Phosphomannosyl Receptors May Participate in the Adhesive Interaction between Lymphocytes and High Endothelial Venules

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ABSTRACT   Normal and malignant lymphocytes can migrate from the bloodstream into lymph nodes and Peyer's patches. This process helps distribute normal lymphocytes throughout the lymphoid system and may provide a portal of entry for circulating malignant cells. An adhesive interaction between lymphocytes and the endothelium of postcapillary venules is the first step in the migratory process. We have recently shown that the simple sugars L-fucose and D-mannose, and an L-fucose-rich polysaccharide (fucoidin), can inhibit this adhesive interaction in vitro. We now report that mannose-6-phosphate, the structurally related sugar fructose-1-phosphate, and a phosphomannan, core polysaccharide from the yeast Hansenula holstii (PPME) are also potent inhibitors. Inhibitory activity was assessed by incubating freshly prepared suspensions of lymphocytes, containing the various additives, over air-dried, frozen sections of syngeneic lymph nodes at 7–10°C. Sections were then evaluated in the light microscope for the binding of lymphocytes to postcapillary venules. Mannose-6-phosphate and fructose-1-phosphate were potent inhibitors of lymphocyte attachment (one-half maximal inhibition at 2–3 mM). Mannose-1-phosphate and fructose-6-phosphate had slight inhibitory activity, while glucose-1-phosphate, glucose-6-phosphate, galactose-1-phosphate, and galactose-6-phosphate had no significant activity (at 10 mM). In addition, the phosphomannan core polysaccharide was a potent inhibitor (one-half maximal inhibition at 10–20 μg/ml); dephosphorylation with alkaline phosphatase resulted in loss of its inhibitory activity. Preincubation of the lymphocytes, but not the lymph node frozen sections, with PPME resulted in persistent inhibition of binding. Neither the monosaccharides nor the polysaccharide suppressed protein synthesis nor decreased the viability of the lymphocytes. Furthermore, inhibitory activity did not correlate with an increase in negative charge on the lymphocyte surface (as measured by cellular electrophoresis). These data suggest that a carbohydrate-binding molecule on the lymphocyte surface, with specificity for mannose-phosphates and structurally related carbohydrates, may be involved in the adhesive interaction mediating lymphocyte recirculation.

In rodent model systems, normal and malignant lymphocytes migrate from the bloodstream into lymph nodes and Peyer's patches (1, 2). This process helps distribute normal lymphocytes throughout the body and may provide a mechanism for the hematogenous dissemination of lymphoid malignancies. Circulating lymphoid cells enter many lymphoid organs by migrating across the structurally distinctive postcapillary venules known as the high endothelial venules or HEV[1] (3, 4).

Both in situ and in vitro studies strongly implicate a specific adhesive interaction between lymphocytes and this specialized endothelium as the initial step in migration (5, 6). In the mouse system, this binding interaction may be responsible for the distinctive recirculatory patterns of T-cells and B-cells,

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D-fructose-6-phosphate; F1P, D-fructose-1-phosphate; PPME, phosphomannosyl-rich, core polysaccharide of mannan derived from the yeast Hansenula holstii; MEM+, minimal essential medium in Earle's salts (without bicarbonate) supplemented with 1 mg/ml bovine serum albumin and buffered with 40 mM Tricine, pH 7.4; I50, concentration for ½ maximal inhibition of binding.

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[1] Abbreviations used in this paper: HEV, high endothelial venule; M6P, D-mannose-6-phosphate; M1P, D-mannose-1-phosphate; F6P,

since the former adhere preferentially to the HEV of peripheral lymph nodes while the latter adhere preferentially to the HEV of Peyer's patches (7). Two groups of investigators have developed antibodies directed at the lymphocyte surface which selectively block the binding of lymphocytes to HEV in frozen sections of lymph nodes (8, 9); in contrast, binding to Peyer's patches is not affected (9). The same specificity has been documented in vivo, where these antibodies inhibit the migration of lymphocytes into lymph nodes without altering the flow of lymphocytes into Peyer's patches (8, 9). Therefore, the selective migration of lymphocytes into various lymphoid organs appears to be due, in part, to specific adhesive molecules on the surfaces of the lymphocytes and the endothelial cells.

