Multiple Carbohydrate Receptors on Lymphocytes Revealed by Adhesion to Immobilized Polysaccharides

Brian K. Brandley, Theodora S. Ross, and Ronald L. Schnaar

Departments of Pharmacology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. Phosphomannan polysaccharides and fucoidan, a polymer of fucose 4-sulfate, have been demonstrated to inhibit adhesion of lymphocytes to tissue sections that contain high endothelial venules (Stoolman, L. M., T. S. Tenforde, and S. D. Rosen, 1984, *J. Cell Biol.*, 99:1535–1540). We have investigated the potential cell surface carbohydrate receptors involved by quantitating adhesion of rat cervical lymph node lymphocytes to purified polysaccharides immobilized on otherwise inert polyacrylamide gels. One-sixth of the lymphocytes adhered specifically to surfaces derivatized with PPME (a phosphomannan polysaccharide prepared from *Hansenula holstii* yeast), whereas up to half of the cells adhered to surfaces derivatized with fucoidan. Several lines of evidence demonstrated that two distinct receptors were involved. Adhesion to PPME-derivatized gels was labile at 37°C (decreasing to background levels within 120 min) whereas adhesion to fucoidan-derivatized gels was stable. Soluble PPME and other phosphomannans blocked adhesion only to PPME-derivatized gels; fucoidan and a structurally related fucan blocked adhesion to fucoidan-derivatized gels. Other highly charged anionic polysaccharides, such as heparin, did not block adhesion to either polysaccharide-derivatized gel. Adhesion to PPME-derivatized gels was dependent on divalent cations, whereas that to fucoidan-derivatized gels was not. The PPME-adherent lymphocytes were shown to be a subpopulation of the fucoidan-adhesive lymphocytes which contained both saccharide receptors. These data reveal that at least two distinct carbohydrate receptors can be found on peripheral lymphocytes.

OUR laboratory has modeled cell–cell recognition by studying sugar-specific adhesion of intact cells to otherwise inert, carbohydrate-derivatized polyacrylamide surfaces. These gels (with covalently linked glycosides or neoglycoproteins) have proved useful in detecting adhesion of hepatocytes, macrophages, and nerve cells to specific carbohydrates (Guarnaccia and Schnaar, 1982; Largent et al., 1984; Blackburn et al., 1986; Brandley and Schnaar, 1986; Schnaar, 1984), and in studying some complex responses of the cells to these surfaces (Guarnaccia et al., 1982; Brandley and Schnaar, 1985). Recent data indicating a role for carbohydrates in lymphocyte–endothelial cell adhesion (Stoolman and Rosen, 1983; Stoolman et al., 1984) suggested that our synthetic surfaces might be used to examine the molecular details of carbohydrate-directed lymphocyte adhesion.

Lymphocytes recirculate from the blood to the lymph by migrating through specialized areas of lymphoid organ capillaries (Gowans, 1959; Gowans and Knight, 1964) termed high endothelial venules (HEVs)[1]. This process is initiated when lymphocytes specifically recognize and adhere to the wall of this specialized endothelium. The recognition is apparently organ specific in that peripheral node and Peyer's patch lymphocyte populations each exhibit preferential binding to their homologous organ HEVs (Butcher et al., 1980). In addition, certain lymphoma cell lines express binding specificity for HEVs from a particular lymphoid organ (Gallatin et al., 1983). Isolated lymphocytes bind in an organ-specific manner to HEVs of fixed or frozen sections of lymphoid organs (Stamper and Woodruff, 1977; Butcher et al., 1980). Using this technology, investigators have implicated carbohydrate residues as recognition markers for adhesion of at least some peripheral lymphocytes to HEVs. Specific inhibition of lymphocyte–HEV binding in vitro by mannose 6-phosphate and the phosphomannan polysaccharide prepared from *Hasenula holstii* yeast (PPME) suggested that mannose phosphate residues were involved (Stoolman and Rosen, 1983; Stoolman et al., 1984). Fucoidan, a fucose 4-sulfate polymer from marine algae, was also a potent inhibitor. Subsequently, direct binding of PPME-conjugated beads to peripheral lymphocytes was demonstrated (Yednock et al., 1984).

The current study reports the use of covalently derivatized polyacrylamide gels to study the adhesion of peripheral lymphocytes to specific carbohydrates. Although surfaces derivatized with simple saccharides did not support lymphocyte adhesion, carbohydrate-specific adhesion to surfaces deriva-

Ms. Ross's current address is Department of Biological Chemistry, Washington University School of Medicine, St. Louis, MO 63110.

1. *Abbreviations used in this paper*: H-DMEM, Hepes-buffered Dulbecco's minimum essential medium; HEV, high endothelial venule; M5-P, phosphorylated pentasaccharide of mannose; PPME, high-molecular-weight yeast phosphomannan core polysaccharide with a mannose/phosphate ratio of 6:1.

tized with PPME or fucoidan was detected. Further characterization revealed that cell adhesion to the two polysaccharides was via separate receptors found to coexist on a subpopulation of lymphocytes.

