS radioanalytical imaging system (AMBIS Systems, Inc., San Diego, CA).

### Enzymatic Treatment of Neutrophils

Neutrophils, suspended in HBSS/10 mM MOPS, pH 7.5 (HBSS/MOPS), were treated twice at 22°C for 10 min with 2 mM DFP to inactivate endogenous serine proteases (1). The cells were then washed with HBSS/MOPS and fixed. Fixed neutrophils ( $7.5 \times 10^6$ /ml) in HBSS/MOPS, were incubated at 37°C with TPCK-trypsin (0.77  $\mu$ M, 41 U/ml) for 10 min or with elastase (40  $\mu$ M, 7.8 U/ml) for 30 min. Control cells were incubated under identical conditions with the same concentrations of enzyme previously inactivated with DFP or buffer alone. After the incubation period, DFP was added to 2 mM and the cells were pelleted at 400 g for 5 min. The cells were resuspended with HBSS/MOPS and DFP was added again to 2 mM. After centrifugation at 400 g for 5 min, the cell pellets were resuspended to  $4 \times 10^6$ /ml in HBSS/HSA/Ca and specific binding of [ $^{125}$ I]GMP-140 was determined. In other experiments, DFP-treated fixed neutrophils ( $1.6 \times 10^7$ /ml) in 0.15 M NaCl, 50 mM acetate, pH 6.0, 10 mg/ml HSA, 9 mM CaCl<sub>2</sub>, 0.05% sodium azide (digestion buffer) were incubated with neuraminidase, endo- $\beta$ -galactosidase, or buffer for varying time periods at 37°C in the presence of 20  $\mu$ M leupeptin, 30  $\mu$ M antipain, 0.64 mM benzamide, and 100 KIU/ml aprotinin. In some incubations 0.5–20 mM of the neuraminidase inhibitor Neu2en5Ac, dissolved in digestion buffer, was included. At these concentrations the pH of the reaction mixture was not affected by the inhibitor. After enzyme treatment the cells were washed twice with cold HBSS/HSA/Ca and resuspended in HBSS/HSA/Ca to  $4 \times 10^6$ /ml before measurement of [ $^{125}$ I]GMP-140 binding. The NDV neuraminidase used was a suspension of virus particles, each of which contains  $\sim 10^3$  neuraminidase molecules, whereas the *V. cholerae* enzyme is in solution.

### Flow Cytometry

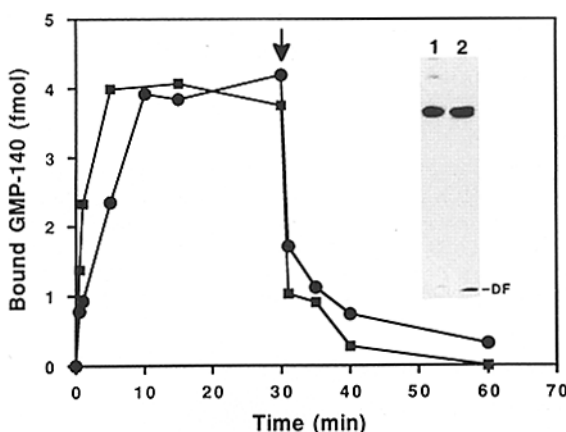
Unstimulated or PMA-stimulated neutrophils were analyzed by direct immunofluorescence by combining 50  $\mu$ l of cells ( $2 \times 10^7$ /ml) with FITC-

anti-Leu 8 (5.5  $\mu$ g/ml), PE-anti-Leu 15 (22  $\mu$ g/ml), FITC-anti-Leu-M1 (4.2  $\mu$ g/ml), or buffer in the presence or absence of either Le<sup>x</sup>-BSA or sialyl-Le<sup>x</sup>-BSA (106  $\mu$ g/ml), and incubated in an ice bath for 30 min. Unstimulated neutrophils were also analyzed by indirect immunofluorescence in which 50  $\mu$ l of neutrophils ( $2 \times 10^7$ /ml) were incubated with 4.2  $\mu$ g/ml of the anti-Le<sup>x</sup> mAb 82H5 in the presence or absence of either Le<sup>x</sup>-BSA or sialyl-Le<sup>x</sup>-BSA (106  $\mu$ g/ml) for 20 min at 4°C, then washed with 1.5 ml HBSS/HSA/Ca. The cells were pelleted at 16,000 g for 10 s, resuspended with 50  $\mu$ l HBSS/HSA/Ca, and then incubated for an additional 20 min at 4°C with FITC-conjugated goat anti-mouse IgG/IgM F(ab)<sub>2</sub> at a final concentration of 11  $\mu$ g/ml. Samples were diluted with 1.0 ml Haema-Line-2 buffer (Baker Instruments, Allentown, PA) and immediately analyzed in a Becton Dickinson FACScan<sup>®</sup> flow cytometer formatted for two-color analysis. The light scatter channels were set on linear gains and the fluorescence channels were set on logarithmic gains. 5,000 fluorescent particles from each sample were analyzed for forward and right angle light scatter and for either FITC or PE fluorescence intensities with ungated acquisition.

