

Direct Demonstration of the Lectin Activity of gp90^{MEL}, a Lymphocyte Homing Receptor

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Abstract. Considerable evidence implicates gp90^{MEL} as a lymphocyte homing receptor mediating lymphocyte attachment to high endothelial venules of lymph nodes in mouse. The protein appears to function as a calcium-dependent, lectin-like receptor as inferred primarily by the ability of specific carbohydrates to block its function and by the presence of a calcium-type lectin domain in its primary sequence. An ELISA assay

is described which provides the first demonstration that the isolated protein has lectin activity and allows a further definition of its carbohydrate specificity. In addition to the monosaccharides mannose-6-phosphate and fructose-1-phosphate, ligand activity is shown for the sulfated glycolipid, sulfatide, and for two sulfated fucose-containing polysaccharides (fucoidin and egg jelly coat) from nonmammalian sources.

IN most mammalian species, lymphocytes in the blood migrate into lymph nodes and other secondary lymphoid organs by first binding to the endothelium of specialized postcapillary venules known as high endothelial venules (HEVs)¹ (reviewed in Berg et al., 1989; Duijvestijn and Hamann, 1989; Rosen, 1989; Stoolman, 1989; Woodruff et al., 1987; Yednock and Rosen, 1989). Over a period of several minutes, the lymphocytes migrate across the endothelium and its associated basement membrane and gain access to the parenchyma of the lymphoid organ where sequestered and processed antigens are encountered. The initiating adhesive interaction is mediated by a lymphocyte receptor, operationally termed a "homing receptor," that interacts with a complementary HEV-ligand expressed on the endothelial cells of HEV. The term "homing" conveys the general finding that different lymphocyte populations show differential adhesive specificities for HEV (reviewed in Butcher, 1986). The term "vascular addressins," which has been applied to the HEV-ligands (Streeter et al., 1988a, b), signifies that these adhesive ligands carry distinctive recognition determinants as a function of the anatomical site of the HEV. A current model of lymphocyte homing (Butcher and Weissman, 1984), postulates that the complement of homing receptors expressed on a particular blood-borne lymphocyte is the primary factor in determining which lymphoid organs it can enter. Adhesion molecules such as LFA-1 appear to be accessory to the homing receptors in that they augment the strength of the lymphocyte-HEV interaction without imparting lymphoid organ selectivity (Hamann et al., 1988; Pals et al., 1988). Two homing specificities, presumably based on two sets of nonoverlapping homing receptor/HEV-ligand

pairs, have been described, directing lymphocyte binding to HEV in peripheral lymph nodes (PNs) and in Peyer's patches (PPs) (Butcher et al., 1980; Jalkanen et al., 1989; Schmitz et al., 1988). Still other "homing specificities" may be involved in lymphocyte extravasation into other lymphoid compartments and into sites of inflammation outside of organized lymphoid organs (Jalkanen et al., 1986; Sackstein et al., 1988). In the mouse, the best characterized homing receptor is gp90^{MEL}, a ≈90-kD surface molecule defined by the mAb MEL-14 (Gallatin et al., 1983). When pretreated with this antibody, lymphocytes are unable to bind to PN HEV but bind normally to PP HEV as measured in an in vitro adherence assay (Stamper and Woodruff, 1976). The occurrence of the MEL-14 mAb-defined epitope on various lymphocyte and lymphoma cell populations correlates with the ability of the cells to bind to PN HEV (Reichert et al., 1983). Moreover, a direct adhesion role has recently been established for gp90^{MEL} by the finding that the isolated native protein (Geoffroy and Rosen, 1989) or a soluble recombinant form of the receptor (Watson et al., 1990) can interact selectively with PN HEV and block subsequent lymphocyte attachment.

A central aspect of the studies of gp90^{MEL} concerns its function as a lectin-like receptor. Early studies revealed that D-mannose-6-phosphate (M6P) and D-fructose-1-phosphate (FIP), when compared to other phosphorylated monosaccharides, selectively inhibit the attachment of lymphocytes to PN HEV in rat (Stoolman et al., 1984), mouse (Yednock et al., 1987b) and human (Stoolman et al., 1987). Additionally, the yeast-derived, M6P-rich phosphomannan monomer core from *Hansenula hostii* known as PPME, and the sulfated, fucose-rich polysaccharide called fucoidin (from brown seaweed) were shown to be potent inhibitors of lymphocyte binding to PN HEV while having little effect on the binding

1. *Abbreviations used in this paper:* FIP, fructose-1-phosphate; HEV, high endothelial venule; M6P, mannose-6-phosphate; PN, peripheral lymph node; PP, Peyer's patch.

