

Phosphomannosyl-derivatized Beads Detect a Receptor Involved in Lymphocyte Homing

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Abstract. Recirculating lymphocytes initiate extravasation from the blood stream by binding to specialized high endothelial venules (HEV) within peripheral lymph nodes (PN) and other secondary lymphoid organs. We have previously reported that lymphocyte attachment to PN HEV is selectively inhibited by mannose-6-phosphate (M6P) and related carbohydrates (Stoolman, L. M., T. S. Tenforde, and S. D. Rosen, 1984, *J. Cell Biol.*, 99:1535–1540). In the present study, we employ a novel cell-surface probe consisting of fluorescent beads derivatized with PPME, a M6P-rich polysaccharide. PPME beads directly identify a carbohydrate-binding receptor on the surface of mouse lymphocytes. In every way examined, lymphocyte attachment to PPME beads (measured by flow cytometry) mimics the interaction of lymphocytes with

PN HEV (measured in the Stamper–Woodruff *in vitro* assay): both interactions are selectively inhibited by the same panel of structurally related carbohydrates, are calcium-dependent, and are sensitive to mild treatment of the lymphocytes with trypsin. In addition, thymocytes and a thymic lymphoma, S49, bind poorly to PPME beads in correspondence to their weak ability to bind to HEV. When the S49 cell line was subjected to a selection procedure with PPME beads, the ability of the cells to bind PPME beads, as well as their ability to bind to PN HEV, increased six- to eightfold. We conclude that a carbohydrate-binding receptor on mouse lymphocytes, detected by PPME beads, is involved in lymphocyte attachment to PN HEV.

LYMPHOCYTES continuously recirculate throughout the widely scattered lymphoid organs of the body, alternating their course between the blood and lymphatic systems. This process of lymphocyte recirculation maximizes the contact of lymphocytes with antigens that are sequestered within secondary lymphoid tissues. Blood-borne lymphocytes initiate extravasation by a highly specific adhesive interaction with the distinctive cuboidal endothelial cells of high endothelial venules or HEV¹ (Gowens and Knight, 1964; Marchesi and Gowens, 1964). HEV are found in secondary lymphoid organs, such as peripheral lymph nodes and gut-associated Peyer's patches, and in sites of chronic inflammation (Ehrlich, 1929; Graham and Shannon, 1972). Once lymphocytes have bound to HEV, they migrate across the endothelium (most likely passing between adjacent endothelial cells) and enter the parenchyma of the lymphoid tissue. The ability of lymphocytes to attach to HEV varies with the developmental stage and functional subclass of the lymphocyte and with the anatomical site of the lymphoid organ (Stamper and Woodruff, 1976; Stevens et al.,

1982; Dailey et al., 1982; Kraal et al., 1983; Reichert et al., 1983; Pals et al., 1986). Lymphocyte–HEV interactions may regulate the representation of lymphocyte classes among the resident populations of lymphoid organs by specifically governing lymphocyte entry into these sites (Butcher et al., 1980; Stevens et al., 1982).

Stamper and Woodruff (1976) have developed an *in vitro* assay that has greatly facilitated the study of lymphocyte–HEV binding and the identification of potential lymphocyte adhesive factors (homing receptors) that mediate this interaction. The *in vitro* assay is based upon the selective attachment of exogenously added viable lymphocytes to HEV that are exposed in cryostat-cut sections of lymphoid organs. Lymphocyte attachment to HEV in this frozen section assay reflects the *in vivo* interaction with remarkable fidelity (Stamper and Woodruff, 1976, 1977; Woodruff et al., 1977; Butcher et al., 1979a, b; Stevens et al., 1982). Employing a slightly modified version of the frozen section assay in rat, we found that the attachment of lymphocytes to peripheral lymph node (PN) HEV is selectively inhibited by D-mannose and L-fucose among an extensive series of neutral monosaccharides (Stoolman and Rosen, 1983). This initial observation was of interest because there are several known examples of carbohydrate-binding receptors that recognize common structural features of L-fucose and D-mannose (Ka-

1. *Abbreviations used in this paper:* FIP, D-fructose-1-phosphate; HEV, high endothelial venule(s); MIP, D-mannose-1-phosphate; M6P, D-mannose-6-phosphate; MNL, mesenteric lymph node lymphocytes; PN, peripheral lymph node; PPME, phosphomannan monoester core from *Hansenula hostii*, Y-2448.

meiyama et al., 1979; Townsend and Stahl, 1981; Stahl and Gordon, 1982).

We further showed that phosphorylation of D-mannose on the sixth carbon increases its inhibitory potency by 25–50-fold (Stoolman et al., 1984). Thus, at a concentration of 10 mM, mannose-6-phosphate (M6P) and fructose-1-phosphate (F1P), a structural analogue of M6P (Kaplan et al., 1977), inhibit lymphocyte attachment to PN HEV by 80–90%, whereas other phosphorylated monosaccharides, such as mannose-1-phosphate (M1P), fructose-6-phosphate, glucose-6-phosphates (1 and 6), and galactose-6-phosphates (1 and 6), have no significant inhibitory activity. In addition, two polysaccharides, PPME (a phosphomonoester mannan fragment containing only mannose and mannose-6-phosphate in a 5:1 ratio) and fucoidin (a sulfated polysaccharide rich in L-fucose), are potent inhibitors of lymphocyte attachment to PN HEV. Other glycoconjugates (charged or neutral) have little or no inhibitory activity. Preincubation experiments, in which lymphocytes or lymph node sections were exposed to inhibitors and then washed before the frozen section assay, indicated that PPME and fucoidin must interact with the lymphocytes rather than the HEV to produce their inhibitory effects. Based on these results, we proposed that a lymphocyte cell surface receptor that recognizes M6P and related sugars is involved in lymphocyte attachment to PN HEV (Stoolman et al., 1984).

In the present study we provide direct evidence for this model. We describe a novel cytochemical probe consisting of small fluorescent beads covalently derivatized with the M6P-rich PPME. Using this probe in conjunction with flow cytometry, we directly establish that mouse lymphocytes express a cell-surface receptor with specificity for M6P-like ligands. Under all conditions tested, the expression and activity of this receptor, as detected with PPME beads, correlate with the ability of lymphocytes to bind to PN HEV. In addition, selection of variants of a lymphoma cell line for their ability to bind PPME beads results in a parallel and stable increase in their ability to bind to PN HEV.

Materials and Methods

Materials

Heparin (H3125), chondroitin sulfate (C3254), kappa carrageenan (C1263), dermatan sulfate (C4259), all phosphorylated monosaccharides, BSA (A4503), cyanogen bromide (C6388), fetuin type III (F2379), hexamethyldisilazane (H4875), trimethylchlorosilane (T4252), trypsin type III (T8253), trypsin inhibitor type II-O (T9253), and 2-mercaptoethanol (M6250) were obtained from Sigma Chemical Co. (St. Louis, MO). Fucoidin was purchased from K & K Laboratories, Inc. (Plainview, NY). The yeast mannans, mnn 1 and mnn 2, were kindly supplied by Dr. C. E. Ballou (Department of Biochemistry, University of California, Berkeley, CA) and PPME, the polyphosphomonoester core from *Hansenula hostii* phosphomannan (Slodki et al., 1973; Kaplan et al., 1978), was a generous gift of Dr. M. E. Slodki (U. S. Department of Agriculture, Northern Regional Research Center, Peoria, IL). Triethylamine (HPLC grade) was obtained from Pierce Chemical Co. (Rockford, IL). Fetuin was desialylated by acid hydrolysis as described by Spiro (1960). Paraformaldehyde was purchased from MCB (Cincinnati, OH). Phthalic acid (gold label) and glutaraldehyde were from Aldrich Chemical Co. (Milwaukee, WI). Disodium EDTA and boric acid were obtained from Mallinckrodt (Paris, KY). Toluidine Blue is distributed by Roboz Surgical Instrument Co. (Washington, DC).