## Results

### Binding of GMP-140 to Neutrophils

To examine the interaction of purified [ $^{125}$ I]GMP-140 with blood cells we exploited the observation that GMP-140, although it is a transmembrane protein, remains soluble in buffer containing Lubrol PX at concentrations well below its critical micellar concentration (CMC). Analytical sedimentation velocity analysis of purified GMP-140 in buffers containing Lubrol PX at or above its CMC (100  $\mu$ M, 0.0058%) indicates that the protein sediments as a homogeneous, monomeric species with a sedimentation coefficient of  $\approx$ 4.5 S. At detergent concentrations comparable to those used in our binding studies (<0.0001%),  $\approx$ 50% of the protein sediments as a monomer and the remainder is a mixture of soluble oligomers of limited size ( $n < 10$ ,  $S < 10$ ) (Moore, K. L., and T. M. Laue, unpublished observations).



**Figure 1.** Time course of [ $^{125}$ I]GMP-140 binding to neutrophils. Unstimulated neutrophils ( $10^6$  cells) were incubated with [ $^{125}$ I]GMP-140 (0.1–0.3 nM) in the presence or absence of 100-fold molar excess unlabeled GMP-140 for the indicated time period at 4°C. Bound and free [ $^{125}$ I]GMP-140 were separated as described in Materials and Methods. The arrow indicates the addition of 100-fold molar excess unlabeled GMP-140. Specific binding in two independent experiments is displayed. The inset shows an autoradiogram of a 7.5% SDS polyacrylamide gel, run under reducing conditions, of [ $^{125}$ I]GMP-140 (lane 1) and bound [ $^{125}$ I]GMP-140 eluted from neutrophils by 100-fold molar excess unlabeled GMP-140 in a separate experiment as described in Materials and Methods. (lane 2). (DF, dye front.)

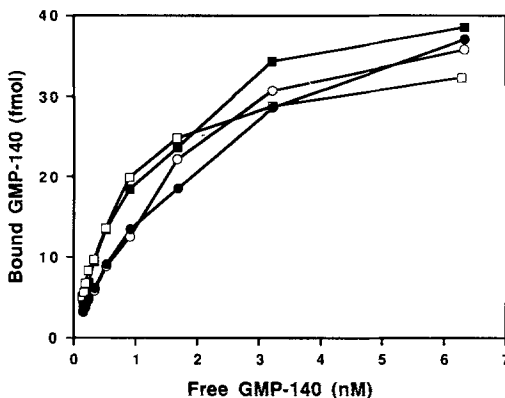
**Table I. Effect of anti-GMP-140 Monoclonal Antibodies or EGTA on [<sup>125</sup>I]GMP-140 Binding to Neutrophils**

Agent added	Final concentration	% Control (n)
Antibody		
G1	10 µg/ml	3 (2)
G2	10 µg/ml	3 (2)
G3	10 µg/ml	29 (2)
S12	10 µg/ml	113 (2)
W40	10 µg/ml	106 (2)
EGTA	5 mM	7 (4)

Neutrophils (10<sup>6</sup>) were incubated with [<sup>125</sup>I]GMP-140 (0.1–0.3 nM) for 1 h at 4°C in the presence of the agent indicated. Bound and free [<sup>125</sup>I]GMP-140 were separated as described in Materials and Methods. Specific binding of [<sup>125</sup>I]GMP-140 is expressed as a percentage of that observed in the absence of antibody or EGTA. All assays were performed in duplicate and the result shown is the mean of the indicated number of independent experiments.

To determine the time course of GMP-140 binding to quiescent neutrophils, 10<sup>6</sup> cells were incubated with 0.1–0.3 nM [<sup>125</sup>I]GMP-140 in the presence or absence of excess unlabeled GMP-140 for varying time periods at 4°C. Specific binding proceeded rapidly, with equilibrium reached between 10 and 15 min (Fig. 1). Binding of [<sup>125</sup>I]GMP-140 was rapidly and completely reversed when 100-fold molar excess unlabeled GMP-140 was added at equilibrium (arrow, Fig. 1).