of lymphocytes to PP HEV (Stoolman and Rosen, 1983; Yednock et al., 1987b; Yednock, T. A., M. S. Singer, Y. Imai, and S. D. Rosen, manuscript in preparation). With PPME-derivatized microbeads as a probe for cell surface receptors, it was demonstrated that the carbohydrate dependency of lymphocyte binding to PN HEV can be accounted for by a calcium-dependent, lectin-like receptor on the lymphocyte surface (Yednock et al., 1987b; Stoolman et al., 1987; Stoolman and Ebling, 1989). Moreover, experiments demonstrating MEL-14 mAb inhibition of PPME bead binding to lymphocytes and the coexpression of PPME bead binding with the MEL-14 epitope on lymphoma variants argued that the receptor, detected by PPME beads, was closely related and probably identical to gp90^{MEL} (Yednock et al., 1987a). In agreement with this prediction, molecular cloning of gp90^{MEL} (Lasky et al., 1989; Siegelman et al., 1989) has revealed a transmembrane protein with a calcium-dependent ("C-type") lectin motif of 117 residues positioned at its extracellular amino terminus. Among the homologous proteins, the best known are the mammalian asialoglycoprotein receptors, the chicken hepatic lectin, and the mannose-binding protein, all of which show calcium-dependent carbohydrate-binding activity (reviewed by Drickamer, 1988). The MEL-14-defined epitope has been shown to map to the amino terminal region of the "lectin" domain (Bowen et al., 1990). Following the sugar-binding motif in the predicted sequence of gp90^{MEL} are an EFG-like motif (34 residues), two identical complement-regulatory motifs of 62 amino acids each, a transmembrane domain, and a short cytosolic tail of 17 amino acids. The arrangement of the three extracellular motifs (lectin/EGF/complement-regulatory) in this murine protein, its human homologue (Bowen et al., 1989; Siegelman and Weissman, 1989; Tedder et al., 1989; Camerini et al., 1989; Tedder et al., 1990; Kishimoto et al., 1990), and in two independent cell adhesion molecules (CAMs) (ELAM-1 [Bevilacqua et al., 1989] and GMP-140/PADGEM [Johnston et al., 1989]) has led to the suggestion of the acronym LEC-CAM as a family designation (Stoolman, 1989). Bevilacqua, M. P., McEver, R. P., and co-workers (Geng et al., 1990) have proposed the name "selectins" for the family. The present study describes an ELISA assay that has been devised to measure the interaction between immunoaffinity-purified gp90^{MEL} and the carbohydrate-based ligand, PPME. The results described herein establish that gp90^{MEL}, as an isolated molecule, exhibits calcium-dependent lectin activity with the expected binding characteristics, including the ability to recognize fucoidin and a structurally related fucan from sea urchin egg jelly coat. The assay further reveals that a sulfated glycolipid of widespread occurrence in mammalian tissues (i.e., sulfatide) possesses ligand activity. It is concluded that the properties displayed by gp90^{MEL} in the ELISA assay account for critical adhesive functions manifested by this receptor at the lymphocyte surface.

Materials and Methods

Reagents

Bovine brain sulfatide [Gal(3-SO₄)β1-Ceramide], G_{M3}, galactosylceramide, cholesterol-3-sulfate, chondroitin sulfate A (bovine trachea), chondroitin sulfate B (porcine skin), chondroitin sulfate C (shark cartilage), colominic acid (*E. coli*), heparan sulfate (bovine intestinal mucosa), hyaluronic acid (human umbilical vein), keratan sulfate (bovine cornea), heparin (porcine in-

testinal mucosa), *N*-acetylneuraminic acid (*E. coli*), sialyllactose (85% α2-3 linkage and 15% α2-6, from bovine colostrum), BSA (3-X crystallized), *p*-nitrophenylphosphate, polyoxyethylene sorbitan monolaurate (Tween 20), and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All of the phosphorylated monosaccharides were from Sigma Chemical Co. and were in the sodium form except for Gal-1-P which was in the potassium form. Polyvinylsulfate (potassium salt) and sodium rhodizonate were from Aldrich Chemical Co. (Milwaukee, WI). An independent sample of brain sulfatide was purchased from Matreya, Inc. (Pleasant Gap, PA). Sulfatide was shown to be homogeneous by thin layer chromatography analysis. G_{M1}, G_{M2}, G_{D1a}, G_{D3}, G_{T1b}, and G_{Q1b} were purchased from Calbiochem-Behring Corp. (La Jolla, CA); and G_{D1b} from Bachem, Inc. (Torrance, CA). Synthetic Gal(6-SO₄)β1-Cer, fucoidin, and heparins (porcine mucosal and bovine lung) were generous gifts from Dr. D. Roberts (Laboratory of Structural Biology, National Institutes of Health, Bethesda, MD). Independent samples of fucoidin were purchased from ICN K&K Laboratories Inc. (Plainview, NY) and Sigma Chemical Co. PPME was purified from a crude yeast mannan preparation kindly provided by Dr. M. E. Slodki (Department of Agriculture, Northern Regional Research Center, Peoria, IL). Sea urchin egg jelly fucan from *S. purpuratus* was a generous gift of Dr. C. Glabe (Department of Molecular Biology and Biochemistry, University of California, Irvine, CA). Sulfate was determined by the barium-rhodizonate method described by Silvestri et al. (1982). Biotinylated-goat anti-rabbit IgG (H+L) and Vectastain ABC-alkaline phosphatase kit were from Vector Laboratories, Inc. (Burlingame, CA). 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and octylβ-D-glucopyranoside were from Boehringer Mannheim (Indianapolis, IN).

The anti-PPME antiserum was prepared by injecting a New Zealand white rabbit with 100 μg of PPME emulsified in 2 ml of Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI). The antigen was administered in multiple intradermal and subcutaneous sites, and the animal was bled 7 wk later.