Preparation of Cell Suspensions

Cell-suspension buffer was either Dulbecco's PBS, or MEM in Earle's salts buffered at pH 7.4 with 40 mM tricine. Except where noted, BSA (1 mg/ml)

was added to the cell-suspension buffer to minimize cell clumping. Peripheral lymphocytes were obtained from mesenteric and peripheral lymph nodes (brachial, cervical, and axillary) of cervically dislocated mice (BALB/c female, 6–10 wk). For thymocyte preparation, the thymus was carefully dissected and cleaned to exclude the parathymic lymph nodes. The lymphoid tissues were teased with fine needles and flushed with cell-suspension buffer. After centrifugation (5 min, 250 g, 4°C) the cells were resuspended in fresh buffer and adjusted to an appropriate concentration of viable (trypan blue-excluding) cells.

Treatment of Cells with Trypsin

The conditions for trypsin treatment were adapted from a report by Woodruff et al. (1977). Lymphocytes (1×10^7 cells/ml) were incubated in PBS \pm 20 μ g trypsin/ml with or without trypsin inhibitor (200 μ g/ml) for 5 min at 37°C. After incubation, the remaining tubes received trypsin inhibitor (200 μ g/ml), and the samples were centrifuged for 10 s in a model B microfuge (Beckman Instruments, Inc., Fullerton, CA). The cells were resuspended in PBS (containing 1 mg/ml BSA) at 4°C, adjusted to a concentration of 1×10^7 /ml, and then immediately employed in the HEV- and bead-binding assays (see below).

HEV Attachment Assay

The procedure, based upon the original Stamper and Woodruff assay (1976), has been described in detail before (Stoolman, et al., 1984). In brief, peripheral lymph nodes were dissected from mice as described above. The tissues were frozen at -160°C and 10- μ m sections were cut on a cryostat (International Equipment Co., Needham Hts., MA; -20°C). The frozen sections were transferred to glass slides in the center of 14-mm wells (formed by an epoxy-coating; Carlson Scientific, Inc., Peotone, IL, cat. No. 100314) and allowed to air-dry at room temperature for 1–3 h. Shortly before the assay, the sections were fixed in freshly prepared paraformaldehyde (1% in 0.1 M Na cacodylate, pH 7.3) for 20 min at 4°C, washed several times in PBS (4°C), and rinsed in the appropriate cell-suspension buffer (4°C). Carbohydrates, to be tested as potential inhibitors, were exposed to lymphocytes at 4°C for 30 min before the addition of the lymphocytes to the sections, and were present throughout the assay period. The slides were positioned on a metal tray (supported by packed ice). Cells were layered onto the slides (110 μ l/well) at a concentration of $3\text{--}10 \times 10^6$ /ml. The slides were then gyrated at 80 rpm for 30 min on a gyratory shaker (model G-24; New Brunswick Scientific Co., Inc., Edison, NJ; 3/4 inch radius of gyration). The cell suspensions were carefully decanted and the sections were refixed in 2.5% glutaraldehyde in PBS at 4°C for 20 min. The slides were then washed in PBS, stained in 0.5% toluidine blue in a 20% solution of ethanol for 15–60 s, rinsed in 100% ethanol, and quickly mounted under glass coverslips with Immu-mount (cat. No. 999X0402; Shandon Southern Instruments Inc., Sewickley, PA).

Quantification of Lymphocyte Attachment

Exogenously added lymphocytes that have bound to a section are easily distinguished from their sectioned counterparts because they stain more intensely (being whole cells) and because they lie above the plane of the section. HEV are readily identified in the stained sections by their prominent basement membranes and distinctive "plump" endothelial cells.

In some experiments, lymphocyte attachment was quantified as described by Stoolman and Rosen (1983). Briefly, lymphocytes that had bound to HEV were counted in ≥ 10 nonoverlapping fields of lymph node cortex (200 \times magnification). The degree of binding was expressed as the mean number of lymphocytes bound to HEV per field. Four to five independent sections were counted for each treatment. In most experiments, an improved method of scoring was employed (Rosen et al., 1985) using a digitizing morphometry system (Bioquant, Nashville, TN). With this technique the index of binding is defined as the total number of lymphocytes bound to 15–30 segments of HEV divided by the combined area of HEV (in units of 10^{-4} μm^2). At least three independent sections were counted for each experimental condition, as indicated in the figure legends.

Preparation of Derivatized Beads

Amino-derivatized polystyrene beads were purchased from Covalent Technology Corp. (Ann Arbor, MI; Covaspheres FX, cat. No. 112030-30, now distributed by Duke Scientific Corp., Palo Alto, CA). PPME, chondroitin sulfate, heparin, and kappa carrageenan were activated with cyanogen bromide employing a procedure adapted from Glabe et al. (1983). 3 mg of car-

bohydrate in 0.3 ml H₂O was added to an equal volume of cyanogen bromide (100 mg/ml H₂O). The pH was adjusted to 11 with 1 N NaOH and maintained between pH 10.5 and 11.5 for 5 min by drop-wise addition of 0.2 N NaOH. The solution was immediately subjected to gel filtration on a 1 × 20 cm column of Sephadex G-75 (superfine) in 0.2 M sodium borate (pH 8.0) at 22°C. 1-ml fractions were collected and small aliquots were assayed for neutral sugar content by the phenol-sulfuric method (Dubois et al., 1956). The three fractions containing the highest concentration of carbohydrate were pooled and immediately mixed with 100 µl of beads (as purchased) per ml of solution. Nonderivatized beads were prepared by exposing beads to borate buffer that lacked carbohydrate. The tubes were then capped and gently rocked overnight at room temperature. Aliquots of the derivatized beads were frozen in liquid nitrogen and stored at -70°C. Cell binding activity (see below) was retained for at least 5 mo under these conditions.

Quantification of the Carbohydrate Substitution on Derivatized Beads

The amount of PPME conjugated to beads was determined by measuring the quantity of mannose released from beads by acid hydrolysis. Derivatized and nonderivatized beads were washed with H₂O and then dried under a stream of nitrogen. The bead samples were treated with 1 N methanolic-HCl at 100°C for 3 h under a nitrogen atmosphere in sealed tubes. The hydrolysis supernatant was removed, neutralized with redistilled pyridine, and dried under a stream of nitrogen. The residue was dissolved in a freshly prepared mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (5:2:1). The TMS-derivatized sugars were then analyzed by gas-liquid chromatography with flame-ionization detection (gas chromatograph, model 3700, Varian Associates, Inc., Palo Alto, CA; 30 meter DBI capillary column, J. W. Scientific, Rancho Cordova, CA). A glucose peak was detected in all bead hydrolysates, corresponding to the ficoll content of Covaspheres FX. This sugar served as an internal standard during the analysis. PPME bead hydrolysates contained an additional peak corresponding to mannose. Mannose detection was related to PPME content by hydrolyzing known amounts of PPME in the presence of nonderivatized beads. This procedure allowed the detection of <0.1 µg of PPME.

The substitution of chondroitin sulfate and heparin on beads was determined by measuring the amount of sulfate released from the derivatized beads by acid hydrolysis. Aliquots of washed beads were suspended in 0.5 N aqueous HCl and heated at 100°C for 60 min. The beads were removed by centrifugation (8,000 g, 5 min in a model B microfuge [Beckman Instruments, Inc.]) and the supernatants were dried under a stream of nitrogen. The residue was suspended in buffer (1.5 mM phthalic acid, adjusted to pH 8.9 with triethylamine) and analyzed for sulfate ions by ion-exchange HPLC (Vydac 3001C column [Western Analytical Products Co., Inc., Temecula, CA], 6000A pump [Waters Associates, Milford, MA], U6K injector, and 441 UV detector). For standards, nonderivatized beads were hydrolyzed in the presence of known quantities of chondroitin sulfate or heparin and the amount of detected sulfate was related to the quantity of the polysaccharide originally present. This procedure allowed the detection of 0.1 µg of heparin or chondroitin sulfate.