To assess whether GMP-140 bound to the neutrophil surface undergoes proteolytic processing, neutrophils were incubated with 2.6 nM [<sup>125</sup>I]GMP-140. Bound radiolabeled protein was eluted with 100-fold molar excess unlabeled GMP-140 and then analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. The eluted radiolabeled protein comigrated with intact [<sup>125</sup>I]GMP-140 (inset, Fig. 1), indicating that the specific binding observed

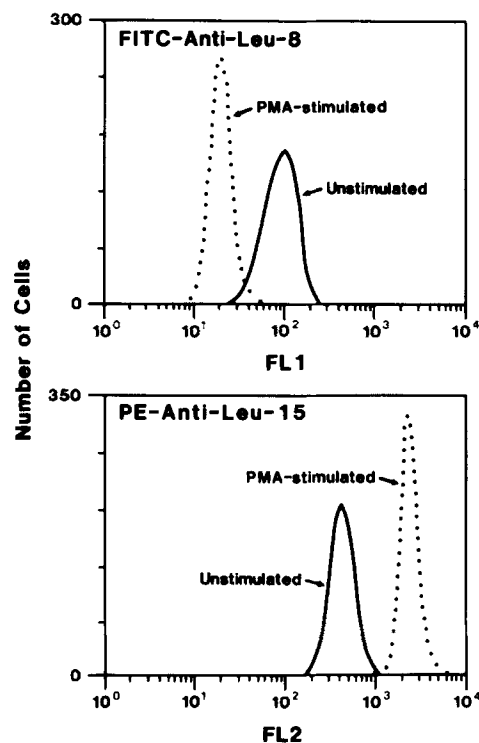


**Figure 2.** Steady-state binding of GMP-140 to neutrophils. Unstimulated and PMA-stimulated neutrophils (10<sup>6</sup> cells) were incubated with [<sup>125</sup>I]GMP-140 (0.1–0.2 nM) in the presence of increasing concentrations of unlabeled GMP-140 (0–6.3 nM) for 1 h at 4°C. Bound and free [<sup>125</sup>I]GMP-140 were separated and specific binding was determined as described in Materials and Methods. Binding data from two paired experiments (circles, squares) are shown. Closed and open symbols indicate data for unstimulated and PMA-stimulated neutrophils, respectively.

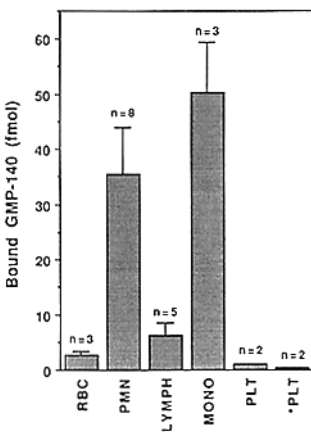
is not due to a minor contaminant in the GMP-140 preparation and that bound GMP-140 is not degraded.

GMP-140-mediated adhesion of neutrophils to thrombin-activated platelets (14, 27) and to thrombin-activated endothelial cells (G. A. Zimmerman, personal communication) requires Ca<sup>2+</sup> and is blocked by some mAbs to GMP-140 (G1, G2, and G3) but not by others (S12, W40) (13, 14). Table I indicates that binding of [<sup>125</sup>I]GMP-140 to neutrophils was also inhibited by chelation of extracellular Ca<sup>2+</sup>. Furthermore, GMP-140 binding was blocked by the mAbs G1, G2, and G3, but not by S12 or W40.

The saturability of GMP-140 binding to unstimulated neutrophils was assessed in competitive binding experiments as described in Materials and Methods. Fig. 2 shows the results of two representative experiments (closed symbols). Specific binding was concentration dependent and saturable at 3–6 nM free GMP-140 with half-maximal binding occurring at ≈1.5 nM GMP-140. At saturation ≈35 fmol of GMP-140 bound to 10<sup>6</sup> neutrophils. Activation of neutrophils with 100 nM PMA had no effect on these binding parameters (Fig. 2, open symbols). In these two paired experiments, half-maximal binding occurred at 1.4 and 2.2 nM for unstimulated cells and 0.9 and 2.1 nM for stimulated cells. Due to the heterogeneous sedimentation behavior of GMP-140 at detergent concentration below the CMC, an accurate estimate of the number of binding sites cannot be determined by Scatchard analysis. If only monomeric GMP-140 interacts with neutrophils, the maximal binding observed (≈35 fmol/10<sup>6</sup> cells) corresponds to ≈20,000 sites per cell. If the oligo-



**Figure 3.** Fluorescence analysis of unstimulated and PMA-stimulated neutrophils. Neutrophils incubated with either 100 nM PMA or diluent for 30 min at 22°C were analyzed for surface expression of Leu 8 antigen (top) and Leu 15 antigen (bottom) by flow cytometry using FITC-anti-Leu 8 and PE-anti-Leu 15, respectively.