ELISA Assay

gp90^{MEL} antigen, purified by immunoaffinity chromatography as previously described (Lasky et al., 1989; Geoffroy and Rosen, 1989), was equilibrated in Dulbecco's PBS (0.68 mM calcium, 0.49 mM magnesium) containing either 1 mM CHAPS or 40 mM octylβ-D-glucopyranoside. The PPME-binding activity of gp90^{MEL} appeared to be more stable in octylβ-D-glucopyranoside than in CHAPS. 10 μl of antigen solution at specified concentrations was diluted into 90 μl of PBS in the wells of Immulon-2 microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). The dilution factor was chosen to reduce the detergent concentration below the critical micellar concentration. Adsorption was allowed to proceed overnight at 4°C. The wells were washed three times with PBS containing 0.1% Tween 20 (PBS-Tween). 200 μl of PBS containing 3% BSA and 0.02% NaN₃ was then added to each well to block nonspecific binding sites. After a 2-h incubation at 22°C with agitation, the wells were washed and then received 100 μl of PPME solution in 3% BSA-PBS or 3% BSA-PBS alone (control for background). After a 1-h incubation at 4°C, the wells were washed to remove unbound polysaccharide and 100 μl of rabbit anti-PPME antiserum (1:100 dilution into 3% BSA in PBS-Tween) was added to each well. This incubation and all subsequent incubations were allowed to proceed at 22°C with agitation. After 1 h of exposure to anti-PPME, the wells were washed and 100 μl per well of biotinylated goat anti-rabbit IgG (1:200 dilution into 3% BSA in PBS-Tween) was added and allowed to react for 1 h. The wells were again washed before the addition of 50 μl per well of Vector ABC-AP reagent in PBS-Tween made according to the manufacturer's specifications. After 30 min, the wells were washed again and color development was initiated by the addition of 100 μl per well of substrate solution (*p*-nitrophenylphosphate, 1 mg/ml in 10% [vol/vol] diethanolamine-HCl buffer, pH 9.8). Optical density readings were made at 405 nm with a (model 3550; BioRad Laboratories, Richmond, CA) microplate reader. Mean values were determined for two to three replicate wells. With PPME added in excess, a saturation level of binding was observed at ≥0.4 μg of gp90^{MEL} per well. In experiments measuring the ability of substances to compete with PPME for binding to immobilized gp90^{MEL}, a concentration of 0.2–0.5 μg per well of gp90^{MEL} was added and the substances (diluted into 50 μl of PBS) were allowed to react with the antigen for 1 h at 4°C. PPME (50 μl, in 6% BSA-PBS) was added to each well, and the plate was incubated for an additional 1 h at 4°C. The plate was then processed as described above. The concentrations of the inhibitors are expressed as final concentrations after addition of the PPME. To test lipids as inhibitors, samples dissolved in chloroform-methanol 1:1 were dried in glass test tubes under a stream of nitrogen, and then suspended in PBS by brief sonication (model D-50; Branson Sonic

Power Co., Chicago, IL). All of the inhibitory effects documented in this study were observed in at least two independent experiments.

In Vitro Lymphocyte-HEV Adherence Assay

The assay used was slightly modified from the original assay of Stamper and Woodruff (1976). This highly validated assay measures the binding of lymphocytes to profiles of HEV exposed in cryostat-cut sections of lymphoid organs. Mouse peripheral lymph nodes (axillary, brachial, and cervical) were sectioned and processed as previously described (Stoolman et al., 1984). Lymphocytes at 1×10^7 cells per ml were pretreated for 30 min at 4°C with the aqueous suspension of lipids and then layered over paraformaldehyde-fixed tissue sections in the continued presence of the lipids. Lymphocyte attachment to HEV was measured by digital morphometry (Rosen et al., 1985) as the number of lymphocytes bound per unit area of HEV. Values were computed as percentages of control binding in the absence of inhibitors. Four to five replicate slides were used for each experimental condition and for the control condition.

Results

Development of an ELISA Assay

Our strategy took advantage of previous work in which transmembrane adhesion proteins have been coated onto plastic wells in a functionally active form (Martin and Springer, 1987; Staunton et al., 1989). Immunoaffinity-purified gp90^{MEL} (>90% pure by SDS-PAGE analysis with silver staining visualization) suspended in either CHAPS or octyl β -D-glucopyranoside detergent was diluted into Dulbecco's PBS (containing calcium and magnesium) to reduce the detergent concentration below the critical micellar concentration and then adsorbed overnight onto Immulon-2 microtiter wells. After washing of the wells and blockage of nonspecific sites with BSA, the wells were exposed to PPME in solution. PPME that bound to gp90^{MEL} in the wells was detected with an anti-PPME rabbit antiserum used in conjunction with a biotinylated second antibody and ABC-

alkaline phosphatase. Quantification was accomplished with a spectrophotometric microtiter plate reader. Fig. 1 A demonstrates that the binding of PPME to immobilized gp90^{MEL} increased with increasing amounts of gp90^{MEL} added to the wells. For a fixed amount of gp90^{MEL}, saturation was approached at a PPME concentration of >10 μ g/ml (Fig. 1 B). Background, defined as the signal in the absence of added PPME, was subtracted from all values in these experiments and those that follow. Typically, this background represented ≤ 25 –50% of the total signal but was very stable within each experiment. Assays carried out with preimmune serum produced ≤ 10 % of the signal seen with the anti-PPME serum. To confirm that gp90^{MEL} and not a contaminant was responsible for the PPME interaction, the effect of the MEL-14 mAb was tested. As shown in Fig. 2, this antibody completely inhibited binding of PPME, whereas the class-matched control antibody anti-T200 (30G12, anti-CD45) had no significant effect. As expected for a calcium-dependent lectin interaction, EGTA at 10 mM, which reduced free calcium to $\sim 10^{-9}$ M, completely blocked the interaction with PPME (Fig. 2). In a quantitative study of the calcium requirement, half-maximal binding of PPME to gp90^{MEL} occurred at $\sim 10^{-5}$ M Ca²⁺ in the presence or absence of 1 mM magnesium (Yednock, T. A., M. S. Singer, Y. Imai, and S. D. Rosen, manuscript in preparation).