Bead-binding Assay

This assay was based on a procedure described by Mirro et al. (1981) in which antibody-derivatized fluorescent beads were employed to detect antigens on intact cells. Bead preparations (carbohydrate-conjugated or nonderivatized) were thawed (see above), diluted with cell-suspension buffer, and centrifuged for 20–30 min (8,000 g) in a model B microfuge at 7°C. The supernatant was removed with aspiration and replaced with fresh cell-suspension buffer. To disperse the bead pellet, the suspension was sonicated for 20 s (Branson Sonic Power Co., Danbury, CT; model 200, microtip probe, 20–30 W continuous power output). The final bead concentration ranged from a 1:20 to a 1:600 dilution of the original (as purchased) suspension (~7.5 × 10¹⁰ beads/ml). The dilution used for each experiment is indicated in the corresponding figure legend. 100 µl of the bead suspension plus an equal volume of suspended cells (1 × 10⁷ cells/ml) were added to individual wells of a flat-bottomed microtiter plate (Linbro/Titertek, 96 wells, 1.0 × 0.6 cm, Flow Laboratories, Inc., McLean, VA, cat. No. 76-203-05). The plate was centrifuged in a TJ-6 table top centrifuge (Beckman Instruments, Inc.; swinging plate-holders, TH-4 rotor) at 1100 rpm (225 g) for 12 min at 4°C. The plate was carefully placed on ice and left undisturbed for 60 min. The contents of each well were resuspended by repeated pipetting and then layered over 0.5 ml PBS, containing 7% BSA and 1 mM so-

dium azide, in 1.5-ml microfuge tubes on ice. The tubes were centrifuged at 1,000 rpm (185 g) for 5 min at 4°C in the TJ-6 centrifuge (swinging-bucket, TH-4 rotor). The supernatant, containing unbound beads, was carefully removed with aspiration. The cells were resuspended in 200 µl of PBS (4°C) by pipetting and were then fixed by the addition of 200 µl of 2% paraformaldehyde in PBS. The samples were analyzed by flow cytometry (see below) within 24 h. In some instances, the final cell pellet was resuspended in 400 µl of PBS without fixative, and the samples were analyzed immediately by flow cytometry. Fixation did not alter the mean fluorescent intensity of single cells decorated with beads.

Fluorescent Cell Analysis

Cells were analyzed with a fluorescence-activated research analyzer (FACS Analyzer; Becton-Dickinson, Paramus, NJ) in the Laboratory for Cell Analysis at the University of California, San Francisco. Electronic gates, based on Coulter volume, were established to restrict the analysis to single cells. The amplifier gain was adjusted such that the fluorescent intensity of a single bead was on the lower end of the logarithmic scale (see legend of Fig. 2). 5,000 single cells were counted for each sample and the data were displayed in histogram form, depicting the amount of fluorescence per cell vs. the number of cells at each fluorescence level. The average fluorescence of the single cell population was electronically integrated and recorded. For comparisons, the analyzer values (on a log scale with a base of 1.0306) were converted to linear values. The control level of PPME bead binding (mean fluorescence) was established with untreated peripheral lymphocytes and was set at 100%. All experimental values represent the average of two 5,000-cell replicate samples from independent tubes. The error bars shown on figures indicate the range of experimental values. For most comparisons, the level of nonderivatized bead binding was subtracted from the level of derivatized bead binding to yield an index of specific bead binding. The degree of specific bead binding was a function of the particular bead preparation and the working dilution of the bead suspension. These factors, however, had a minimal effect on the level of nonderivatized bead binding, which was <1 bead/cell in all experiments (see Fig. 2). For comparison of separate experiments, each figure legend indicates the bead dilution and the degree of specific PPME bead binding (relative to nonderivatized beads).

Studies with the Lymphoma Cell Line, S49

S49 cells (T cell lymphoma, ATCC No. TIB 28) were obtained from the Cell Culture Facility at the University of California, San Francisco. The cells were grown in suspension at 37°C with 5% atmospheric CO₂ in DME-H16 medium, containing 3 g/liter glucose, 3.7 g/liter NaHCO₃, 0.11 g/liter pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin, supplemented with 10% heat-inactivated horse serum. The cells were subcultured every 3–4 d by a 1/14 dilution (maximum density of 10⁶ cells/ml).

Bead-attachment Assays. All bead-attachment assays with S49 were performed in modified growth medium containing 10 mM Hepes rather than NaHCO₃ and 2% heat-inactivated horse serum. The cells were exposed to a 1/100 dilution of beads as described above, washed by centrifugation through heat-inactivated horse serum, and analyzed without fixation.

Selection for S49 Cells That Bind High Numbers of PPME Beads. All procedures were carried out under sterile conditions (beads were washed with sterile modified growth medium). After exposure to a 1/100 dilution of beads (see above), 7–10 identical samples were pooled and the cells were spun through 3 ml of heat-inactivated horse serum to remove unbound beads. The cells were resuspended in modified growth medium to a density of 5 × 10⁶ cells/ml and then immediately subjected to sorting (FACS 440; Becton-Dickinson). 25,000–50,000 of the brightest 1–4% of the cells were collected and then resuspended in 5 ml of conditioned growth medium (normal S49 cells were grown to a density of 5 × 10⁵ cells/ml and the medium was collected by filtration through a sterilization filter, pore size 0.22 µm, and then mixed 1:1 with fresh medium), supplemented with 10⁻⁵ M 2-mercaptoethanol. The selected cells were incubated as above, reaching confluency within 7–10 d and were then maintained normally.

Selection for Cells That Bind Low Numbers of PPME Beads. Back-selection was performed essentially as described above except that the cells were exposed to a 10-fold higher concentration of PPME beads, and cells that failed to bind beads were selected by FACS for expansion in culture.

Quantitative Comparison of the S49 Cell Lines for HEV-binding Activities. For accurate comparison of the ability of the selected S49 variants to bind to HEV, a method similar to that described by Butcher et al. (1979a) was employed. Immediately before the assay the different S49 variants were mixed with identical aliquots of mesenteric lymph node lymphocytes (MNL). The final cell concentrations were 3 × 10⁷ S49 cells/ml and 6 ×

10^6 MNL/ml. The HEV-binding assay was then performed and the sections were fixed and stained as described above. S49 cells have an average diameter of 20 μm and can be readily distinguished from normal mouse lymphocytes (diameter of 6–8 μm). The number of S49 cells bound to 20–30 HEV was divided by the number of bound MNL to establish an adherence ratio, referenced to the common MNL population. This ratio was used for comparisons of the various S49 variants.

Results

PPME Beads As a Probe for the Lymphocyte Cell Surface

As shown in Fig. 1, the M6P-rich polysaccharide, PPME, potently inhibited lymphocyte binding to PN HEV in mouse, a result that we have previously found in rat (Stoolman et al., 1984). 50% inhibition was achieved at 5 $\mu\text{g}/\text{ml}$ of PPME (5 nM polysaccharide or 4 μM M6P equivalents). Because of this considerable activity and the fact that PPME has a well-defined composition, we chose it as the basis of a cell surface cytochemical probe. Initially, we employed fluoresceinated PPME prepared by the technique of Glabe et al. (1983). We could not, however, detect binding of this soluble probe to the cell surface of lymphocytes by either fluorescence microscopy or flow cytometry. To augment sensitivity, we developed a solid-phase probe (see Materials and Methods) consisting of PPME covalently coupled to intensely fluorescent polystyrene beads (0.6 μm in diameter). The derivatized beads contained from 2.8×10^{-17} to 13.6×10^{-17} g of PPME per bead, depending upon the concentration of PPME used for coupling (see legend to Fig. 7). This range of substitution corresponded to 17–82 PPME molecules or 13,000–64,000 M6P moieties per bead.

As visualized by fluorescence microscopy (Fig. 2), PPME beads bound to the surface of peripheral lymphocytes (teased out of mesenteric lymph nodes) in significantly greater numbers than nonderivatized beads. Using flow cytometry to quantify bead binding (Fig. 2), we found that PPME beads bound six to eightfold better than nonderivatized beads to peripheral lymphocytes. The distribution of PPME bead binding to peripheral lymphocytes was typically bimodal (Fig. 2), with $\sim 40\%$ of the lymphocytes binding PPME beads to

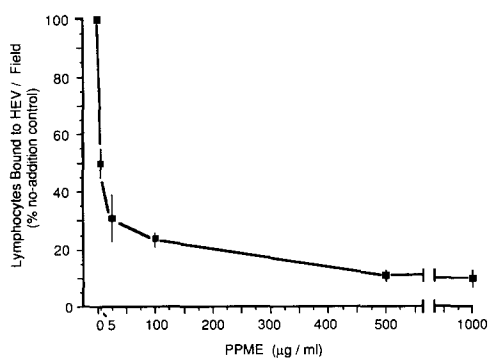


Figure 1. Effect of PPME on lymphocyte attachment to mouse PN HEV. Peripheral lymphocytes were exposed to sections of PN HEV in the presence of the indicated concentration of PPME (x-axis). The y-axis represents the number of lymphocytes attached to HEV per microscopic field ($200\times$) of lymph node cortex. At least 10 fields were counted for each section. Values are expressed as a percentage of binding in the no-addition controls. Means and SEMs are based on three to four independent replicate sections.

the same extent as nonderivatized beads (an average of <1 bead/cell) and the remaining 60% binding an average of 18 beads/cell (with a range of 2–100).