**Figure 4.** Binding of [ $^{125}$ I]GMP-140 to various blood cells. Human neutrophils (PMN), monocytes (MONO), lymphocytes (LYMPH), and erythrocytes (RBC) ( $10^6$  cells) and quiescent (PLT) or thrombin-activated platelets (\*PLT) ( $10^8$  cells) were incubated with [ $^{125}$ I]GMP-140 (2.4 nM) in the presence or absence of 100-fold molar excess of unlabeled GMP-140 for 1 h at 4°C. Bound and free [ $^{125}$ I]GMP-140 were separated as described in Materials and Methods. All assays were performed in quadruplicate. Specific binding is expressed as femtomoles GMP-140 bound/ $10^6$  cells, except for binding to platelets, which is expressed as femtomoles GMP-140 bound/ $1.5 \times 10^7$  cells (mean  $\pm$  SD).

druplicate. Specific binding is expressed as femtomoles GMP-140 bound/ $10^6$  cells, except for binding to platelets, which is expressed as femtomoles GMP-140 bound/ $1.5 \times 10^7$  cells (mean  $\pm$  SD).

mers also bind, the actual number of sites may be considerably lower. Despite limitations in quantitative interpretation, the data demonstrate that neutrophil activation does not alter the apparent affinity or number of binding sites for GMP-140.

The state of activation of the cells was analyzed by measuring surface expression of both the Leu 8 antigen and Leu 15 antigen (CD11b/CD18) using flow cytometry. Fig. 3 shows fluorescence histograms of unstimulated and PMA-stimulated neutrophils stained with FITC-anti-Leu 8 (*top*) and PE-anti-Leu 15 (*bottom*). These data confirm previous reports that cellular activation downregulates surface expression of the Leu 8 antigen but upregulates expression of the Leu 15 antigen (22, 24). The inverse modulation of these two adhesive receptors by PMA contrasts with the lack of effect of PMA on specific binding of [ $^{125}$ I]GMP-140 to neutrophils. To ensure that GMP-140 did not activate neutrophils during the assay period, as assessed by these criteria, unstimulated neutrophils were incubated with GMP-140 (40–50 nM) or buffer for 60 min at 4°C, followed by measurement of Leu 8 antigen and Leu 15 antigen expression by flow cytometry. Under these conditions GMP-140 had no effect on either Leu 8 or Leu 15 antigen expression when compared with sham-treated controls (data not shown).

To determine if the interaction of GMP-140 with neutrophils was affected by fixation, unstimulated and PMA-stimulated neutrophils were fixed with 1% paraformaldehyde, then incubated for 1 h at 4°C with near saturating concentrations of [ $^{125}$ I]GMP-140 (2.4–2.6 nM). Fixation did not alter the amount of GMP-140 specifically bound to either unstimulated or stimulated neutrophils when compared with sham-treated controls ( $n = 3$ , data not shown).

### Binding of GMP-140 to Other Blood Cells

Neutrophils, erythrocytes, lymphocytes, monocytes, or platelets were incubated with 2.4 nM [ $^{125}$ I]GMP-140 in the presence or absence of excess unlabeled GMP-140. GMP-140 bound specifically to monocytes as well as neutrophils, but not to erythrocytes or to unstimulated or thrombin-stimulated platelets (Fig. 4). Very low levels of binding to unfractionated peripheral blood lymphocytes were observed. This may represent specific interactions of GMP-140 with a subset

of lymphocytes or with a small number of contaminating monocytes.