Effects of Monosaccharides and Polysaccharides

Our previous results established that M6P and FIP were selective inhibitors of *in vitro* lymphocyte binding to PN HEV as compared to mannose-1-phosphate, fructose-6-phosphate and several other phosphorylated monosaccharides. When tested in the ELISA assay, these monosaccharides exhibited a similar inhibitory profile. 50% inhibition of PPME binding to gp90^{MEL} (Fig. 3) was achieved at 7 mM for both

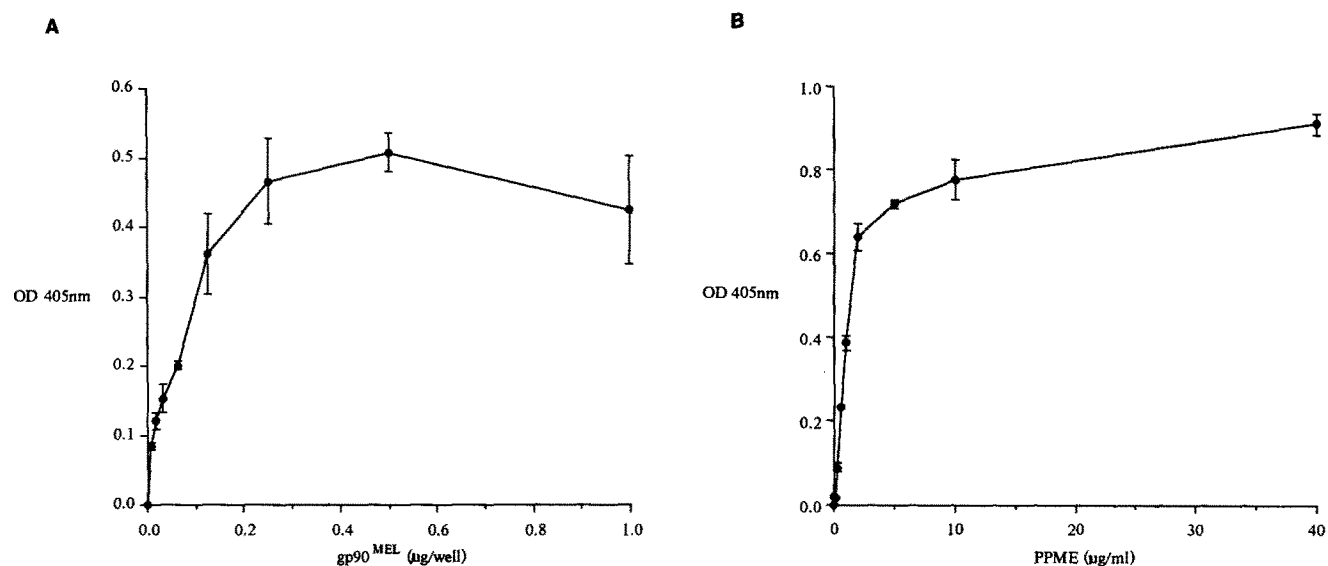


Figure 1. Binding of PPME to gp90^{MEL}. (A) The indicated amounts of gp90^{MEL} were allowed to bind to the wells of a microtiter plate and then tested for PPME binding (final concentration of 20 μ g/ml) as described in Materials and Methods. The y-axis indicates the optical density readings at 405 nm after subtraction of the background (no added PPME condition). The values represent means of duplicate determinations. Error bars denote half-ranges of variation. (B) Variable amounts of PPME were bound to a fixed amount of immobilized gp90^{MEL} (0.4 μ g added per well). The error bars indicate SEMs based upon three replicate determinations.

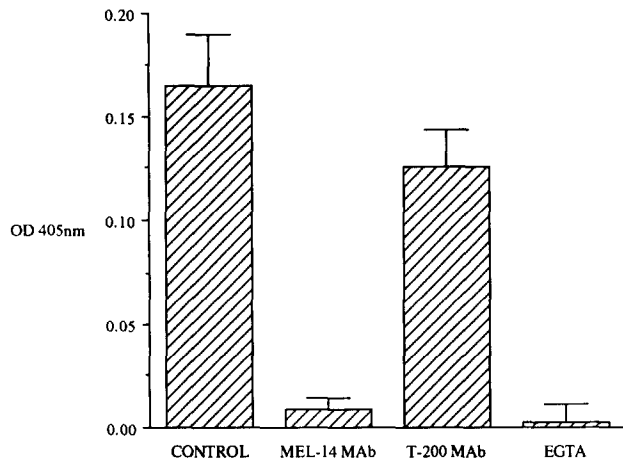


Figure 2. Effects of MEL-14 mAb, T-200 mAb, and EGTA on PPME binding to gp90^{MEL}. Before addition of 50 μ l of PPME (2 μ g/ml) to immobilized gp90^{MEL}, 50 μ l of MEL-14 mAb (50 μ g/ml), T-200 mAb (50 μ g/ml), 20 mM EGTA or 3% BSA-PBS alone were added to the wells and allowed to incubate for 1 h at 4°C. The error bars indicate SEMs based upon three replicate determinations. The concentration of added gp90^{MEL} was 0.5 μ g per well.