In a previous report (10), we suggested that an L-fucose/D-mannose specific lectin on the surface of rat lymphocytes participates in the attachment to HEV in vitro. This hypothesis was based on the observation that L-fucose, the structurally related simple sugar D-mannose (both contain an axial 2-OH and an equatorial 4-OH), and the sulfated, L-fucose-rich polysaccharide fucoidin inhibit lymphocyte binding. In contrast, an extensive series of simple sugars and polysaccharides fail to inhibit. Finally, Spangrude et al. (11) recently reported that fucoidin blocks the migration of lymphocytes into lymph nodes in vivo. This finding suggests that the receptor for fucoidin characterized in vitro participates in the normal migration of circulating lymphocytes.

The present study extends these initial findings by documenting the inhibitory activity of several phosphorylated carbohydrates, incuding D-mannose-6-phosphate (M6P), D-fructose-1-phosphate (F1P), and a phosphomannosyl-rich, core polysaccharide derived from a yeast mannan (PPME). These data provide further evidence that a carbohydrate-binding molecule on the lymphocyte surface may be involved in adhesion. The possible relationship between this receptor and previously described phosphomannosyl receptors is discussed.

MATERIALS AND METHODS

Chemicals: Monosaccharides were obtained from Sigma Chemical Co. (St. Louis, MO). Fucoidin (from K & K Labs, Plainview, NY) and the hydrolysis product of *Hansenula holstii* phosphomannan (PPME; kindly provided by Dr. M. E. Slodki, Northern Regional Research Laboratory, Peoria, IL) were used without further purification unless indicated. The mannans from the mnn1 and mnn2 yeast mutants were generously provided by Dr. C. E. Ballou, University of California at Berkeley. Heparin (H3125), chondroitin sulfate (C3254), dextran sulfate (D7515), mannan (M7504), and bovine serum albumin (A4378) were obtained from Sigma Chemical Co.

Preparation of Sections: A nonspecific antigenic stimulus (0.1–0.2 ml of a 1:1 emulsion of Freund's complete adjuvant and sheep erythrocytes) was administered subcutaneously (to ether-anesthetized, 180 g, female, Sprague-Dawley rats) in the region of the superficial cervical lymph node chains. This procedure increased both the number and the volume of lymph nodes recovered (compared to untreated animals) without altering the characteristics of the adhesive interaction (data not shown).

5–7 d after the challenge, animals were killed and the cervical lymph nodes were frozen and sectioned as previously described (10). For some experiments, freshly cut frozen sections were air-dried onto slides at room temperature and then fixed (0.5–1% freshly prepared paraformaldehyde in 0.1 M Na-cacodylate buffer, pH 7.3, 4°C, 20 min), washed (phosphate-buffered saline, pH 7.4, 4°C; two dips in three changes of buffer) and used immediately. The slides remained in phosphate-buffered saline until used in the binding assay (<30 min) to prevent dehydration. Sections prepared in this manner showed reduced nonspecific binding (at sites other than the HEV) and enhanced specific binding activity (Fig. 1). Fixation did not alter the characteristics of the adhesive interaction since the rank-order-of-potencies for the mannose phosphates were unchanged (Table I).
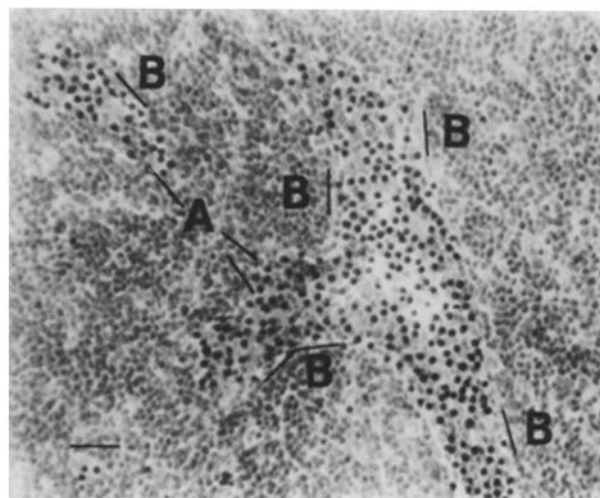

FIGURE 1 Lymphocyte binding to fixed sections. In the absence of inhibitors, lymphocytes (A) attach primarily to the HEV (the perimeter boundaries of which are defined by bars designated with B). Lymphocyte binding to non-HEV sites is 50–100-fold lower than that to HEV. Bar, 42 μm. × 200.