Carbohydrate Inhibition of Lymphocyte Attachment To PPME Beads and To PN HEV

Fig. 3 shows the effects of a panel of phosphorylated monosaccharides at 10 mM on the attachment of PPME beads to mouse lymphocytes. M6P and FIP were the only sugars that produced significant inhibition at this concentration. Neither sugar affected the binding of nonderivatized beads to lymphocytes (not shown). This result indicates that PPME beads bind to the lymphocyte surface through a receptor that recognizes M6P and structurally related sugars.

The same phosphorylated monosaccharides (10 mM) were tested for their effects on the attachment of lymphocytes to mouse PN HEV (Fig. 3). As in the PPME bead assay, M6P and FIP inhibited lymphocyte binding by 80–90%, whereas the other phosphorylated monosaccharides had little or no inhibitory activity.

When compared in a dose-response study, M6P was approximately fourfold more potent than MIP as an inhibitor of lymphocyte attachment to PPME beads (Fig. 4). MIP was more effective than the unrelated sugar, galactose-6-phosphate, which had no more inhibitory activity than sodium phosphate. Almost the same quantitative relationship existed between M6P and MIP when these sugars were compared as inhibitors of lymphocyte binding to PN HEV (Fig. 4). As previously described (Stoolman and Rosen, 1983), lymphocyte attachment to PN HEV is sensitive to increased ionic strength of the assay medium. The inhibitory effects of sodium phosphate and galactose-6-phosphate on PPME bead binding correspond to those expected in the HEV assay, based on the ionic strength of these compounds.

When a series of glycoconjugates was tested in the two assays, further parallels were observed (Fig. 5). Both interactions were inhibited by fucoidin and PPME. Fucoidin, at 10 $\mu\text{g}/\text{ml}$, completely eliminated lymphocyte attachment to PPME beads as well as to PN HEV. At 1 mg/ml, PPME inhibited mouse lymphocyte attachment to PPME beads and to PN HEV by 80–90%. No activity was found with the mnn 1 and 2 yeast mannans that differ from PPME in quantity and linkage of their phosphate moieties (Ballou and Racchke, 1974). Several other glycoconjugates also had no significant inhibitory activity.

We refer to the lymphocyte cell surface receptor, detected by PPME beads, as a carbohydrate-binding receptor because of its selective interactions with related carbohydrate structures (i.e., M6P, FIP, and the M6P-rich polysaccharide PPME). This designation must be considered tentative, however, until the actual biological ligand for this receptor is demonstrated to be carbohydrate in nature. Furthermore, the fact that M6P is the most potent inhibitory monosaccharide for this receptor implies that the postulated biological ligand resembles, but does not necessarily contain, M6P (see Discussion).

Correlation between Lymphocyte Attachment To PPME Beads and To PN HEV

As shown above, M6P, FIP, PPME, and fucoidin inhibit the attachment of lymphocytes to PN HEV in close correspon-

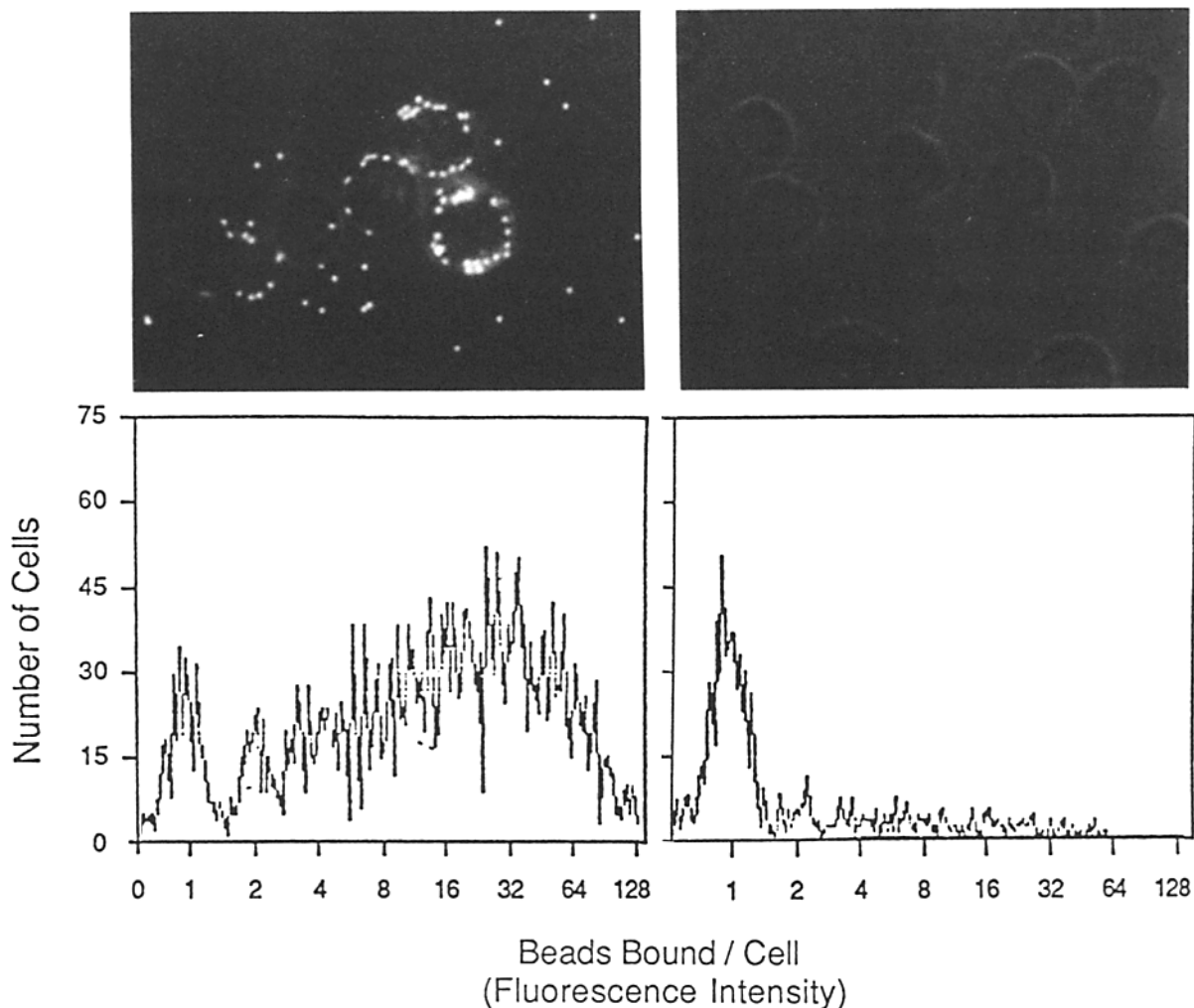


Figure 2. PPME-bead and nonderivatized-bead binding to the surface of mouse peripheral lymphocytes. Fluorescent beads derivatized with PPME bind in large numbers to the surface of lymphocytes (*left micrograph*) as viewed by epifluorescence microscopy (optics set for fluorescein). In contrast, nonderivatized beads bind poorly to lymphocytes (*right micrograph*). Below each micrograph is a representative fluorescence histogram generated by flow cytometry analysis of corresponding samples of bead-reacted lymphocytes. This analysis excluded the contribution from dead cells and cell aggregates (PPME beads did not promote cell aggregation). The x-axis (logarithmic scale) indicates the number of beads bound per lymphocyte, based on fluorescence intensity. The y-axis indicates the number of lymphocytes at each level of fluorescence. The mean number of beads bound per cell is directly proportional to the mean fluorescence intensity of the lymphocyte population. The PPME bead signal is six-eightfold higher than the signal produced by nonderivatized beads. These results are reproducible with usually <10% variation between replicate samples. In most experiments, the level of nonderivatized-bead binding was subtracted from the level of derivatized-bead binding to yield an index of specific bead binding.

dence with their inhibition of lymphocyte binding to PPME beads. To address further the possible involvement of the carbohydrate-binding receptor in lymphocyte attachment to PN HEV we compared lymphocyte attachment to PPME beads and to HEV under a variety of experimental conditions.