M6P and F1P, respectively, while M1P and F6P required two- to threefold higher concentrations. Galactose-1-phosphate, galactose-6-phosphate, and glucose-1-phosphate were equivalent to F6P (not shown). These differences among the phosphorylated monosaccharides were statistically significant and were consistently observed in several experiments with different batches of sugars. Neutral monosaccharides (D-mannose, L-fucose, D-fucose, D-fructose, and D-galactose) were inactive in this assay at >400 mM (data not shown). Also, sialic acid as free 5 *N*-acetylneuraminic acid (Neu5Ac) or as sialyllactose was three- to fivefold less active on a molar basis than M6P (not shown) and thus was comparable to F6P and the other relatively inactive phosphorylated monosaccharides.

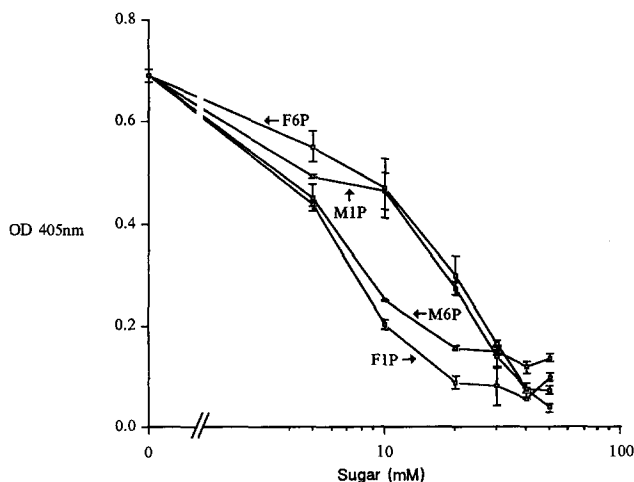


Figure 3. Effects of phosphorylated monosaccharides on PPME binding to gp90^{MEL}. The abscissa indicates the final sugar concentration. The concentration of added gp90^{MEL} was 0.3 μ g/well and the amount of PPME was 0.1 μ g/well. Values are means of duplicate determinations. The error bars denote half ranges of variation.

Among the charged polysaccharides, fucoidin and sea urchin egg jelly fucan (a sulfated fucose homopolysaccharide) were both very potent inhibitors on a mass basis in the ELISA assay (Fig. 4 A). With three different preparations of fucoidin in several experiments, 50% inhibition was observed at 0.1–0.4 μ g/ml, equivalent to 1–4 nM, based on an average molecular weight of 100,000 D (Roberts and Ginsberg, 1988). Hence, on a molar basis, fucoidin was six orders of magnitude more potent than M6P. The egg jelly fucan, with a molecular weight >10⁶ D was even more active than fucoidin per unit molecule. Other charged polysaccharides, including chondroitin sulfates A, B, and C, heparan sulfate, keratan sulfate, hyaluronic acid, and colominic acid (a homopolymer of α 2–8 linked sialic acid) were at least 3,000-fold less active per unit weight than fucoidin or egg jelly fucan. On the basis of sulfate concentration, fucoidin and egg jelly fucan were also three orders of magnitude more effective than the other sulfated polysaccharides (Fig. 4 B) as well as polyvinylsulfate, a highly sulfated hydrocarbon. A direct comparison of the sulfate content of the various sulfated compounds established that sulfate content was not correlated with inhibitory efficacy (Table I).

Effects of Glycolipids

Preliminary experiments (True, D. D., unpublished observations) had suggested that microspheres coated with sulfatide (i.e., Gal(3-SO₄) β 1-Cer) could bind to mouse lymphocytes. Since this interaction was partially inhibited (\sim 50%) by MEL-14 mAb and by EDTA, it seemed likely that sulfatide possessed ligand activity for gp90^{MEL}. To explore this possibility further, we determined whether brain sulfatide in an aqueous suspension could inhibit the binding of PPME to gp90^{MEL} in the ELISA assay. Sulfatide at 10 μ g/ml almost completely prevented PPME binding to immobilized gp90^{MEL} (Table II) with 50% inhibition at \sim 1 μ g/ml or 1 μ M SO₄ (Fig. 4). Gal(6-SO₄) β 1-Cer, cholesterol 3-SO₄, galactosylceramide (Gal β 1-Cer), and several gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1b}) had no activity at the same concentration and much less, if any, activity when tested at a 10-fold higher concentration (Table II). Additionally, the gangliosides G_{M2}, G_{M3}, G_{D3}, and G_{Q1b} showed little or no activity at 100 μ g/ml.

Effect of Sulfatide on Lymphocyte Binding to HEV

Consistent with the inhibitory effect of sulfatide on gp90^{MEL} in the ELISA assay, this glycolipid also blocked lymphocyte attachment to PN HEV in a dose-dependent manner in the *in vitro* adherence assay, whereas Gal(6-SO₄)-Cer and cholesterol-3-SO₄ displayed minimal activity (Fig. 5). None of the lipids adversely affected the viability of the test lymphocytes, as determined by trypan blue exclusion.