TABLE I

	Unfixed sections	Fixed sections
Control	100 ± 10	100 ± 13
Mannose-6-P	7 ± 4	23 ± 7
Mannose-1-P	90 ± 27	90 ± 21

Inhibitory activity using unfixed and fixed frozen sections. Lymphocytes (2 × 10^7 for unfixed sections; 1 × 10^6 for fixed sections) were incubated with sugars (4°C; 10 mM; 30 min) then applied to sections (7°C; 80 rpm; 50 min). Sections were then fixed, washed, stained and examined for binding activity as described in Materials and Methods. The mean ± the SEM for four replicates (unfixed sections) or eight replicates (fixed sections) are reported.

Binding Assay: The procedure, based on an assay originally devised by Stamper and Woodruff (5), has been described in detail elsewhere (10). In brief, freshly prepared lymphocyte suspension (from the cervical lymph nodes of adult Sprague-Dawley rats), containing various additives, were layered onto either air-dried or fixed, 10 μm sections of peripheral lymph node. After a 15–50-min incubation (at 7–10°C with rotary agitation), the cell suspensions were decanted and the sections were fixed in glutaraldehyde (3% for 20 min at 4°C). The sections were gently washed to remove nonadherent cells and then specific binding to the HEV was determined as described in the next section.

Potential inhibitors (the mono- and polysaccharides) were added to the cell suspensions 15–30 min before the start of the binding assays. The final cell concentrations were 1–2 × 10^7 cells/ml when unfixed sections were used and 1–5 × 10^6 cells/ml when fixed sections were used. Inhibitors were present throughout the subsequent binding incubation unless specifically indicated.

Quantitation of Binding: Binding activity was quantified, in a single-blind fashion, as described previously (10) by determining the average number of lymphocytes bound to the HEV in × 200 microscopic fields (based on counts of multiple fields covering the entire section). To facilitate the comparison among experiments conducted on different days or using different lymph node sections, the binding under each experimental condition was expressed relative to the binding of the control cells as follows: Binding relative to control = (HEV-bound test cells per × 200 microscopic field)/(HEV-bound control cells per × 200 microscopic field) × 100.

Individual data points represent the mean and standard error of the mean (SEM) for measurements on three to eight separate sections taken from various regions of the lymph node. When calculated in this fashion, SEMs for individual data points ranged from 10 to 20%.

Alkaline Phosphatase Digestion: PPME (400 μg/ml) in 10 mM Tris-HCl, pH 8.0, was digested with *E. coli* alkaline phosphatase (Sigma Chemical Co.) at 320 μg/ml (4 U/mg) for 24 h at 37°C with or without 100 mM KH$_2$PO$_4$. Na-azide (0.02%) was added to retard bacterial growth during the incubation. Samples were dialyzed into MEM+ for testing in the binding assay. Phosphate assays (12) were done on samples dialyzed into 10 mM Tris-HCl, pH 8.0.

Preincubation Experiments: Assays were carried out in which either the lymphocytes or the HEV sections were preincubated with PPME before the quantitation of binding. Fixed lymph node sections, a rapid cell washing procedure, and a short binding assay (15 min as opposed to 50 min) were employed. For preincubation of lymphocytes, a suspension containing 10^7 cells/ml was exposed at 4°C to PPME (200 µg/ml), mnn1 mannan (200 µg/ml) or medium for 30 min, washed quickly (three 10-s washes using a Beckman Model B microcentrifuge [Beckman Instruments, Inc., Palo Alto, CA]), and tested for binding activity. The rapid washing procedure resulted in a 900-fold dilution of the PPME. For preincubation of the HEV, fixed sections were pretreated with either PPME (200 µg/ml) or medium at 7°C for 30 min, washed to achieve a 900-fold dilution of the polysaccharide, and assayed for binding activity.

Protein Synthesis: The rate of protein synthesis in lymphocytes was determined by measuring the incorporation of [³H]leucine (ICN Pharmaceuticals Inc., Irvine, CA; Cat. No. 20032; 120 Ci/mM) into TCA-precipitable counts as previously described (10).