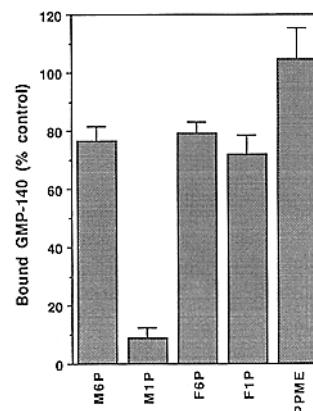
### Effect of Soluble Carbohydrates on GMP-140 Binding

Adhesion of lymphocytes to HEVs mediated by the peripheral lymph node homing receptor (Mel 14/Leu 8 antigen) is inhibited by the phosphorylated monosaccharides, fructose-1-phosphate and mannose-6-phosphate, and by the mannose-6-phosphate-rich polysaccharide, PPME (53, 64). Fig. 5 demonstrates that the same concentrations of these sugars did not markedly inhibit binding of [ $^{125}$ I]GMP-140 to neutrophils. In contrast, binding was almost completely inhibited by 10 mM mannose-1-phosphate, which does not block lymphocyte adhesion to HEV (64). This suggests that the carbohydrate structures recognized by GMP-140 and the peripheral lymph node homing receptor are distinct. The actual receptor for GMP-140 is unlikely to be mannose-1-phosphate, because high concentrations of the sugar are required to inhibit [ $^{125}$ I]GMP-140 binding and mannose-1-phosphate has not been described in N- or O-linked glycans.

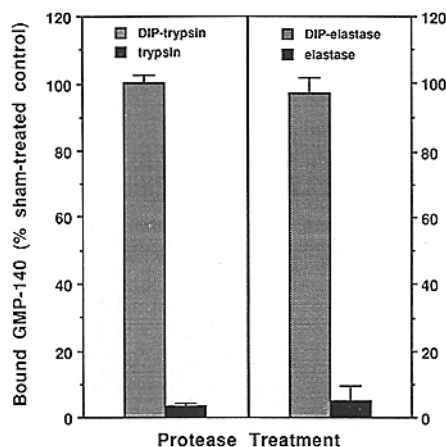
### Structural Features of the Neutrophil Receptor for GMP-140

DFP-treated, fixed neutrophils were incubated with various enzymes before measurement of [ $^{125}$ I]GMP-140 binding. Treatment with trypsin or elastase decreased specific GMP-140 binding to 4 and 5%, respectively, when compared with sham-treated cells, whereas DFP-inactivated enzymes had no effect on GMP-140 binding (Fig. 5). These data indicate that GMP-140 binds either to a cell surface protein(s) and/or to structure(s) associated with a protein.

DFP-treated fixed neutrophils were also treated with neuraminidase from either *V. cholerae*, which cleaves  $\alpha$ 2-3-,  $\alpha$ 2-6-, and  $\alpha$ 2-8-linked sialic acids (43), or the Newcastle disease virus, which cleaves only  $\alpha$ 2-3- and  $\alpha$ 2-8-linked sialic acids (38, 43). After 10–30 min of incubation with 0.1–0.2 U/ml of *V. cholerae* or NDV neuraminidase, specific GMP-140 binding decreased to  $28 \pm 9$  and  $52 \pm 9$ % (mean  $\pm$  SD,  $n = 7$ ), respectively, when compared with sham-treated controls (Fig. 6). To minimize the possibility that this effect

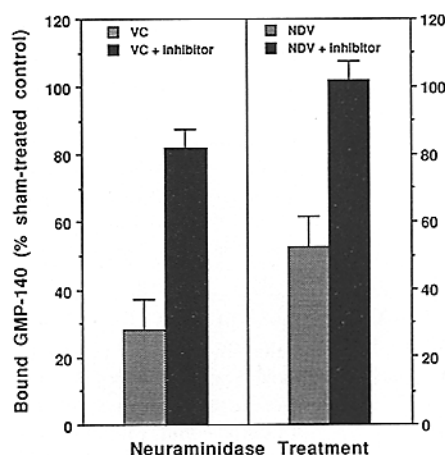


**Figure 5.** Effect of soluble carbohydrates on [ $^{125}$ I]GMP-140 binding to neutrophils. Human neutrophils ( $10^6$  cells) were incubated with [ $^{125}$ I]GMP-140 (0.2–0.3 nM) and mannose-6-phosphate (M6P), mannose-1-phosphate (M1P), fructose-6-phosphate (F6P), fructose-1-phosphate (F1P), or PPME in the presence or absence of 100-fold molar excess unlabeled GMP-140 for 1 h at 4°C. Phosphorylated monosaccharides were present at 10 mM, whereas PPME was present at 100  $\mu$ g/ml. All assays were performed in triplicate. Specific binding of [ $^{125}$ I]GMP-140 is expressed as a percentage of binding in the absence of added carbohydrate (mean  $\pm$  SD,  $n = 3$ ).