Materials and Methods

Materials

The following chemicals were obtained from the sources indicated: glucose, galactose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fructose, and L-fucose (Pfanstiehl Laboratories, Waukegan, IL); glucose 6-phosphate, mannose 6-phosphate, L-fucose 1-phosphate, fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, sialic acid, mannan (M-7504), dextran (D-1390), dextran sulfate (D-6001, 500 kD), fucoidan (from *Fucus vesiculosus*, F-5631), carrageenans k (C-1263), i (C-4014), and l (C-3889), heparin (H-3125), chondroitin 4-sulfate (type A, C-4134) and chondroitin 6-sulfate (type C, C-4384), bovine serum albumin (BSA, A4503) and protease (P-5147) (Sigma Chemical Co., St. Louis, MO); acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine (Polysciences, Inc., Warrington, PA). Sodium [^{3}H]borohydride was from New England Nuclear, Boston, MA). All other reagents were of the highest quality and were obtained through standard sources. Aminohexyl glycosides and glycosylated BSA (neoglycoproteins) were prepared by published methods (Lee et al., 1976; Weigel et al., 1979). Purified M5-P (a phosphorylated pentasaccharide of mannose) and PPME (the phosphomannan core fragment) from the yeast *Hansenula holstii* phosphomannan (Bretthauer et al., 1973), were generous gifts of Dr. J. Distler (University of Michigan, Ann Arbor, MI), or were prepared from crude phosphomannan (see below [Slodki et al., 1973]) kindly supplied by Dr. M. Slodki (Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, IL). Sea urchin (*S. purpuratus*) egg jelly fucan, a polymer of fucose sulfate (Glabe et al., 1982), was a generous gift from Dr. C. Glabe (University of California, Irvine, CA).

Hepes-buffered Dulbecco's minimum essential medium (H-DMEM) was prepared as described previously (Guarnaccia and Schnaar, 1982), with the addition of 2 mg/ml BSA. Hepes-buffered saline contained 135 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 25 mM Hepes, adjusted to pH 7.4 with NaOH. Lysis buffer contained 0.1 M potassium phosphate buffer, pH 7.0, and 0.1% Triton X-100.

Fucoidan Preparation

Commercially obtained fucoidan was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mg/ml protease and incubated for 4 h at 37°C. The resulting solution was dialyzed against running water for 12 h, then against distilled water for 24 h. The dialysate was applied to a Sepharose 4B gel filtration column (1 × 45 cm) and eluted with water, and the high-molecular-weight carbohydrate peak was collected. After chilling to 0°C and adding cold ethanol to a final concentration of 70%, the polysaccharide was precipitated by the dropwise addition of saturated aqueous NaCl. The precipitate was collected by centrifugation, washed by centrifugation in cold 100% ethanol, and dried under vacuum. The resulting material was free of measurable protein, and was derivatized directly with hexanediamine (see below). A portion of the resulting derivative was subjected to acid hydrolysis (1 M HCl, 100°C, 3 h), and the resulting sugar(s) reduced with [^{3}H]$NaBH_4$ (Takasaki and Kobata, 1978). The reduced sugars were analyzed by HPLC on a Spherogel TSK DEAE column (Altex Scientific, Inc., Berkeley, CA, 0.75 × 7.5 cm) in a gradient of borate buffers (Lee, 1972) at a flow rate of 0.6 ml/min.

PPME Preparation

PPME is the core phosphomannan resulting from mild acid hydrolysis of *H. holstii* phosphomannan (Slodki, 1962). It was prepared from the crude phosphomannan by the methods of Slodki (personal communication [Slodki et al., 1973]). Phosphomannan from *H. holstii* (1 g) was dissolved in 100 ml of water, and strong cation-exchange resin (BioRad Laboratories, Richmond, CA, AG-50W, H^+ form) was added to adjust the pH to 2.5. The mixture was stirred at 100°C for 1 h, cooled, and filtered. The filtrate was adjusted to pH 11 by addition of saturated barium hydroxide, and the barium salt of PPME was precipitated by the addition of an equal volume of 95% ethanol and cooling at 4°C overnight. The precipitate was collected by centrifugation and dissolved in water, and the barium was removed with cation-exchange resin (H^+ form). After filtration, the resulting solution was dialyzed against distilled water for 24 h and evaporated under vacuum.