Surface Charge Determinations: The increment in negative charge density on the lymphocyte surface, due to the binding of charged carbohydrates, was determined by measuring the electrophoretic mobility of cells in the presence of sugars and polysaccharides. The mobilities of individual cells per sample were measured in a Zeiss cytopherometer (Carl Zeiss Co., New York, NY) equipped with a rectangular microelectrophoresis chamber (700 µm depth) and a Zn/ZnSO₄ electrode assembly. The measurements were made at 7.0 +/− 0.1°C in MEM+ containing various phosphorylated sugars or charged polysaccharides (pH 7.4). The ionic strength of the MEM+ with or without added polysaccharides was 0.156; the addition of 10 mM D-mannose-1-phosphate (M1P) or M6P increased the ionic strength to 0.176. For each sample the cellular electrophoretic mobility was determined from a series of 40–60 velocity measurements on individual cells at two stationary levels within the microelectrophoresis chamber. All measurements were made with the polarity of the applied electric field (6.0 V/cm) in alternate directions, thereby canceling out any effect of mechanical fluid drift on the net electrophoretic velocity. Surface charge density (C/m²) was calculated from the electrophoretic mobility (µm × s⁻¹/V × cm⁻¹) using the Gouy-Chapman equation (13).
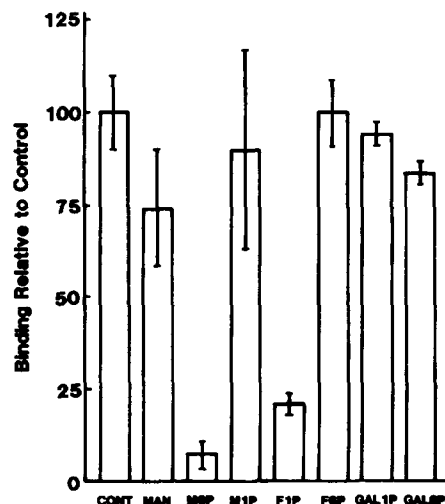
FIGURE 2 Lymphocyte attachment in the presence of various monosaccharides (10 mM). CONT, no-added-sugar control; MAN, D-mannose; M6P, D-mannose-6-phosphate; M1P, D-mannose-1-phosphate; F1P, D-fructose-1-phosphate; F6P, D-fructose-6-phosphate; GAL1P, D-galactose-1-phosphate; GAL6P, D-galactose-6-phosphate. Freshly prepared lymphocyte suspensions were preincubated with sugar (4°C; 15 min) and incubated over unfixed, frozen sections (7°C; 80 rpm; 50 min). Sections were fixed, washed, stained, and examined for binding activity as described previously (10). Each point depicts the mean and SEM of four replicates. Glucose phosphates (1 and 6) showed no significant inhibitory activity at a concentration of 10 mM (data not shown).

RESULTS

Specific, phosphorylated carbohydrates were potent inhibitors of the lymphocytes-HEV adhesive interaction in the frozen section assay. At 10 mM, the structurally related sugars M6P and F1P produced 80–90% inhibition of binding. In contrast, D-mannose-1-phosphate (M1P), D-fructose-6-phosphate (F6P), the D-galactose phosphates (1 and 6) and the D-glucose phosphates (1 and 6) had little or no effect (Fig. 2). M6P produced one-half maximal inhibition of binding (I50) at 2–3 mM (Fig. 3). Therefore, phosphorylation at the six position increased the potency of mannose 25–50-fold (I50 = 150 mM for D-mannose; see reference 10).

We reported previously that increasing the ionic strength of the incubation buffer results in a dose-dependent inhibition of lymphocyte binding (10). A 50% inhibition of binding required a 30% increase in ionic strength. In contrast, M6P or F1P inhibit binding by >80% at concentrations that increase the ionic strength by only 13%. Furthermore, all the sugar phosphates tested increased ionic strength to approximately the same degree and yet only M6P and F1P had significant inhibitory activity (at 10 mM). Thus a change in ionic strength alone cannot account for the inhibitory activity of the phosphorylated sugars.