**Figure 6.** Effect of proteolytic enzymes on [ $^{125}$ I]GMP-140 binding to neutrophils. Fixed neutrophils were treated with trypsin, DIP-trypsin, elastase, or DIP-elastase as described in Materials and Methods. Cells were incubated with [ $^{125}$ I]GMP-140 (0.1–0.3 nM) in the presence or absence of 100-fold excess unlabeled GMP-140 for 1 h at 4°C. Bound and free GMP-140 were separated as described in Materials and Methods. All assays were performed in quadruplicate. Specific binding of [ $^{125}$ I]GMP-140 is expressed as a percentage of binding to sham-treated controls (mean  $\pm$  SD,  $n = 3$ ).

was due to either endogenous neutrophil proteases or protease contamination in the neuraminidase preparations, neutrophils were treated with DFP before fixation to inactivate endogenous serine proteases, and the neuraminidase incubations were performed in the presence of 10 mg/ml HSA as well as several protease inhibitors. The specificity of the neuraminidase effect was further demonstrated by the ability of a competitive neuraminidase inhibitor, Neu2en5Ac (34, 35), to prevent the neuraminidase-induced reduction in GMP-140 binding to neutrophils (Fig. 6). Neu2en5Ac inhibited the effect of neuraminidase in a dose-dependent manner with an  $IC_{50}$  of 2.5 mM (data not shown). These data suggest that the neutrophil receptor for GMP-140 contains sialic acid residues that are essential for function. Neutrophils contain both  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acids, but  $\alpha$ 2-8 linkages have not been detected (11, 12, 50). Partial loss



**Figure 7.** Effect of neuraminidase treatment on [ $^{125}$ I]GMP-140 binding to neutrophils. Fixed neutrophils were treated with 0.2 U/ml of either *V. cholerae* (VC) or Newcastle disease virus (NDV) neuraminidase as described in Methods, either in the presence or absence of 20 mM Neu2en5Ac. Cells were then incubated with [ $^{125}$ I]GMP-140 (0.1–0.3 nM) in the presence or absence of 100-fold excess unlabeled GMP-140 for 30 min at 4°C. For cells treated with NDV neuraminidase, 1 mM Neu2en5Ac was also included during the binding assay. Bound and free GMP-140 were separated as described in Materials and Methods. All assays were performed in quadruplicate. Specific binding of [ $^{125}$ I]GMP-140 is expressed as a percentage of binding to sham-treated controls (mean  $\pm$  SD).

of GMP-140 binding after treatment with NDV neuraminidase implies that at least some of the sialic acid linkages in the receptor are of the  $\alpha$ 2-3 type. The greater inhibition observed using the *V. cholerae* enzyme may mean that  $\alpha$ 2-6 linkages are also required for receptor function. Alternatively, the essential linkages may all be  $\alpha$ 2-3 but are not equally accessible to hydrolysis by the NDV enzyme, which is part of an intact virus. Finally, substitutions on the sialic acids (30, 43) may have impeded the efficient action of the neuraminidases (58).

Myeloid cells, in contrast to erythroid and lymphoid cells, are rich in fucosylated polylactosaminoglycans which can terminate in  $\alpha$ 2-3- or  $\alpha$ 2-6-linked sialic acids (reviewed in reference 10). These structures are present on both neutrophil glycoproteins and glycolipids (11, 12, 50). To examine the possible role of these glycans in GMP-140 recognition, we treated cells with endo- $\beta$ -galactosidase, which hydrolyzes the  $\beta$ 1-4 linkage between galactose and N-acetylglucosamine (Gal $\beta$ 1-4GlcNAc) in unbranched polylactosaminyl side chains of glycoproteins (44, 45). Pretreatment of fixed neutrophils with up to 0.2 U/ml of *E. freundii* endo- $\beta$ -galactosidase for 30 min, or up to 0.4 U/ml of *B. fragilis* endo- $\beta$ -galactosidase for 60 min, had no effect on specific binding of GMP-140 (data not shown). The lack of effect of these enzymes on binding suggests that the GMP-140 recognition structure is not on a polylactosamine side chain. Alternatively, the relevant side chain(s) may not be susceptible to enzymatic hydrolysis under these conditions. Resistance to hydrolysis by endo- $\beta$ -galactosidase may occur if the glycans are not accessible to the enzyme on the surface of an intact neutrophil. Highly fucosylated and/or branched polylactosaminoglycans may also be resistant to hydrolysis by this enzyme (44, 45); however, branched polylactosaminoglycans are not present in neutrophils (10).