Carbohydrate-derivatized Polyacrylamide Gels

PPME and fucoidan were derivatized with aminohexyl extensions at their reducing ends as described previously for glycosaminoglycans (Raja et al., 1984) to allow covalent attachment to polyacrylamide gels. Briefly, polysaccharides were treated with sodium borohydride to linearize the reducing end sugar, which was then oxidized under mild conditions (60 mM $NaIO_4$, 1 h, 0°C). Treatment of the resulting aldehydo-terminated polysaccharides with hexanediamine under reducing conditions led to the desired aminohexyl derivatives. The ratio of carbohydrate (McKelvy and Lee, 1969) to primary amine (Lee, 1978) was 34:1 for PPME and 52:1 for fucoidan. The derivatives were immobilized on polyacrylamide surfaces in the bottom of 96-well plastic dishes using a succinimidyl ester immobilization reagent, N-6 (the *N*-succinimidyl ester of 6-acrylamidohexanoic acid [Pless et al., 1983]). Polysaccharide derivatives (up to 60 mg) were added to an aqueous solution (8 ml) of 20% (wt/vol) acrylamide, 1.25% (wt/vol) bisacrylamide, 0.13% (vol/vol) tetramethylethylenediamine, and 25 mM KH_2PO_4, pH 7.0. Crystalline N-6 (0.2 mg/mg polysaccharide) was added and the solution was allowed to incubate at ambient temperature for 15 min (during which time the primary amino groups became alkylated by N-6, creating an acrylyl derivative of the polysaccharide). The solution was deaerated, ammonium sulfate (0.18% wt/vol) was added, and aliquots were polymerized in 96-well dishes under deaerated water as described previously (Guarnaccia and Schnaar, 1982). After polymerization, excess active esters were eliminated by treatment of the gels with 50 mM ethanolamine in Hepes buffer, pH 8, for 2 h. The resulting gels were washed in distilled water, then with 0.15 M NaCl supplemented to 5% with isopropanol (to retard bacterial growth), and stored in the latter solution at 4°C. Acid hydrolysis of the resulting derivatized gels (6 N HCl, 100°C, 1 h) followed by colorimetric analysis of the released sugar (McKelvy and Lee, 1969) revealed incorporation of 25–50% of the polysaccharide into the gels. Polysaccharide immobilization was stable, with loss of <2% per week of storage. Aminohexyl glycosides (Weigel et al., 1979) and neoglycoproteins (Lee et al., 1976) of glucose, galactose, mannose, and GlcNAc were covalently immobilized in polyacrylamide gels as described previously (Guarnaccia and Schnaar, 1982; Pless et al., 1983; Schnaar, 1984).

Preparation of Lymphocytes

Male rats (Sprague-Dawley) were anesthetized with sodium pentabarbitol and the cervical lymph nodes were excised. The nodes were washed in ice-cold H-DMEM and teased apart in fresh buffer. The resulting cell suspension was triturated 5–10 times and passed through a Nitex 25 μm filter (cat. No. 3-25/6, Tetko Inc., Elmsford, NY), and cells were collected by centrifugation (200 *g*, 5 min, 4°C), and washed three times by centrifugation in ice-cold H-DMEM. The resulting population was typically >85% viable (by trypan blue exclusion), with only trace red blood cell contamination. Cervical lymph nodes from one rat yielded 1.2–2 × 10^8 lymphocytes. Monoclonal antibody typing of rat cervical lymph node cells prepared in a similar manner (Dallman et al., 1984) revealed that 55–60% of the cells were T lymphocytes (mAb W3/13 and/or mAb MRC OX19 positive) and 40–45% were B lymphocytes (mAb MRC OX12 positive).

Adhesion Experiments

Cell adhesion to derivatized gels was measured as described previously (Guarnaccia and Schnaar, 1982). Briefly, lymphocytes were diluted to 3.3 × 10^6 cells/ml in H-DMEM and pipetted onto gels in microtiter wells (150 μl of cell suspension per well). In some experiments soluble sugars, sugar phosphates, or polysaccharides were added to the medium in which the cells were diluted before addition of cells to the wells. The plate was centrifuged upright (50 *g*, 5 min) at 4°C to bring the cells into contact with the gel surface. Incubations at the indicated times and temperatures were begun immediately at the end of this centrifugation. After the desired incubation the wells were immersed in Hepes-buffered saline at 0°C, sealed with plastic tape, inverted, and centrifuged (while inverted) for 10 min at the detachment force indicated for each experiment. The wells were frozen in dry ice while inverted, and the gels (with adherent cells) were cut from the well bottoms and placed in 1 ml of lysis buffer. Lactate dehydrogenase in the lysate was measured (Schnaar et al., 1978) and compared to that from an aliquot of the cell suspension to determine the percentage of added cells that had adhered. Background adhesion (which ranged from 5–20% of the cells added) was

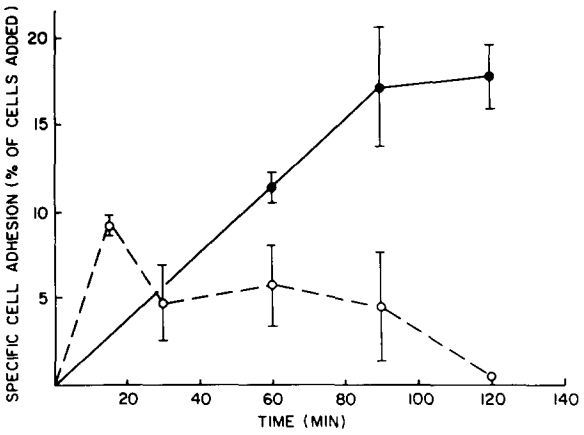

Figure 1. Specific adhesion of rat lymphocytes to PPME-derivatized gels. Rat lymphocytes were suspended in H-DMEM and incubated at either 8°C (●) or 37°C (○) on gels covalently derivatized with PPME (2.5 mg/ml gel) as described in the text. After incubation at the indicated temperature and for the indicated time, nonadherent cells were removed using a detachment force of 50 *g* and adherent cells were quantitated (as described in the text). The data are expressed as specific adhesion, with background adhesion (in the presence of 250 μg/ml soluble PPME) of 12–17% subtracted. Data are presented as the mean ± SEM of data from five to seven experiments (8°C) or one to three experiments (37°C) performed in triplicate.