In light of the M6P effect we tested several yeast mannans for inhibitory activity. The mnn1 and mnn2 mannans failed to inhibit at 1 mg/ml (data not shown). In contrast, PPME, derived from the yeast *Hansenula holstii*, was a potent inhibitor producing one-half maximal inhibition of binding at 10–20 µg/ml (~10⁻⁸ M; Fig. 4). In addition, the inhibitory activity of PPME appeared to plateau at concentrations >100 µg/ml (Fig. 4). In several experiments, the PPME-resistant binding ranged from 20 to 40% of the control value.
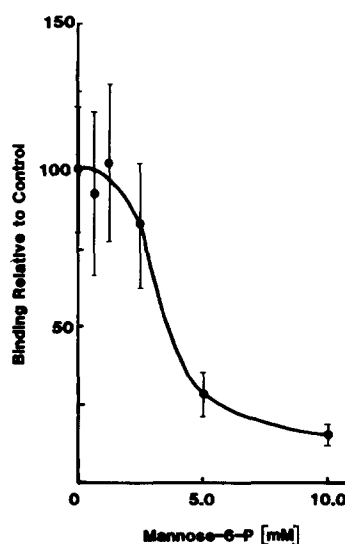
FIGURE 3 Dose-response curve for mannose-6-phosphate. Assay conditions as described in Fig. 2. Each point depicts the mean and SEM of four replicates.

PPME consists entirely of mannose and phosphate (6:1 molar ratio). The exposed phosphate residues are esterified to the 6 position of mannose (14, 15). These residues are critical to the inhibitory activity of PPME, since digestion with alkaline phosphatase reduced both its phosphate content and inhibitory activity (Fig. 5). An excess of free phosphate in the digestion mixture prevented the removal of phosphate (via product inhibition) and preserved the activity of PPME.

Preincubation experiments, in which a procedure maximized for speed and sensitivity was used, revealed that the lymphocyte was the primary target for the action of PPME. Preexposure to PPME significantly reduced the attachment
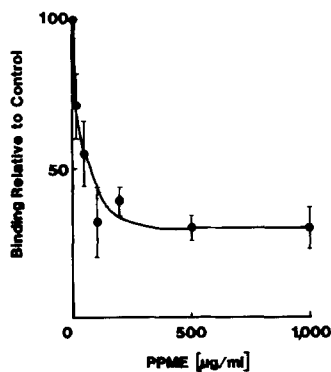
FIGURE 4 Dose-response curve for PPME. Assay conditions are described in Fig. 2. Each point depicts the mean and SEM of five replicates. Similar results were obtained in three independent experiments.
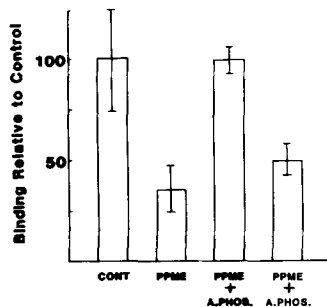

FIGURE 5 Inhibitory activity of PPME after removal of exposed phosphate residues. PPME was digested with *E. coil* alkaline phosphatase (as described in Materials and Methods) with or without 100 mM phosphate present. This procedure resulted in a 50% decrease in phosphate content of PPME; 100 mM phosphate prevented the removal of phosphate from PPME. Treated and untreated PPME (200 μg/ml) were assayed for inhibitory activity as described in Fig. 2. Each point depicts the mean and SEM of four replicates.
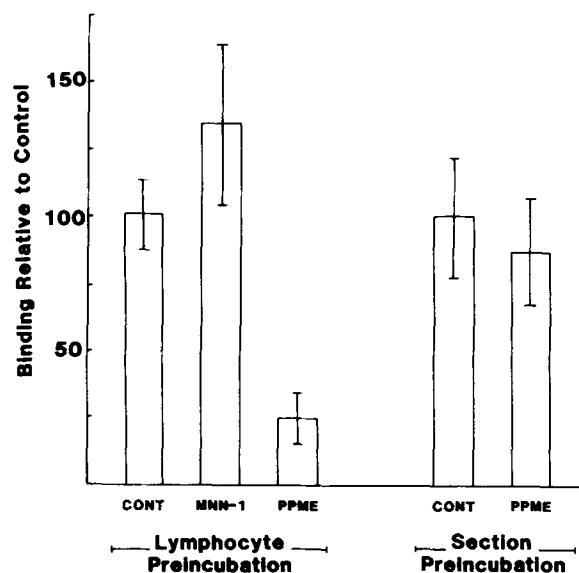

FIGURE 6 Persistent inhibition of binding after preincubation of lymphocytes with PPME. Each point depicts the mean and the SEM of five replicates.

of lymphocytes relative to both the control and the mnn1-mannan-treated cells (Fig. 6). In contrast, no significant inhibition of binding was detected when sections were pretreated in a similar manner (Fig. 6). Both the preincubations and the binding assay were conducted at temperatures that inhibit endocytosis (4°C and 10°C, respectively). In addition, increasing the duration of the wash procedure (from 1 to 15 min), without changing either the overall dilution factor or the duration of the binding assay, significantly reduced the inhibition by PPME. These data suggest that PPME binds reversibly to a cell-surface receptor involved in adhesion.