Thymocytes vs. Peripheral Lymphocytes. Thymocytes are immature T cells that do not recirculate in vivo and bind poorly to HEV in vitro (Stamper and Woodruff, 1976; Butcher et al., 1979a). We found that thymocytes bound poorly to PPME beads as well as to PN HEV (Fig. 6); thymocyte binding was 10% that of peripheral lymphocytes in both assays. Furthermore, S49 cells (a lymphoma cell line having thymocyte-like characteristics) also bound poorly to PPME beads (see legend to Fig. 11) and to PN HEV (see leg-

end to Fig. 12). These results are significant because they establish an additional correlation between PPME bead- and HEV-binding and also demonstrate that PPME beads constitute a selective probe that does not bind indiscriminately to all cell surfaces.

Beads derivatized with chondroitin sulfate or heparin showed very different binding characteristics (Fig. 7). These bead types, when substituted with carbohydrate to at least the same degree as PPME beads, bound as poorly as nonderivatized beads to peripheral lymphocytes. However, chondroitin sulfate beads and especially heparin beads exhibited significant binding to thymocytes. Heparin beads also bound in high numbers to the S49 cell line (see legend to Fig. 11). These results establish the selectivity of the interaction of PPME with lymphocytes; merely coating beads with a nega-

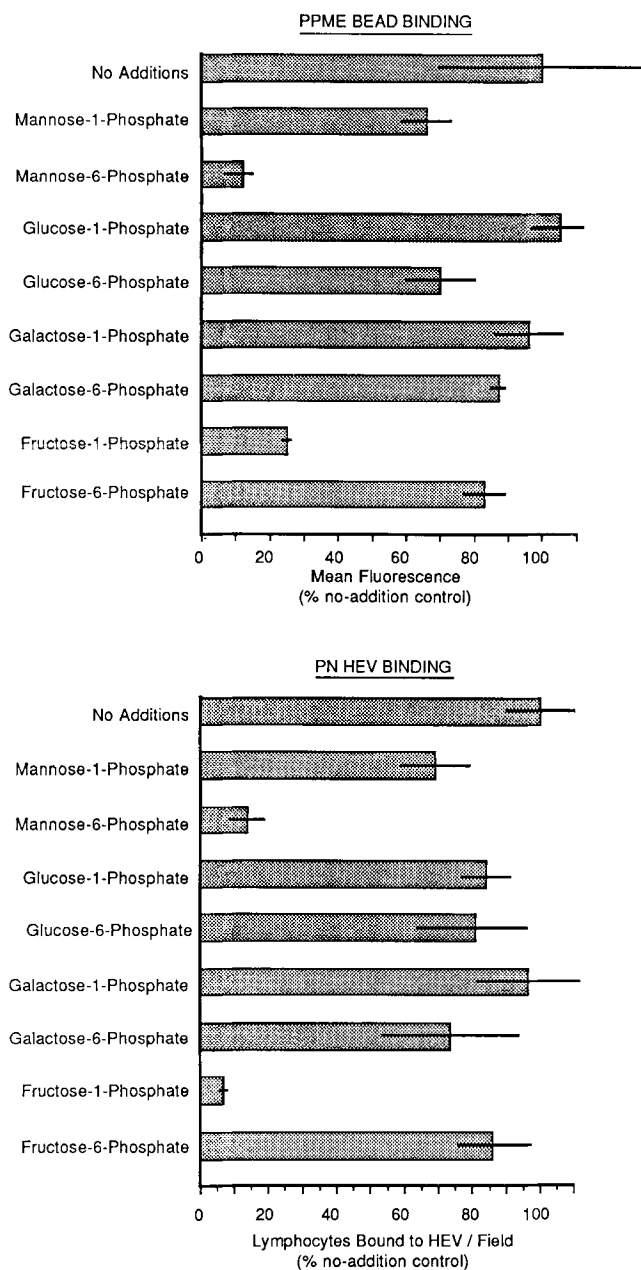


Figure 3. Effect of phosphorylated monosaccharides on the attachment of mouse peripheral lymphocytes to PPME beads and to PN HEV. (*Top*) Lymphocytes were exposed to beads (diluted 1:20) in the presence of the indicated sugar (at 10 mM). The x-axis represents the level of PPME-bead binding, expressed as a percentage of the specific binding of PPME beads to lymphocytes with no additions (see legend to Fig. 2). PPME-bead binding was threefold higher than that of nonderivatized beads, which was not affected by any of the sugars. (*Bottom*) Lymphocytes were exposed to sections of mouse PN in the presence of the indicated sugar (at 10 mM). The x-axis represents the number of lymphocytes attached to HEV per microscopic field (200 \times) of lymph node cortex. This value is expressed as a percentage of lymphocyte binding to HEV in the no-addition control. Means and SEMs are based on three to five independent replicates.

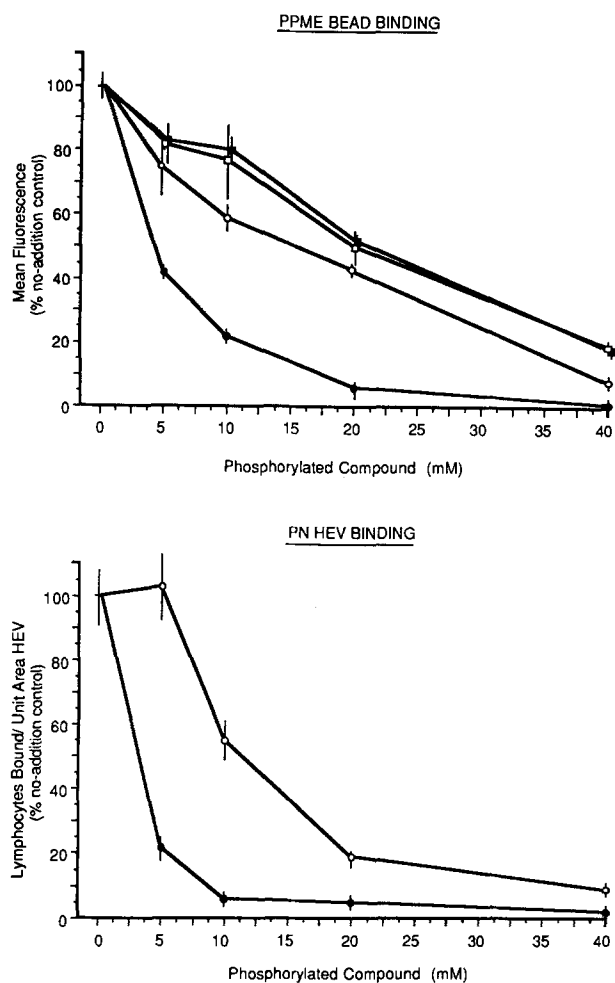


Figure 4. Effect of phosphorylated compounds on the attachment of mouse peripheral lymphocytes to PPME beads and to PN HEV. (*Top*) Lymphocytes were exposed to beads (diluted 1:200) in the presence of M1P (open circles), M6P (closed circles), galactose-6-phosphate (open squares), and disodium phosphate (closed squares) at varying concentrations (indicated on the x-axis). The y-axis represents PPME-bead binding, expressed as a percentage of the specific binding of PPME beads to lymphocytes with no additions. The degree of PPME-bead binding (with no additions) was eightfold higher than that of nonderivatized beads, which was not affected by the highest concentration of the inhibitors. (*Bottom*) Lymphocytes were exposed to PN HEV in the presence of M1P (open circles) and M6P (closed circles) at the concentrations indicated on the x-axis. The y-axis indicates the number of lymphocytes bound per unit area of HEV. These values are expressed as a percentage of lymphocyte binding to HEV in the no-addition control. Means and SEMs are based on five independent replicates.

tively charged polysaccharide does not result in an affinity for the lymphocyte surface. It is also noteworthy that the binding of heparin beads to thymocytes was not affected by 10 mM M6P (not shown), indicating that this sugar does not interfere with all bead-cell interactions.