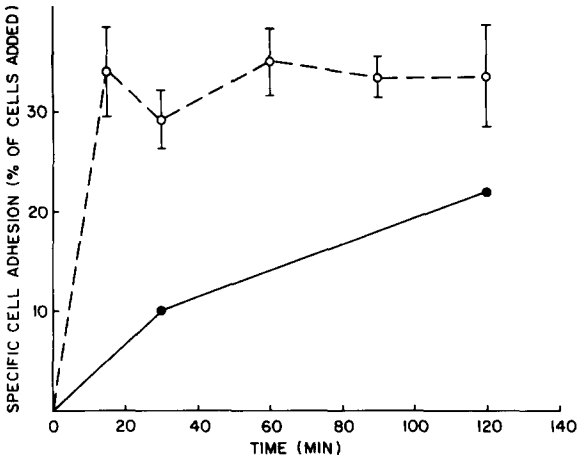

Figure 2. Specific adhesion of rat lymphocytes to fucoidan-derivatized gels. Rat lymphocytes were suspended in H-DMEM and incubated at either 8°C (●) or 37°C (○) on gels covalently derivatized with fucoidan (2.0 mg/ml gel) as described in the text. After incubation at the indicated temperature and for the indicated time, nonadherent cells were removed using a detachment force of 150 *g* and adherent cells were quantitated (as described in the text). The data are expressed as specific adhesion, with background adhesion (in the presence of 250 μg/ml of soluble fucoidan) of 10–20% subtracted. Data are presented as the mean ± SEM of data from 1 experiment (8°C) or 2–13 experiments (37°C) performed in triplicate.

measured on gels derivatized with BSA, or in the presence of excess soluble fucoidan (for adhesion to fucoidan-derivatized gels) or PPME (for PPME-derivatized gels).

In some experiments nonadherent cells from one set of gels were centrifuged onto a second set of gels to determine relationships between adherent and nonadherent subpopulations. This was accomplished by a modification of the microtiter well technique (McClay et al., 1981) using double-sided tape to seal plates to each other (D. R. McClay, personal communication). After incubation of cells on the first set of gels (as described above), the plate was inverted above the second plate of gels and attached using double-sided tape. Centrifugation at 50 *g* for 10 min resulted in movement of any nonadherent cells to the fresh gel surface. After a second incubation the wells were again inverted and centrifuged at 50 *g* for 10 min. After freezing in dry ice (as above), the second set of gels was removed and adherent cells were determined by using the lactate dehydrogenase assay described above.

Results

Lymphocytes (isolated from rat cervical lymph nodes) adhered specifically to polyacrylamide gels derivatized with the phosphomannan polysaccharide, PPME (Fig. 1) or the fucose 4-sulfate polymer, fucoidan (Fig. 2). Gels derivatized with aminohexylglycosides of glucose, galactose, mannose, or *N*-acetylglucosamine or with various neoglycoproteins (glucose-, galactose-, mannose-, or *N*-acetylglucosamine-BSA) did not support adhesion (data not shown). When optimal levels of the saccharides were immobilized (2.5 mg PPME/ml gel; 2.0 mg fucoidan/ml gel), the maximum specific cell adhesion to PPME-derivatized gels ranged from 13 to 29% of the cells added (average 18%) whereas a maximum of 25–51% of added lymphocytes adhered specifically to fucoidan-derivatized gels (average 35%). Half-maximal adhesion to each saccharide occurred when ~1 mg/ml saccharide was immobilized, and no significant additional adhesion occurred when the immobilized saccharide level was raised to nearly 5 mg of saccharide/ml gel (data not shown).

As described below, many characteristics of lymphocyte adhesion to the two polysaccharide-derivatized gels varied significantly, demonstrating the involvement of two distinct carbohydrate receptors.

Effect of Temperature

Lymphocyte adhesion to PPME-derivatized gels was optimal at lower temperatures (Fig. 1). The number of cells adhering to 8°C increased to maximal levels during the first 90 min of contact with the derivatized surface and was stable for at least 2 h. In contrast, at 37°C PPME-directed cell adhesion was maximal at early incubation times, then decreased to background levels within 2 h. Adhesion to fucoidan-derivatized gels had quite different kinetics and temperature dependence (Fig. 2). At 37°C adhesion reached maximum levels within 15 min, and was stable at this higher temperature for at least 2 h (the strength of adhesion actually increased with time at 37°C, data not shown). At 8°C adhesion to fucoidan-derivatized gels was slow, and did not reach maximum levels within 2 h (the longest incubation tested).