The activities of the phosphorylated sugars and PPME were not due to a generalized toxic effect on the lymphocytes, since maximally inhibiting concentrations of these substances neither inhibited the rate of protein synthesis (see Materials and Methods) nor reduced trypan blue exclusion. Furthermore, inhibition cannot be attributed solely to an increase in the negative charge on the surface of the lymphocytes. Cellular electrophoresis measurements indicated that the charge density on the cell surface varied considerably in the presence of the various substances tested (Fig. 7). However, there was no correlation between the magnitude of the negative charge density on the lymphocyte surface and the level of binding to HEV. For example, dextran sulfate (25 μg/ml) failed to inhibit binding and yet resulted in the greatest increase in charge density. In contrast, M6P (10 mM) was a potent inhibitor but produced the smallest increase in charge density.

DISCUSSION

In summary, M6P, the structurally related sugar F1P and the M6P-rich core phosphomannan PPME are potent inhibitors
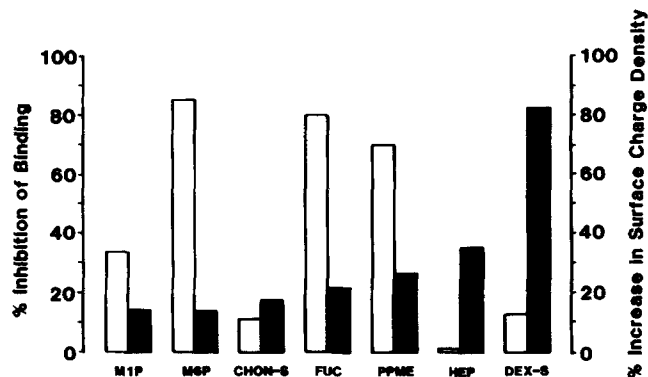

FIGURE 7 Percent inhibition of binding activity versus percent increase in negative surface charge density on the lymphocyte. Clear bars depict binding activity in the presence of carbohydrates; solid bars depict charge density on the cell surface. % Inhibition of binding = 1 − (binding in the presence of sugar)/(binding in the absence of sugar) × 100. % Increase surface charge density = (charge density in presence of sugar)/(charge density in absence of sugar) − 1 × 100. M1P (mannose-1-phosphate, 10 mM); M6P (mannose-6-phosphate, 10 mM); CHON-S (chondroitin sulfate, 25 μg/ml); FUC (fucoidin, 10 μg/ml); PPME (core phosphomannan from *H. holstii*, 200 μg/ml); HEP (heparin, 25 μg/ml); DEX-S (dextran sulfate, 25 μg/ml). Inhibitory activities pooled from published data (Results and ref. 10) in which the SEM ranged from 10 to 20%. Surface charge determined by cellular electrophoresis as described in Materials and Methods. Solid bars depict means of measurements made on 40–60 individual cells. SEM ranged from 3–4%. Based on a Student's *t* test, the difference in surface charge in the presence of M1P and M6P was not statistically significant. In contrast, the differences between the phosphorylated simple sugars and the various polysaccharides are significant (P < 0.01). In addition, the differences between fucoidin or PPME on the one hand, and heparin or dextran sulfate on the other, are statistically significant (P < 0.01).

of lymphocyte binding to the HEV of rat peripheral lymph nodes in vitro. Inhibitory activity does not result from either a generalized metabolic insult to the lymphocyte or a nonspecific increase in the density of negative charge at the cell

surface. Although steric interference with lymphocyte adhesion cannot be ruled out for the high molecular weight polysaccharides (fucoidin and PPME), such a mechanism seems unlikely for the monosaccharide inhibitors. These data suggest that a phosphomannosyl-binding receptor (lectin-like receptor) on the lymphocyte surface is involved in the attachment of lymphocytes to peripheral node HEV.