Trypsin Sensitivity. Woodruff et al. (1977) demonstrated that brief treatment of rat lymphocytes with low concentrations of trypsin destroys their ability to bind to PN HEV. Using similar conditions, we found that pretreatment of mouse lymphocytes with trypsin completely prevented their attachment to PPME beads as well as their binding to PN

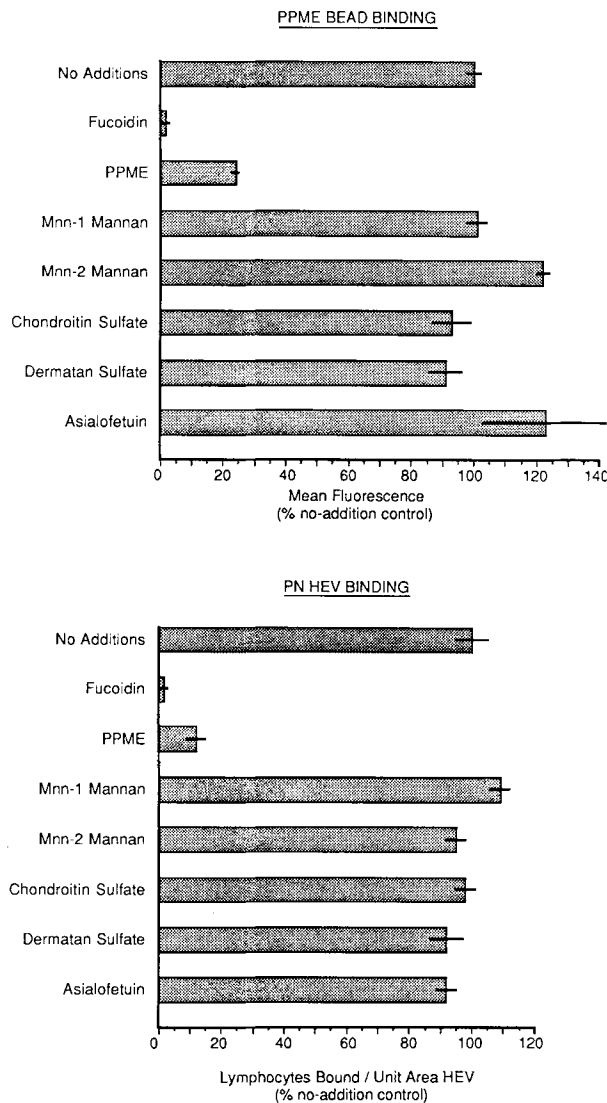


Figure 5. Effect of glycoconjugates on the attachment of mouse peripheral lymphocytes to PPME beads and to PN HEV. (*Top*) Lymphocytes were exposed to beads (diluted 1:600) in the presence of 10 $\mu\text{g}/\text{ml}$ of fucoidin or 1 mg/ml of each of the other compounds indicated on the y-axis. The x-axis indicates the level of PPME-bead binding (expressed as a percentage of specific PPME-bead binding to lymphocytes with no additions). The level of PPME-bead binding (with no additions) was twice that of nonderivatized beads, which was not affected by any of the inhibitors. (*Bottom*) Lymphocytes were exposed to sections of mouse PN in the presence of indicated compounds (fucoidin at 10 $\mu\text{g}/\text{ml}$, all others at 1 mg/ml). The x-axis represents the number of lymphocytes bound per unit area HEV. These values are expressed as a percentage of lymphocyte binding in the no-addition control. Means and SEMs are based on three to four independent replicates.

HEV (Fig. 8). When the cells were exposed to trypsin plus excess trypsin inhibitor, binding to PPME beads and to PN HEV remained at control levels.

Divalent Cation Requirements. EDTA prevented mouse lymphocyte attachment to PN HEV (Fig. 9), a result first demonstrated in the rat by Woodruff et al. (1977). Correspondingly, lymphocyte attachment to PPME beads was also completely eliminated by EDTA (Fig. 9). Both binding interactions were restored in the presence of excess Ca^{++} ,

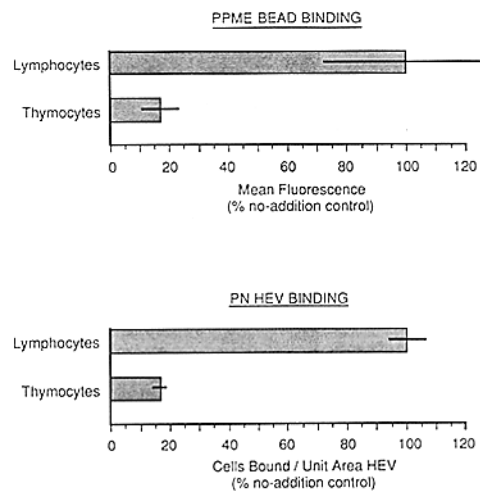


Figure 6. Attachment of peripheral lymphocytes and thymocytes to PPME beads and to PN HEV. (*Top*) Peripheral lymphocytes and thymocytes were exposed to beads (diluted 1:20) and the level of specific PPME-bead binding to each cell type is indicated on the x-axis (expressed as a percentage of the specific binding of PPME beads to lymphocytes). The level of PPME bead binding to lymphocytes was six times the level of nonderivatized-bead binding to either cell type. (*Bottom*) Lymphocytes and thymocytes (each at 7×10^6 cells/ml) were exposed to sections of mouse PN. The x-axis indicates the number of cells bound per unit area HEV (expressed as a percentage of the level of lymphocyte binding). The means and SEMs are based on five to six independent replicates.

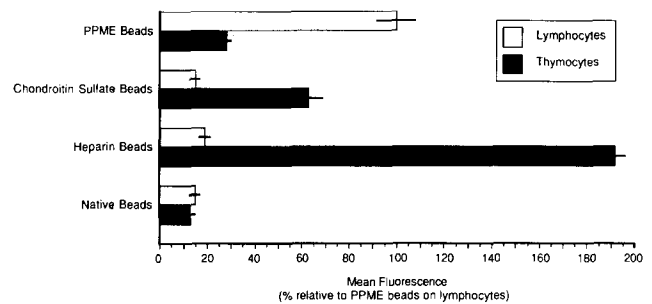


Figure 7. Attachment of peripheral lymphocytes and thymocytes to nonderivatized beads and to carbohydrate-derivatized beads. Lymphocytes (stippled bars) and thymocytes (dark bars) were exposed to the indicated type of bead (diluted 1:200). The degree of bead binding to each cell type is indicated on the x-axis (expressed as a percentage of the fluorescent signal produced by lymphocytes that were exposed to PPME beads). In a separate experiment the degree of carbohydrate substitution per bead was related to the ability of the beads to bind to lymphocytes. PPME beads, prepared by the standard procedure, contained 14×10^{-17} g PPME/bead and bound nine times better than nonderivatized beads to lymphocytes. When the concentration of cyanogen bromide-activated PPME was reduced tenfold, the substitution on the beads was 3×10^{-17} g/bead. The specific binding of these beads was one-third of that of the standard PPME bead preparation. Beads substituted with 4 or 16×10^{-17} g/bead of heparin or with 10, 55, or even $3,200 \times 10^{-17}$ g/bead of chondroitin sulfate bound no better than nonderivatized beads to lymphocytes.

but not Mg^{++} . Therefore, lymphocyte attachment to PPME beads, as well as to PN HEV, requires Ca^{++} . Lymphocyte attachment to nonderivatized beads was not affected by the removal of divalent cations.