Sugar Specificity

The specificity of lymphocyte adhesion to polysaccharide-derivatized gels was tested by adding potential carbohydrate inhibitors to the adhesion medium (Fig. 3, Table I). Whereas soluble PPME reduced adhesion to PPME-derivatized gels to near background levels (adhesion to underivatized acrylamide was typically 8% of the cells added), it was ineffective at reducing adhesion to fucoidan-derivatized gels. Conversely, soluble fucoidan blocked adhesion to fucoidan-derivatized gels, but not to PPME-derivatized gels. The effects of other carbohydrates as inhibitors were consistent with the in-

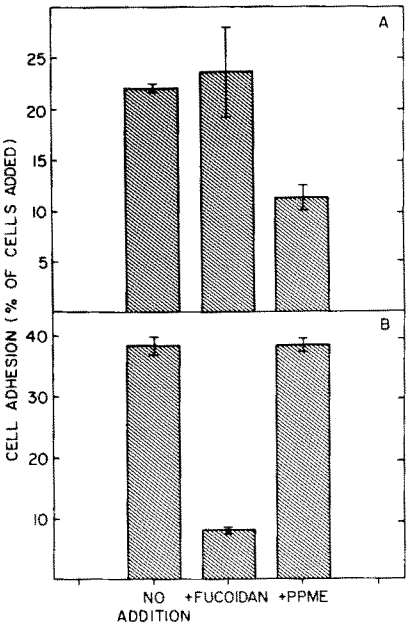

Figure 3. Inhibition of carbohydrate-directed lymphocyte adhesion by soluble polysaccharides. Rat lymphocytes were suspended in H-DMEM and incubated on polyacrylamide gels covalently derivatized with (*A*) PPME (2.5 mg/ml gel) or (*B*) fucoidan (2.0 mg/ml gel). Cells were incubated under optimal conditions (60 min at 8°C on PPME-derivatized gels, or 90 min at 37°C on fucoidan-derivatized gels) in the presence or absence of soluble PPME or fucoidan (250 μg/ml) as indicated. After the desired incubation, nonadherent cells were removed using a detachment force of 50 *g* (PPME-derivatized gels) or 150 *g* (fucoidan-derivatized gels), and adherent cells were quantitated as described in the text. Data are present as the mean ± SEM of (*A*) triplicate or (*B*) quadruplicate determinations.

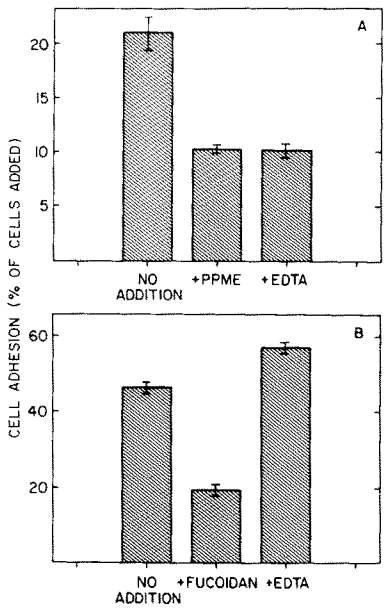

Figure 4. Effect of removal of divalent cations on carbohydrate-directed lymphocyte adhesion. Rat lymphocytes were suspended in H-DMEM, or in H-DMEM lacking calcium and magnesium and supplemented with 1 mM EDTA (as indicated). Cells were incubated on polyacrylamide gels covalently derivatized with (*A*) PPME (2.5 mg/ml gel) or (*B*) fucoidan (2.0 mg/ml gel) under optimal conditions (60 min at 8°C on PPME-derivatized gels, or 90 min at 37°C on fucoidan-derivatized gels) in the presence or absence of soluble PPME or fucoidan (250 μg/ml) as indicated. After the desired incubation, nonadherent cells were removed using a detachment force of 50 *g* (PPME-derivatized gels) or 150 *g* (fucoidan-derivatized gels) and adherent cells quantitated as described in the text. Data are presented as the mean ± SEM of (*A*) triplicate or (*B*) quadruplicate determinations.

Table I. Lymphocyte Adhesion to Fucoidan-derivatized Gels in the Presence of Potential Carbohydrate Inhibitors

Carbohydrate added*	Concentration necessary for 50% inhibition of adhesion to gels	
	PPME-derivatized	Fucoidan-derivatized
	μg/ml	*μg/ml*
Polysaccharides		
PPME	50	(>1,000)
M5-P	100	(>1,000)
Fucoidan	(>500)	25
Egg jelly fucan	ND	35
Dextran sulfate	(>500)	100
Heparin	(>500)	(>1,000)
Mannan	(>500)	(>1,000)
Chondroitin 4-sulfate	ND	(>1,000)
Chondroitin 6-sulfate	ND	(>1,000)
Dextran	ND	(>1,000)
	mM	*mM*
Anionically charged saccharides		
Mannose 6-phosphate	50	(>50)
Glucose 1-phosphate	(>50)	50
Glucose 6-phosphate	(>50)	50

ND, not determined.
* Other carbohydrates tested for inhibition (at 50 mM) of fucoidan-directed adhesion without effect were fucose 1-phosphate, fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, sialic acid, Glc, Gal, Man, fucose, Fru, GlcNAc, and GalNAc.