The apparent difference in the maximal inhibitory activities of PPME (60% at 200 μg/ml; see Fig. 4) and M6P (80–90% at 10 mM; see Figs. 2 and 3) may reflect the difference in the ionic strengths of these solutions. As we reported previously (10), a 13% increase in ionic strength alone can produce a 10–30% reduction in the attachment of lymphocytes to HEV in vitro. Therefore, the difference in the ionic strengths of the disodium M6P and PPME containing solutions, rather than an intrinsic difference in the inhibitory potencies of these compounds, may account for the fact that a 10 mM solution of the former (ionic strength of 0.176) inhibits binding to a greater extent than a 200 μg/ml solution of the latter (ionic strength of 0.156).

The plateau in PPME-sensitive adhesion suggests that lymphocytes may use more than one adhesive mechanism to bind to the HEV of peripheral lymph nodes. The lymphocyte suspensions employed in our studies are heterogeneous; therefore, PPME-sensitive adhesion may be restricted to a specific subset of the cells derived from peripheral lymph nodes. Whether this subset conforms to a functional class (T-cells, B-cells, etc.) or includes a mixture of cell types remains to be determined. We are currently investigating this question by quantitating the expression of the cell-surface phosphomannosyl receptor on functional subsets of lymphocytes and by comparing the inhibitory activity of PPME in cell suspensions enriched for each of these populations.

The phosphomannosyl specificity of the putative "adhesive lectin" on lymphocytes is similar to that described for membrane receptors implicated in the intracellular targeting of M6P-containing acid hydrolases to the lysosome. These phosphomannosyl-specific receptors are widely distributed in mammalian tissues on both the plasma and internal membranes (16–20). This receptor has also been implicated in the absorptive pinocytosis of M6P-containing glycoproteins and polysaccharides in the human diploid fibroblast (21, 22).

The attachment of lymphocytes to peripheral node HEV and the absorptive pinocytosis of M6P-containing acid hydrolases are both inhibited by M6P, F1P, and PPME (Figs. 2 and 4; and references 17, 23). M1P, glucose-6-phosphate, galactose-6-phosphate, and alkaline phosphatase-treated PPME show relatively little or no activity (Figs. 2 and 5; and references 17, 23). A further parallel is that L-fucose and D-mannose inhibit in both systems (10, 17), although the neutral monosaccharides are substantially less active than their phosphorylated counterparts. In contrast to these apparent similarities is the recent report that fucoidin, the most potent inhibitor of lymphocyte-HEV binding thus far identified (10), does not inhibit the attachment of a M6P-containing α-mannosidase (derived from the slime mold *Dictyostelium discoideum*) to the surface of intact, rabbit alveolar macrophages (24). Additional studies are required to determine the structural and functional relationships between the putative adhesive lectin on the lymphocyte surface and the phosphomannosyl receptors described above.

Ligatin is a plasma membrane protein that appears to act as a receptor ("baseplate") for the attachment of peripheral glycoproteins in a variety of tissues (25). Ligatin and its associated glycoproteins may play a role in the intercellular adhesion of embryonic chick neural retina cells (26). Ligatin has also been shown to bind to phosphorylated monosaccharides; however, the carbohydrate-binding specificity of ligatin varies with the tissue source complicating its comparison with the putative adhesive factor on lymphocytes (27).

The precise contribution of the phosphomannosyl receptor—whether it is the acid hydrolase receptor, a form of ligatin, or some other cell surface lectin—to the adhesive interaction between lymphocytes and peripheral lymph node HEV remains to be determined. One possibility is that the receptor forms a direct bridge to the HEV by binding to exposed glycocojugates (containing M6P or structurally related carbohydrates) on the luminal surface of the HEV. Alternatively, the phosphomannosyl receptor on the lymphocyte may serve as a baseplate for another adhesive molecule with actually forms the bridge to the HEV. A further possibility is that the phosphomannosyl receptor is not involved directly in attachment, but regulates the activity of an adhesive molecule(s) elsewhere on the cell surface. Experiments are currently underway to determine which model may apply.

Finally, further study is required to define the relationship between the phosphosmannosyl receptor and lymphocyte membrane antigens implicated as adhesive factors in binding to HEV. Chin et al. (8) have described a polyclonal antibody that blocks the binding of lymphocytes to HEV of rat peripheral lymph nodes. This antibody recognizes a set of polypeptides on the surface of rat lymphocytes. Gallatin et al. (9) have employed a monoclonal antibody to identify analogous molecules on mouse lymphocytes. It remains to be determined whether these antigens are phosphomannosyl receptors, ligands for a phosphomannosyl receptor, or possibly elements of an entirely independent adhesive mechanism.

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