Discussion

Our previous studies had indicated a close relationship between the calcium-dependent, lectin-like receptor detected on the lymphocyte cell surface by PPME and the protein (i.e., gp90^{MEL}) recognized by the adhesion-blocking mAb, MEL-14. The finding of a C-type lectin domain at the extracellular terminus of gp90^{MEL} lent strong support to the contention that the relationship was one of identity. Here, we establish identity by showing directly that immunoaffinity-

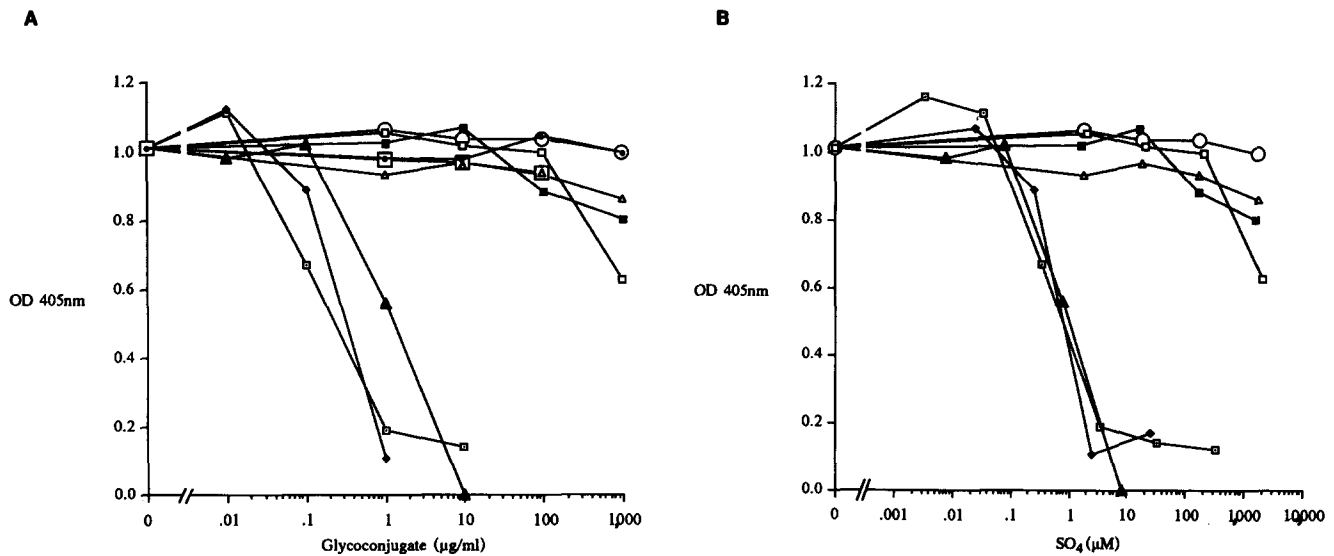


Figure 4. Effects of polysaccharides and sulfatide on PPME binding to gp90^{MEL}. The following glycoconjugates were tested for their ability to compete the binding of PPME (0.1 µg/well) to immobilized gp90^{MEL} (0.2 µg/well): (□) egg jelly fucan; (◆) fucoidin (Sigma Chemical Co.); (■) chondroitin sulfate B; (○) chondroitin sulfate C; (◻) heparan sulfate; (▲), sulfatide; (△) keratan sulfate; (□) hyaluronic acid; and (●) colominic acid. Chondroitin sulfate A was also tested and found to be inactive at 1,000 µg/ml (not shown). Values are means of duplicate determinations. The half ranges of variation were typically <8% of the means. (A) Glycoconjugate concentrations are given as µg/ml. (B) Concentrations are specified on the basis of sulfate concentration as determined for each glycoconjugate by the barium-rhodizonate method.

purified gp90^{MEL}, immobilized on plastic, can bind to PPME with the predicted characteristics. As determined in the ELISA assay, the interaction is saturable, calcium-dependent, and selectively inhibited by MEL-14 mAb. With respect to sugar inhibition, M6P and F1P are two to threefold more active than M1P and F6P in competing PPME binding to gp90^{MEL}, in agreement with the relative activities of these sugars in blocking both PPME bead binding to lym-

phocytes and lymphocyte attachment to PN HEV (Yednock et al., 1987b). Fucoidin, a fucose-rich polysaccharide with sulfation primarily on the 4-position of fucose (DeAngelis and Glabe, 1987), is substantially more potent than PPME as an inhibitor of lymphocyte attachment to PN HEV (Yednock et al., 1987b; Stoolman et al., 1984). Heretofore, it has not been clear whether this polysaccharide achieves inhibition of lymphocyte attachment to HEV by binding to the same subsite of gp90^{MEL} or even to the same receptor as PPME (Brandley et al., 1987; Yednock et al., 1987a). We have now shown that fucoidin directly blocks PPME binding to gp90^{MEL} in the ELISA assay and thus appears capable of a direct interaction with this lymphocyte receptor. The sea urchin egg jelly fucan, a homopolysaccharide of L-fucose with sulfation on the C-4 position of the sugar and hence clearly related to fucoidin (DeAngelis and Glabe, 1987), is even more active as an inhibitor. Testing of several other charged polysaccharides (hyaluronic acid, colominic acid, heparins, chondroitin sulfates, and keratan sulfate) and a sulfated hydrocarbon, many of which have charge densities comparable to or exceeding that of the fucans (Table I; DeAngelis and Glabe, 1987), establishes that the inhibitory effects of the fucans are highly selective and cannot merely be explained on the basis of charge density.

Table I. Sulfate Contents and Inhibitory Activities of Sulfated Macromolecules

Substance	SO ₄	Concentration for 50% inhibition
	nmol/µg	µg/ml
sulfatide (Matreya, Inc.)	0.7	ND
sulfatide (Sigma Chemical Co.)	0.8	0.7-1.3
cholesterol-3-sulfate	1.2	>100
chondroitin sulfate A	1.4	>1,000
chondroitin sulfate B	1.6	>1,000
chondroitin sulfate C	1.8	>1,000
keratan sulfate	1.8	>1,000
fucoidin (Sigma Chemical Co.)	2.1	ND
heparan sulfate	2.1	>1,000
fucoidin (ICN K&K Laboratories)	2.5	0.1-0.4
heparin (porcine intestinal)	2.8	100
heparin (bovine lung)	3.0	19
heparin (porcine mucosa)	3.0	110
egg jelly fucan	3.4	0.2
polyvinylsulfate	6.0	>1,000

The substances listed were analyzed for sulfate content. The values represent means of duplicate determinations. The half-range variations were <2% of the means in all cases. Substances are listed in order of increasing sulfate content. Concentrations for 50% inhibition in the ELISA assay were determined from the dose-response curves of Fig. 4 and from dose-response curves not shown.