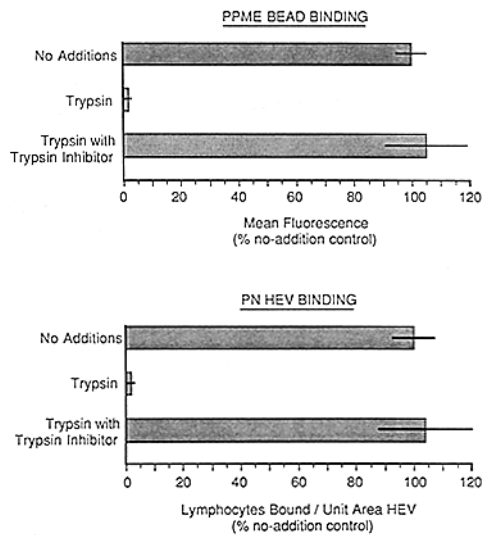


Figure 8. Binding of trypsin-treated lymphocytes to PPME beads and to PN HEV. Mouse peripheral lymphocytes were treated with either PBS with no additions, PBS with 20 μg trypsin/ml, or PBS containing 20 μg trypsin/ml plus 200 μg trypsin inhibitor/ml (as described in Materials and Methods). (*Top*) Control and treated lymphocytes were exposed to beads (diluted 1:20) and the degree of specific PPME binding is represented on the x-axis (expressed as a percentage of binding to PBS-treated lymphocytes). The control level of PPME-bead binding was fourfold higher than the level of nonderivatized-bead binding, which was not affected by any of the treatments. (*Bottom*) Treated lymphocytes were exposed to sections of mouse PN. The x-axis represents the number of lymphocytes bound per unit area of HEV (expressed as a percentage of binding in the PBS-treated control). Means and SEMs are based on five replicate samples.

Selection of S49 Cell Line Variants for PPME-Bead Binding

The Interaction of PPME Bead-selected S49 Variants with PPME Beads and with Other Types of Carbohydrate-Derivatized Beads. S49 cells were exposed to PPME beads and the brightest 1–4% of the population was selected by FACS for expansion in culture. Fig. 10 shows that with each round of selection the ability of the cells to bind PPME beads increased. After seven rounds of selection, the resulting cell line, S49-PB^{HI}, bound to PPME beads about eightfold better than the parental line. Two additional rounds of selection further increased PPME bead binding activity by twofold. The ability of S49-PB^{HI} to bind PPME beads was stable through at least 43 wk of continuous culture, representing \sim 330 cell generations.

PPME-bead binding was effectively reduced by a serial back-selection procedure. S49-PB^{HI} cells were exposed to a high concentration of PPME beads, and nonfluorescent cells were selected and returned to culture. After three rounds of back selection, the resulting cell line (termed S49-PB^{LO}) bound PPME beads only slightly better than the original S49 line (Fig. 10).

To determine the specificity of the selection techniques, the cell lines were tested against other types of carbohydrate-conjugated beads. The parental S49 cells bound high numbers of beads conjugated with heparin and moderate numbers of beads conjugated with the sulfated galactan, k-carrageenan (see legend to Fig. 11). As shown in Fig. 11,

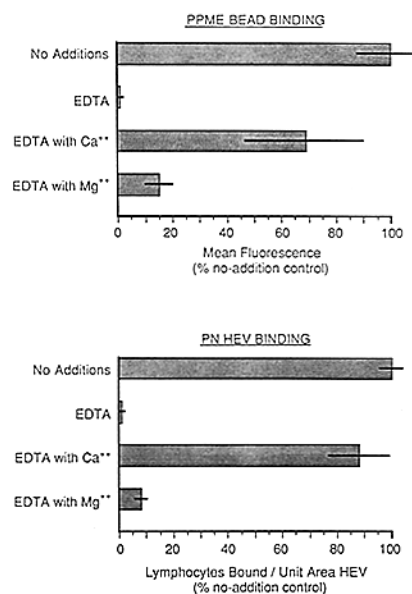


Figure 9. Divalent cation requirement for lymphocyte binding to PPME beads and to PN HEV. Peripheral lymphocytes were exposed to beads (diluted 1:20) and to cryostat-cut sections of PN in either buffer (PBS with 1 mg/ml BSA) with no additions, buffer with 2 mM EDTA, buffer with 2 mM EDTA and 4 mM CaCl_2 , or in buffer with 2 mM EDTA and 4 mM MgSO_4 . (*Top*) The x-axis represents the level of PPME-bead binding to lymphocytes (expressed as a percentage of the specific binding of PPME-beads to lymphocytes with no additions). The level of PPME-bead binding (under control conditions) was fourfold higher than the level of nonderivatized-bead binding, which was not significantly affected by any of the conditions. (*Bottom*) The x-axis represents the number of lymphocytes bound per unit area of HEV (expressed as a percentage of binding in the no-addition control). Means and SEMs are based on five replicate samples.

S49-PB^{HI} showed no significant change in binding to either heparin or galactan beads. The level of heparin and galactan bead binding also remained unaltered in the back-selected line, S49-PB^{LO}. In addition, the low-level binding to nonderivatized beads did not change during either positive or negative sorting procedures. Therefore, selection with PPME beads directly affected the ability of S49 cells to interact with PPME beads, but did not alter their ability to bind to beads coated with other negatively charged polysaccharides or to nonderivatized beads.

The Interaction of the Selected S49 Cells with HEV. Fig. 12 demonstrates that selection for cells that bind well to PPME beads resulted in an increased ability to bind to PN HEV. S49-PB^{HI} bound to PN HEV sixfold better than the parental S49 line, while HEV-attachment of the back-selected line was reduced almost completely to the original level. These results establish that expression of the carbohydrate-binding receptor (detected by PPME beads) is intimately associated with the ability of the cells to bind to PN HEV.

It is significant that the variants selected for PPME bead binding did not exhibit altered ability to attach to Peyer's patch HEV (Fig. 12), a mucosal system with a cell recognition specificity distinct from that of the peripheral node interaction (Butcher et al., 1980; Stevens et al., 1982; Gallatin et al., 1983; Kraal et al., 1983; Chin et al., 1984; Rassmus-

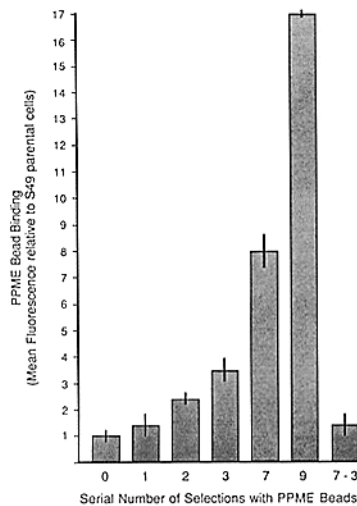


Figure 10. Attachment of PPME beads to PPME-bead-selected variants of the S49 cell line. S49 was subjected to nine serial rounds of selection for PPME-bead binding with FACS as described in Materials and Methods. Several of the resulting cell line variants were maintained for at least 1 mo in continuous culture to stabilize their phenotypic characteristics and were then tested for PPME-bead binding. These variants are designated on the x-axis by their number in the selection process (the parental S49 cell line equals 0). As described in the text, the cell line obtained after seven rounds of sorting is referred to as S49-PB^{HI}. This cell line was subjected to three serial rounds of back selection with PPME beads (see Materials and Methods) and the resulting variant is indicated on the x-axis as sort number 7-3. This back selected line is referred to in the text as S49-PB^{LO}. All cells were exposed to a 1:100 dilution of PPME beads or to nonderivatized beads, as described in Materials and Methods. The low level of binding exhibited by nonderivatized beads was approximately the same for each of the cell lines (see Fig. 11) and was subtracted from the level of PPME-bead binding to determine the degree of specific PPME-bead binding. Specific PPME-bead binding for each cell line is expressed on the y-axis relative to the value for S49 parental cells (normalized to unity).

sen et al., 1985; Pals et al., 1986). This finding implies that the binding of lymphocytes to PP HEV does not involve a M6P-like specificity. In support of this inference, we have found that PPME does not inhibit the attachment of lymphocytes to PP HEV (unpublished observation).

Discussion

Using PPME beads in conjunction with flow cytometry, we have demonstrated directly that mouse peripheral lymphocytes express a cell surface receptor that can bind PPME (a polysaccharide that contains only mannose and M6P). Competition studies with free sugars and polysaccharides established that this receptor recognizes M6P and related carbohydrates. Fucoidin also produces potent inhibition of PPME-bead attachment.²

Beads substituted with heparin, chondroitin sulfate, or a sulfated galactan bound no better than nonderivatized beads to the surface of peripheral lymphocytes. In contrast, thymocytes, a nonrecirculating population of lymphocytes, bound

2. The basis for fucoidin's activity is not understood since there is no obvious structural relationship between fucose-4-sulfate, a major constituent of fucoidin, and M6P. This issue is further addressed in the companion study.