terpretation of different receptors for the two polysaccharides (Table I). Selective inhibition of adhesion to PPME-derivatized gels was found when a phosphomannan pentasaccharide (M5-P), or mannose 6-phosphate were added. In contrast, dextran sulfate and glucose 1- and 6-phosphates selectively blocked adhesion to fucoidan-derivatized gels. The most potent inhibitors of adhesion to fucoidan-derivatized gels were fucoidan itself, and another fucose 4-sulfate polymer, sea urchin egg jelly fucan (Glabe et al., 1982). It is notable that other highly charged polysaccharides (including heparin) and monosaccharide phosphates were ineffective at blocking adhesion to either PPME- or fucoidan-derivatized gels. Dextran sulfate (a highly sulfated synthetic derivative of dextran) inhibited adhesion to fucoidan-derivatized gels, having one-fourth the inhibitory potency as fucoidan or egg jelly fucan.

Dependence on Divalent Cations

Another characteristic of adhesion to the two polysaccharides which differed was its dependence on divalent cations. When adhesion was performed in H-DMEM lacking calcium and magnesium and supplemented with 1 mM EDTA, adhesion to PPME-derivatized gels was reduced to background levels, whereas adhesion to fucoidan-derivatized gels was unaffected (Fig. 4).

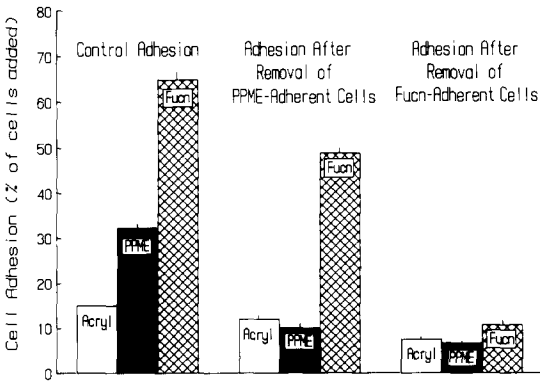

Figure 5. Rat lymphocytes were suspended in H-DMEM and incubated at 8°C for 90 min on underivatized polyacrylamide gels (control adhesion, *left set of bars*), gels derivatized with PPME (2 mg/ml, *center set of bars*), or gels derivatized with fucoidan (2 mg/ml, *right set of bars*) as described in the text. Replicate sets of wells preincubated on each gel type were inverted onto fresh wells containing control gels (Acryl, *open bars*), PPME-derivatized gels (*solid bars*), or fucoidan-derivatized gels (Fucn, *cross-hatched bars*) and attached with double-sided tape. Cells remaining nonadherent after the first incubation were centrifuged onto the second set of gels at 50 *g* for 10 min at 4°C, and incubated an additional 90 min at 8°C. After the second incubation, the wells were reinverted and centrifuged at 50 *g* for 10 min at 4°C to remove cells that had not adhered to the second set of gels. While still inverted, the wells were frozen in dry ice and cells adherent to the second set of gels were quantitated as described in the text. Data are from quadruplicate determinations, and are presented as the mean ± SEM.

Relationship between PPME-adherent and Fucoidan-adherent Lymphocyte Subpopulations

After incubation on either control (underivatized polyacrylamide), PPME-derivatized, or fucoidan-derivatized gels, nonadherent cells were centrifuged onto a second gel surface in order to determine the relationship between adherent and nonadherent subpopulations. The results (Fig. 5) demonstrate that the PPME-adherent cells are a subpopulation of the fucoidan-adherent cells which have receptors for both saccharides. Preincubation on fucoidan-derivatized gels eliminated binding both to fucoidan- and PPME-derivatized gels, whereas precincubation on PPME-derivatized gel eliminated adhesion to PPME-derivatized gels and reduced adhesion to fucoidan-derivatized gels by an amount equivalent to the loss of PPME-adherent cells. Thus, approximately half of the cells are competent to bind to fucoidan-derivatized gels, and one-third of those cells were also competent to bind to PPME-derivatized gels.

Discussion

Complex carbohydrates on one cell surface and complementary carbohydrate binding proteins (lectins) on apposing cells have been implicated in cell–cell recognition and adhesion in several cell systems (Brandley and Schnaar, 1986). Recent studies measuring adhesion of lymphocytes to isolated HEVs (through which lymphocytes migrate) suggested a role for complex carbohydrates in lymphocyte recirculation (Stoolman and Rosen, 1983; Stoolman et al., 1984). This led us to study direct binding of lymphocytes to well-defined carbohydrate-derivatized surfaces. Similar surfaces have been used successfully to examine specific adhesion and postadhesion responses of several cell types (Guarnaccia et al., 1982; Guarnaccia and Schnaar, 1982; Largent et al., 1984; Schnaar, 1984; Blackburn et al., 1986; Brandley and Schnaar, 1986; Brandley and Schnaar, 1985). Previous results (Stoolman et al., 1984) indicated that adhesion of lymphocytes to HEVs was inhibited by PPME (a high-molecular-weight yeast phosphomannan core polysaccharide with a mannose/phosphate ratio of 6:1) and by fucoidan (a polymer of fucose 4-sulfate), although these polysaccharides are not closely structurally related. We report that rat peripheral lymphocytes adhere with carbohydrate specificity to otherwise nonadhesive polyacrylamide gels covalently derivatized with either PPME or fucoidan. The characteristics of the adhesion demonstrate the involvement of two separate lymphocyte cell-surface carbohydrate receptors that coexist on a subpopulation of lymphocytes.