**Table II.** Effect of Anti-Le $^x$  mAb or Neoglycoproteins on [ $^{125}$ I]GMP-140 Binding to Neutrophils

Agent added	Final concentration	% Control*
Antibody†		
82H5	400 $\mu$ g/ml	112
Neoglycoprotein§		
Le $^x$ -BSA	6.5 $\mu$ M	96
Sialyl-Le $^x$ -BSA	6.5 $\mu$ M	92

\* Bound and free [ $^{125}$ I]GMP-140 were separated as described in Materials and Methods. Specific binding of [ $^{125}$ I]GMP-140 is expressed as a percentage of sham-treated controls. All assays were performed in duplicate and the result shown is the mean of two independent experiments.

† Neutrophils ( $4 \times 10^6$ /ml) were preincubated with mAb for 15 min at 4°C. A 250- $\mu$ l aliquot ( $10^6$  cells) was then transferred to 250  $\mu$ l of HBSS/HSA/Ca containing 0.1–0.2 nM [ $^{125}$ I]GMP-140 in the presence or absence of >100-fold molar excess of unlabeled GMP-140.

§ [ $^{125}$ I]GMP-140 (0.1–0.2 nM) in the presence or absence of >100-fold excess of unlabeled GMP-140 was preincubated with neoglycoproteins at the indicated concentration for 10 min at 4°C before addition of neutrophils.

The structures Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc (Le<sup>x</sup> or CD15), and Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc (sialyl-Le<sup>x</sup>) are present as nonreducing termini on polylactosaminoglycans isolated from human neutrophil glycopeptides (11, 50). Le<sup>x</sup>, which is also present on neutrophil glycolipids (12), is recognized by a number of mAbs specific for neutrophils and monocytes (18, 47, 49, 54, 57). Our neuraminidase and protease studies indicate that Le<sup>x</sup> per se cannot constitute the recognition structure for GMP-140. In support of this conclusion, we found that preincubation of neutrophils with 400  $\mu$ g/ml of the anti-Le<sup>x</sup> mAb 82H5 had no effect on binding of [<sup>125</sup>I]GMP-140 (Table II). To further examine whether Le<sup>x</sup> or sialyl-Le<sup>x</sup> are involved in GMP-140 recognition, we measured binding of [<sup>125</sup>I]GMP-140 to neutrophils in the presence of the multivalent neoglycoproteins Le<sup>x</sup>-BSA and sialyl-Le<sup>x</sup>-BSA. Neither conjugate diminished [<sup>125</sup>I]GMP-140 binding (Table II). Under these conditions the conjugates were in >10<sup>4</sup>-fold molar excess over [<sup>125</sup>I]GMP-140. To examine whether the Le<sup>x</sup>-BSA was functional, we used flow cytometry to test its ability to inhibit binding of two anti-Le<sup>x</sup> mAbs to neutrophils. Under conditions in which the conjugate was in  $\approx$ 350-fold molar excess over antibody (see Materials and Methods), Le<sup>x</sup>-BSA inhibited 82H5 and anti-Leu-M1 binding by 75 and 95%, respectively, whereas sialyl-Le<sup>x</sup>-BSA had no effect (data not shown). These results confirm that the Le<sup>x</sup>-BSA conjugate is functional, since it inhibited binding of both anti-Le<sup>x</sup> antibodies at concentrations well below those which had no effect on [<sup>125</sup>I]GMP-140 binding. The data also confirm the specificity of the two mAbs, since Le<sup>x</sup>-BSA, but not sialyl-Le<sup>x</sup>, inhibited antibody binding. A similar analysis of the functional efficacy of sialyl-Le<sup>x</sup>-BSA was not performed because an antibody to sialyl-Le<sup>x</sup> was not available. Nevertheless, the concentrations of both neoglycoproteins used in these studies were well in excess of those capable of blocking cell-cell adhesion and receptor-ligand interactions in other systems (8, 42). These results provide substantial evidence that the Le<sup>x</sup> structure is not involved in GMP-140 binding. They also suggest that sialyl-Le<sup>x</sup> is either not a recognition structure for GMP-140 or that other carbohydrate and/or protein components are required to enhance its affinity and/or avidity for GMP-140.