In an attempt to identify additional carbohydrate-based inhibitors of the PPME/gp90^{MEL} interaction, we tested a series of glycolipids in the ELISA assay. Sulfatide [Gal(3-SO₄)β1-Cer], which preliminary experiments indicated might interact with gp90^{MEL}, is now confirmed to be active. In contrast, cholesterol-3-sulfate and the synthetic glycolipid, Gal(6-SO₄)β1-Cer are substantially less active. The presence of the sulfate moiety is critical to sulfatide's activity, since galactosylceramide (Galβ1-Cer) is inactive. The functional relevance of these observations with the ELISA assay

Table II Effects of Lipids on PPME Binding to gp90^{MEL}

Experiment	Inhibitor	$\mu\text{g/ml}$	OD 405 nm
1	None	—	0.596 ± 0.025
	Sulfatide (Sigma Chemical Co)	10	0.030 ± 0.005
	Sulfatide (Matreya, Inc)	10	0.019 ± 0.006
	Galactosylceramide	10	0.668 ± 0.021
	Gal(6-SO ₄) β 1-Cer	10	0.612 ± 0.016
	Cholesterol 3-SO ₄	10	0.583 ± 0.014
2	None	—	0.291 ± 0.065
	Sulfatide (Sigma Chemical Co)	10	0.023 ± 0.018
	G _{M1}	10	0.208 ± 0.014
	G _{D1a}	10	0.328 ± 0.017
	G _{D1b}	10	0.256 ± 0.053
	G _{T1b}	10	0.287 ± 0.017
3	None	—	0.362 ± 0.064
	Sulfatide (Matreya, Inc)	100	0.0
	G _{M1}	100	0.310 ± 0.026
	G _{M2}	100	0.248 ± 0.042
	G _{M3}	100	0.378 ± 0.019
4	None	—	0.431 ± 0.047
	Sulfatide (Matreya, Inc)	10	0.0
	G _{D1a}	100	0.331 ± 0.025
	G _{D1b}	100	0.313 ± 0.011
	G _{T1b}	100	0.257 ± 0.026
	G _{D3}	100	0.555 ± 0.021
	G _{Q1b}	100	0.288 ± 0.032

The substances were tested in the ELISA assay as described under Materials and Methods. The errors denote SEMs based upon three replicate determinations. Structures of Glycolipids: Galactosylceramide, Gal β 1-Cer, Sulfatide, Gal(3-SO₄) β 1-Cer, G_{M1}, Gal β 1-3GalNAc β 1-4[Neu5Ac α 2-3]Gal β 1-4Glc β 1-1Cer, G_{D1a}, NeuAc α 2-3Gal β 1-3GalNAc β 1-4[NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer, G_{D1b}, Gal β 1-3GalNAc β 1-4[NeuAc α 2-8NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer, G_{T1b}, NeuAc α 2-3Gal β 1-3GalNAc β 1-4[NeuAc α 2-8NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer, G_{M2}, GalNAc β 1-4[NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer, G_{M3}, NeuAc α 2-3Gal β 1-4Glc β 1-1Cer, G_{D3}, NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc β 1-1Cer, G_{Q1b}, NeuAc α 2-8NeuAc α 2-3Gal β 1-3GalNAc β 1-4[NeuAc α 2-8NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer

is borne out by direct lymphocyte adhesion assays. Sulfatide, like PPME and fucoidin, inhibits lymphocyte attachment to PN HEV in the Stamper-Woodruff in vitro adherence assay, while Gal(6-SO₄) β 1-Cer and cholesterol-3-sulfate are essentially inactive at the concentrations tested. On a molar basis, Gal(3-SO₄) β 1-Cer is over three orders of magnitude more potent than M6P in both the in vitro adherence assay and the ELISA assay. It seems probable that the enhanced efficacy of sulfatide (monomeric molecular weight of 807) relative to M6P and FIP derives, at least in part, from its micellar organization in aqueous solution. Similarly, the high avidity of PPME for immobilized gp90^{MEL} (Fig 1 B) is likely attributable to the multivalent presentation of M6P residues in the polysaccharide.