Adhesion of lymphocytes to PPME-derivatized gels has characteristics similar to those reported for lymphocyte binding to HEVs (Woodruff et al., 1977) (and PPME-derivatized beads [Yednock et al., 1984, 1985]). PPME is a well-characterized polysaccharide consisting solely of mannose and mannose 6-phosphate residues (Pittsley et al., 1961; Slodki, 1962; Jeanes and Watson, 1962). Lymphocyte adhesion to gels derivatized with purified PPME was stable only at low temperatures, as is adhesion to HEVs. Lymphocyte adhesion to PPME-derivatized gels was also dependent on divalent cations, as is lymphocyte adhesion (initially) to HEVs (Woodruff et al., 1977) and to PPME-derivatized beads (Yednock et al., 1984). Adhesion to both PPME-derivatized gels (and HEVs) was blocked by mannose 6-phosphate and more potently by phosphomannans.

Purified fucoidan and sea urchin egg jelly fucan were used to demonstrate the carbohydrate specificity of fucoidan-directed lymphocyte adhesion. In contrast to PPME, commercially available fucoidan is a crude preparation containing proteins and sugars other than fucose (Medcalf and Larsen, 1977). Purification by protease treatment and chromatography, however, resulted in a preparation essentially free of protein, and having fucose as its major saccharide (>80% by HPLC) with a smaller amount of galactose present (<10%). Significantly, there was no detectable mannose in this preparation. Although it is not possible to rule out the presence of a minor contaminant that may be responsible for the adhesion detected, a highly purified fucose 4-sulfate polysaccharide from another source (sea urchin egg jelly fucan) was equally potent in blocking fucoidan-directed lymphocyte adhesion, whereas other highly sulfated polysaccharides (heparin, chondroitins) were without effect. The highly sulfated carrageenans (galactose 3,6-anhydrogalactose copolymers with more than one sulfate per disaccharide) actually enhanced adhesion to fucoidan-derivatized gels (data not shown), perhaps owing to their ability to activate lymphocytes (Ishizaka et al., 1980; Kolb et al., 1981). The only anionic polysaccharide that inhibited adhesion was the synthetically sulfated dextran sulfate (having up to three sulfates per sugar residue). This was not unexpected, because dextran sulfate has been reported to bind to lymphocyte surfaces and increase their surface anionic charge (Parish and Snowden, 1985; Stoolman et al., 1984). In total, the data suggest

fucoidan-directed adhesion was not simply due to anionic charge, but was carbohydrate specific.

The ability of either phosphomannans or fucoidan to block lymphocyte adhesion to HEVs in vitro (Stoolman et al., 1984), and the subsequent demonstration of a phosphomannan receptor apparently involved in that adhesion (the Mel-14 antigen [Yednock et al., 1985]), suggested that binding of phosphomannans and fucoidan may have been through that same receptor. However, highly purified PPME (added at 10-fold the concentration necessary to inhibit binding to HEVs or PPME-derivatized gels) did not reduce fucoidan-directed lymphocyte adhesion. Similarly, mannose 6-phosphate, (at fivefold the concentration necessary to inhibit HEV binding) and M5-P were ineffective in inhibiting fucoidan-directed adhesion. Additional evidence for multiple receptors was the finding that removal of divalent cations did not diminish fucoidan-directed lymphocyte adhesion, although it eliminated PPME-directed adhesion. A novel assay developed by Dr. D. R. McClay, Duke University, allowed us to analyze the relationship between PPME-adherent and fucoidan-adherent cells. As shown in Fig. 5, when the lymphocyte population was depleted of fucoidan-adherent cells (50% of the cell population), no cells competent to bind to PPME remained. Similarly, when PPME-adherent cells were removed (17% of the population) adhesion to fucoidan-derivatized gels was reduced by an equivalent amount. These data indicate that approximately one-sixth of the cervical lymph node lymphocytes have receptors for both PPME and fucoidan, approximately one-third of the lymphocytes have fucoidan receptors but no PPME receptors, and approximately half of the lymphocytes do not have receptors for either saccharide.