## Discussion

Our findings indicate that GMP-140 binds rapidly, reversibly, and saturably to receptors on human neutrophils. The  $K_d$  and number of these receptors could not be determined precisely because of the heterogeneous sedimentation behavior of GMP-140 at detergent concentrations below the CMC for Lubrol PX. However, it is clear that neither the density nor the apparent affinity of the receptors is altered by stimulation of cells with the pharmacological agonist PMA. The receptors still interact with GMP-140 when neutrophils are chilled to 4°C or fixed with paraformaldehyde (this study and 13). These properties contrast with those of the  $\beta$ 2 leukocyte integrins, where at least some adhesive functions require metabolically active cells and/or cellular activation (2, 25, 31, 39, 41, 59).

Purified GMP-140 binds to monocytes as well as neutrophils, but not to lymphocytes or erythrocytes, supporting previous observations that GMP-140 mediates adhesion of stimulated platelets only to neutrophils and monocytes (27).

Furthermore, neither quiescent nor activated platelets bind GMP-140. The observation that thrombin-stimulated platelets, which express GMP-140 on their surface, do not bind [<sup>125</sup>I]GMP-140, suggests that homotypic interactions do not occur between GMP-140 molecules on opposing cells.

Another selectin, the Mel 14/Leu 8 antigen, is present on neutrophils and monocytes (23, 56) and has been suggested to be a candidate receptor for GMP-140. However, two lines of evidence indicate that the Leu 8 antigen is not a GMP-140 receptor. First, it is expressed on 70% of peripheral blood lymphocytes (23, 56), which do not bind GMP-140 (this study) or rosette-activated platelets (27). Second, it is rapidly shed from the neutrophil (22, 24) and lymphocyte (21, 56) surface as a consequence of cellular activation. In contrast, stimulation of neutrophils with PMA does not alter the number or apparent affinity of receptors for GMP-140, although it does down-regulate surface expression of the Leu 8 antigen.

Neuraminidase pretreatment of neutrophils markedly diminishes GMP-140 binding, a clear parallel to the ability of neuraminidase pretreatment of HEVs of peripheral lymph nodes to prevent Mel 14/Leu 8 antigen-dependent lymphocyte adhesion (40). However, the effects of soluble carbohydrates on GMP-140 binding are different from those observed for lymphocyte binding to peripheral lymph node HEVs. This suggests that the carbohydrate structures on the GMP-140 receptor are distinct from those on the receptors for the Mel 14/Leu 8 antigen. The effect of neuraminidases indicates that the molecules recognized by GMP-140 contain  $\alpha$ 2-3- and perhaps  $\alpha$ 2-6-linked sialic acids essential for function. It is also possible that the critical sialic acid residues carry substitutions (43, 58) that may be required for GMP-140 recognition. Our findings are consistent with the hypothesis that the lectin-like domain of GMP-140 binds to oligosaccharides containing terminal sialic acids. A less likely possibility is that removal of sialic acids affects the conformation of other functionally important regions of the receptor.

The loss of GMP-140 binding after protease treatment of neutrophils indicates that the receptor is a glycoprotein rather than a glycolipid. This conclusion is strengthened by observations that accessibility of glycolipids to antibody binding is actually increased after protease treatment of intact cells (51, 62). Binding of GMP-140 to its receptor may be due to both protein-protein and protein-carbohydrate interactions. Alternatively, GMP-140 may bind strictly to an oligosaccharide, but the protein core of the receptor may present the oligosaccharide in the configuration optimal for binding.

A possible oligosaccharide target for GMP-140 binding is a polylactosaminoglycan modified by terminal sialic acid. Our data indicate that Le<sup>x</sup> and sialyl-Le<sup>x</sup>, which are terminal structures on neutrophil polylactosaminoglycans (34), are not sufficient to mediate GMP-140 binding. However, we cannot exclude the possibility that one of these glycans, particularly sialyl-Le<sup>x</sup>, constitutes part of a more complex recognition structure for GMP-140. If so, it may be part of a polyfucosylated lactosaminoglycan that is resistant to hydrolysis by endo- $\beta$ -galactosidase.

When platelets and endothelial cells are stimulated with receptor-mediated agonists such as thrombin, GMP-140 is redistributed within minutes from the membranes of secretory storage granules to the plasma membrane (3, 16, 32,



52). The current study suggests that, once GMP-140 is expressed on the cell surface, it will bind rapidly and reversibly to its receptor on leukocytes. These properties should allow it to mediate rapid, regulated targeting of neutrophils and monocytes to sites of acute inflammation or hemorrhage.

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