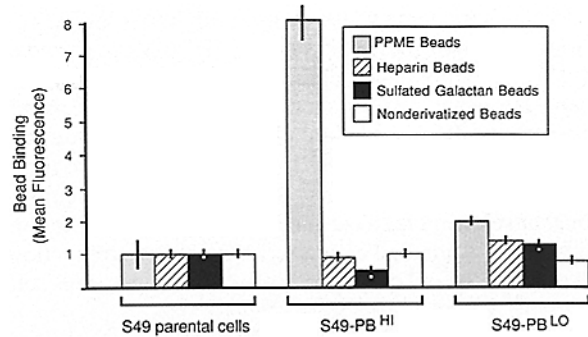


Figure 11. Attachment of S49, S49-PB^{HI}, and S49-PB^{LO} to PPME beads, heparin beads, sulfated galactan beads, and nonderivatized beads. Cells were exposed to a 1:100 dilution of the indicated bead type as described in Materials and Methods. The levels of bead binding exhibited by S49 parental cells were as follows (flow cytometry fluorescence values on a linearized scale): nonderivatized beads, 1.3 ± 0.1 ; PPME beads, 1.9 ± 0.2 ; heparin beads, 17.6 ± 0.1 ; galactan beads, 5.4 ± 0.5 . For each bead type the parental S49 level of binding was normalized to unity and the binding exhibited by S49-PB^{HI} and S49-PB^{LO} cells was expressed relative to this value. In comparison, the level of bead binding exhibited by peripheral lymphocytes measured in the same experiment were as follows: nonderivatized beads, 2.3 ± 0 ; PPME beads, 8.4 ± 0.6 ; heparin beads, 2.3 ± 0 ; galactan beads, 2.9 ± 0.2 .

PPME beads poorly but bound both heparin beads and chondroitin sulfate beads strongly. S49, a thymic lymphoma, also bound PPME beads weakly, while binding large numbers of heparin and sulfated-galactan beads. Thus, the interaction of peripheral lymphocytes with PPME is highly selective. Furthermore, these observations raise the possibility that carbohydrate-derivatized fluorescent beads (e.g., heparin beads) might be useful in the identification and characterization of other cell surface carbohydrate-binding or glycosaminoglycan-binding receptors.

Our previous indirect evidence suggested the existence of a PPME-binding receptor on lymphocytes that is involved in the attachment of lymphocytes to PN HEV. The PPME bead probe allowed this hypothesis to be tested by direct analysis of the lymphocyte cell surface. Under all conditions examined, lymphocyte attachment to PPME beads was found to mimic the interaction between lymphocytes and PN HEV. In brief, lymphocyte attachment to PPME beads and to PN HEV was calcium dependent, trypsin sensitive, and selectively inhibited by M6P, PPME, and fucoidin. Thymocytes

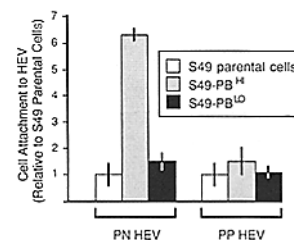


Figure 12. Attachment of S49, S49-PB^{HI}, and S49-PB^{LO} to PN HEV and PP HEV. Cell suspensions were exposed to sections of peripheral lymph nodes and Peyer's patches, and the degree of lymphoma cell binding to HEV was quantified as described in Materials and Methods. Binding of S49 parental cells to both types of HEV was set at unity and the levels of binding exhibited by S49-PB^{HI} and S49-PB^{LO} were expressed relative to these values. For comparison, the degree of binding to PN HEV exhibited by S49 parental cells was $5.6 \pm 0.9\%$ of the value for peripheral lymphocytes measured in the same experiment.

and a thymic lymphoma (S49) showed minimal binding activity toward both PPME beads and PN HEV. Moreover, the affinity of S49 lymphoma cells for PN HEV was markedly increased by serial selection of variants for PPME bead binding activity. Back selection with PPME beads returned PPME bead binding and PN HEV binding activities to their original low levels. To our knowledge, this is the first demonstration that selection for a carbohydrate-binding receptor on the surface of mammalian cells alters their ability to function in a well-defined and physiologically significant cell-cell adhesive interaction.

The precise relationship between the lymphocyte receptor described herein and the phosphomannosyl receptors implicated in transport of acid hydrolases from the Golgi to the lysosome has yet to be resolved. A wide variety of cell types possess a 215-kD M6P-specific receptor which is concentrated within the Golgi cisternae and associated structures (Sahagian et al., 1981; Brown and Farquhar 1984a; Geuze et al., 1984), while a few cell types also express the receptor on the cell surface (Kaplan et al., 1977; Brown and Farquhar, 1984a, b; Shepherd et al., 1984). The interaction of PPME beads with lymphocytes differs from the ligand-receptor interactions described for the 215-kD receptor in several important respects. The latter activity is not inhibited by fucoidin (Shepherd et al., 1984), is much more selective (>100-fold) for M6P than M1P (Kaplan et al., 1977; Shepherd et al., 1984), and does not require divalent cations (Rome et al., 1979; Hoflack and Kornfeld, 1985a, b). A newly described M6P receptor, presumably also involved in sorting of lysosomal enzymes, does have a requirement for divalent cations, but exhibits no preference for calcium over magnesium (Hoflack and Kornfeld, 1985a, b). These differences suggest that the carbohydrate-binding receptor, implicated by our studies in lymphocyte binding to PN HEV, is distinct from the presumptive lysosomal enzyme sorting receptors. Furthermore, on the basis of carbohydrate-binding specificity and cell-type distribution, there is no apparent relationship between the carbohydrate-binding receptor described in this report and lymphocyte cell surface receptors identified by fluorescently labeled neoglycoproteins (Kieda et al., 1977).

PPME beads constitute a novel probe for investigating the mechanism of lymphocyte attachment to HEV because they appear to measure the activity (i.e., carbohydrate-binding activity) of a receptor involved in this interaction. The exact role of the carbohydrate-binding receptor in lymphocyte attachment to PN HEV is not known at present. The simplest model would predict that this lymphocyte surface receptor mediates adherence to PN HEV by interacting directly with M6P or a M6P-like ligand associated with the HEV. Evidence for direct participation of M6P residues on PN HEV is lacking since treatment of lymph node sections with high concentrations of alkaline phosphatase or alpha-mannosidase does not affect lymphocyte attachment (Rosen, S. D., and M. S. Singer, unpublished observations). However, an essential role for sialic acid on PN HEV is indicated, as sialidase treatment of peripheral lymph node sections abolishes subsequent lymphocyte binding (Rosen et al., 1985). It is possible that the PN HEV attachment site contains a recognition determinant that is mimicked by M6P and that sialic acid is either part of this structure or it modulates the activity of the structure. Alternatively, the carbohydrate-binding recep-

tor on the surface of lymphocytes may not interact directly with the HEV, but instead may associate with other molecules (integral or peripheral membrane components) on the lymphocyte surface to form an active "homing receptor complex." Similarly, the attachment site on PN HEV may be comprised of multiple components, one or more of which is sialylated. Clearly, further work is required to define the number of molecular elements involved in this adhesive interaction and to determine the exact relationship between the carbohydrate-binding receptor described herein and the sialylated component on PN HEV.

A major question concerns the relationship of the carbohydrate-binding receptor identified with PPME beads to other putative homing receptors described in rat (Chin et al., 1982, 1983; Rasmussen et al., 1985) and in mouse (Gallatin et al., 1983). For both species, antibodies have been described that specifically prevent lymphocyte binding to PN HEV *in vivo* and *in vitro*. In mouse, an adhesion-blocking monoclonal antibody (MEL-14) recognizes a single 80-90-kD glycoprotein on the lymphocyte surface (Gallatin et al., 1983; Siegelman et al., 1986). In a companion study (Yednock et al., 1987) we have employed PPME beads to demonstrate that the MEL-14 antigen is closely related if not identical to the lectin-like receptor described in this report.

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