The C-type lectin domain of gp90^{MEL} is almost certainly involved in the binding of the intact receptor to PPME. In addition to the sugar selectivity of the interaction and the requirement for calcium (two hallmarks of a C-type lectin), PPME binding is blocked by the MEL-14 mAb, which detects an epitope in the lectin domain of gp90^{MEL} (Bowen et al, 1990). Based on their activity in competing PPME binding in the ELISA assay, it seems likely that the fucans as well as sulfatide also interact with the lectin domain of gp90^{MEL},

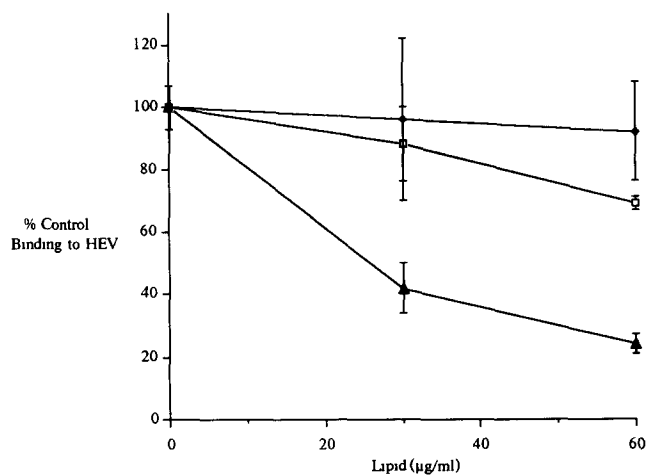


Figure 5 Effects of sulfated lipids on lymphocyte attachment to PN HEV. Lymphocytes, exposed to lipids at the indicated concentrations, were tested for their ability to attach to PN HEV in the Stamper-Woodruff in vitro adherence assay as described in Materials and Methods. Binding is computed as a percentage of the control value determined in the absence of added lipid. Error bars denote SEMs computed from four to five replicate tissue sections. The symbols are (▲) sulfatide, (□) Gal(6-SO₄) β 1-Cer, and (◆) cholesterol-3-SO₄.

but these predictions remain to be confirmed in direct binding assays.

It would be satisfying to account for the most active carbohydrates in the ELISA assay on the basis of some common structural motif. M6P and FIP do share structural features, which appear to account for their highly selective binding to lysosomal sorting receptors (Tong and Kornfeld, 1989). However, in the case of the gp90^{MEL} the difference in potency between these sugars and the other phosphorylated sugars is not sufficiently great to warrant the proposal of a detailed model. Moreover, our finding that fucans and sulfatide are highly active relative to other charged polysaccharides and glycolipids does not justify modeling on the basis of their constituent sugars (i.e., fucose-4-sulfate and galactose-3-sulfate), since the potential contributions of glycosidic linkage and multivalency to the activity of these glycoconjugates are not known at present. The eventual identification of monosaccharides or structurally-defined oligosaccharides with potent and selective inhibitory activities will greatly facilitate efforts to elucidate the critical features of carbohydrate structure required for recognition by gp90^{MEL}.

Our previous work has shown that gp90^{MEL} functions as an adhesion molecule by binding directly to the endothelium of PN HEV through an interaction with an as yet uncharacterized HEV-ligand. It is also known that the lectin activity of gp90^{MEL} is required for its interaction with the endothelium (Geoffroy and Rosen, 1989; Watson et al, 1990). Consequently, inhibitory sugars for gp90^{MEL} (e.g., M6P, FIP, etc.) are presumed to be structural mimics of a carbohydrate recognition determinant present on the HEV-ligand. Since the inhibitory sugars are all anionic, it is strongly suspected that the actual recognition determinant is a negatively charged carbohydrate. Additionally, the fact that the C-type lectin motif of gp90^{MEL} contains significantly more basic amino

acids than any other member of this lectin family is consistent with the possibility of a negatively charged sugar ligand (Lasky et al., 1989). In this regard, it is intriguing that the PN HEV-ligand is inactivated by sialidases or by pretreatment of the HEV with *Limax flavus* agglutinin, a sialic acid-specific lectin (Rosen et al., 1989; Rosen et al., 1985; True, D. D., M. S. Singer, L. A. Lasky, and S. D. Rosen, manuscript submitted for publication). Despite the fact that free sialic acid (Neu5Ac) and various glycosidically-linked forms of sialic acid (e.g., sialyllactose, colominic acid, and gangliosides) do not compete effectively in the ELISA assay, it remains to be determined whether sialic acid (perhaps in an unusual form or linkage) is a recognition determinant of the HEV-ligand. (Sialic acid has considerable informational potential in that it occurs in nature in a multitude of different forms and linkages [Schauer, 1985].)

In view of the activity of sulfatide and sulfated fucans in the ELISA assay, sulfated glycoconjugates must also be considered as possible candidates for the HEV-ligand. Interestingly, HEVs are notable for their high level of sulfate incorporation into macromolecules, but little information is available about the biochemical nature of these sulfated macromolecules (Andrews et al., 1982, 1983). Among the prominent sulfated macromolecules found in higher cells are sulfated glycosaminoglycans. However, the results obtained with the ELISA assay indicate that the most commonly occurring forms of these glycosaminoglycans (heparin, heparan sulfate, keratan sulfate, chondroitin sulfate) are not directly involved as HEV-ligands. Although sulfatide is present in various mammalian tissues (reviewed in Roberts, 1987; Roberts and Ginsberg, 1988), there is no information on its occurrence in HEV. However, the HEV-ligand for gp90^{MEL} is unlikely to be a glycolipid, since the binding sites on PN HEV for a soluble recombinant form of gp90^{MEL} are not substantially affected by extraction of the tissue with chloroform-methanol-H₂O solvent mixtures (Rosen, S. D., M. S. Singer, and L. A. Lasky, unpublished observation). An immunologically defined candidate for the HEV-ligand of gp90^{MEL} is the antigen recognized by the MECA-79 mAb (Streeter et al., 1988b). This antibody reacts preferentially with PN HEV and blocks lymphocyte attachment to the HEV. Future experiments must address whether the MECA-79 antigen or an associated molecule contains a carbohydrate determinant that is recognized by gp90^{MEL}. The identification and structuring of this postulated carbohydrate represent extremely important areas for future investigation.

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