The presence of phosphomannan residues on mammalian glycoconjugates (Distler et al., 1979; Lang et al., 1984), and their potential role in recognition phenomena both in lymphocyte homing (Stoolman et al., 1984) and glycoprotein sorting (Sly and Fischer, 1982) has been established. However, the relevance of fucoidan-directed lymphocyte adhesion is speculative. Whereas fucose sulfate polymers have been shown to act as recognition markers in sperm-egg binding in the sea urchin (Glabe et al., 1982), fucose sulfate-containing glycoconjugates have not been widely reported in vertebrates. Although the presence of as yet undescribed fucose sulfate on the HEV is not ruled out (Kent et al., 1978), we favor an alternate hypothesis. In many vertebrate glycoconjugates, fucose and sialic acid coexist on the same carbohydrate chain (Ledeen and Yu, 1982). When molecular models of some of these chains are compared with molecular models of fucoidan, it is apparent that the spatial relationship between the fucose ring and the sulfate anionic charge in fucoidan can mimic the relationship between fucose and the anionically charged carboxylic acid of sialic acid. Such a relationship was postulated by Rosen, et al. (1985) to explain the finding that pretreatment of HEV sections with neuraminidase specifically inhibited lymphocyte binding. While these studies are suggestive, only characterization of endogenous glycoconjugates of HEV which support lymphocyte adhesion will clarify this point further.

Although the molecular mechanisms which underlie cell-cell recognition and adhesion in lymphocytes remain largely unknown, in vitro adhesion assays (Stamper and Woodruff, 1977; Butcher et al., 1980), and inhibition of such adhesion by carbohydrates (Stoolman and Rosen, 1983; Stoolman et al., 1984; Yednock et al., 1984, 1985) and monoclonal antibodies (Gallatin et al., 1983; Yednock et al., 1985) have begun to reveal some of the molecules involved in these complex processes. The demonstration that lymphocytes bind to chemically defined surfaces derivatized with specific carbohydrates offers an additional tool to probe these interactions.

The authors sincerely thank Dr. M. Slodki for the gift of *H. holstii* phosphomannan, Dr. J. Distler for kindly providing purified phosphomannans, Dr. C. Glabe for providing sea urchin egg jelly fucan, and Dr. D. McClay for sharing his new technology for analysis of adhesive and nonadhesive subpopulations. We also thank William Seidel for material support and Paula Manzuk for manuscript preparation.

This work was supported by National Institutes of Health grants HD-20527 and HD-14010 and American Cancer Society Faculty Award FRA-280 (to Dr. Schnaar) and National Research Service Award CA 08012 (to Dr. Brandley). Ms. Ross was a participant in the Student Intern Program of the Biology Department of Kalamazoo College, Kalamazoo, MI.

Received for publication 24 March 1986, and in revised form 4 March 1987.

References

Blackburn, C. C., P. Swank-Hill, and R. L. Schnaar. 1986. Gangliosides support neural retina cell adhesion. *J. Biol. Chem.* 261:2873–2881.

Brandley, B. K., and R. L. Schnaar. 1985. Phosphorylation of extracellular carbohydrates by intact cells: chicken hepatocytes specifically adhere to and phosphorylate immobilized *N*-acetyl glucosamine. *J. Biol. Chem.* 260: 12474–12483.

Brandley, B. K., and R. L. Schnaar. 1986. Review: cell-surface carbohydrates in cell recognition and response. *J. Leukocyte Biol.* 40:97–111.

Bretthauer, R. K., G. J. Kaczorowski, and M. J. Weise. 1973. Characterization of a phosphorylated pentasaccharide isolated from Hasenula holstii NRRL Y-2448 phosphomannan. *Biochemistry.* 12:1251–1256.

Butcher, E. C., R. G. Scollay, and I. L. Weissman. 1980. Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. *Eur. J. Immunol.* 10:556–561.

Dallman, M. J., M. L. Thomas, and J. R. Green. 1984. MRC OX-19: a monoclonal antibody that labels rat T lymphocytes and augments in vitro proliferative responses. *Eur. J. Immunol.* 14:260–266.

Distler, J., V. Hieber, G. Sahagian, R. Schmickel, and G. W. Jourdian. 1979. Identification of mannose 6-phosphate in glycoproteins that inhibit the assimilation of β-galactosidase by fibroblasts. *Proc. Natl. Acad. Sci. USA.* 76:4235–4239.

Gallatin, W. M., I. L. Weissman, and E. C. Butcher. 1983. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature (Lond.).* 304:30–34.

Glabe, C. G., L. B. Grabel, V. D. Vacquier, and S. D. Rosen. 1982. Carbohydrate specificity of sea urchin sperm bindin: a cell surface lectin mediating sperm-egg adhesion. *J. Cell Biol.* 94:123–128.

Gowans, J. L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol. (Lond.).* 146:54–69.

Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. Lond. B Biol. Sci.* 159:257–282.

Guarnaccia, S. P., M. S. Kuhlenschmidt, C. W. Slife, and R. L. Schnaar. 1982. Hepatocyte adhesion to immobilized carbohydrates. II. Cellular modification of the carbohydrate surface. *J. Biol. Chem.* 257:14293–14299.

Guarnaccia, S. P., and R. L. Schnaar. 1982. Hepatocyte adhesion to immobilized carbohydrates. I. Sugar recognition is followed by energy-dependent strengthening. *J. Biol. Chem.* 257:14288–14292.

Ishizaka, S., T. Hasuma, S. Otani, and S. Morisawa. 1980. Lymphocyte activation by purified carrageenan. *J. Immunol.* 125:2232–2235.

Jeanes, A., and P. R. Watson. 1962. Periodate-oxidized phosphomannan Y-2448: structural significance of its reaction with alkali. *Can. J. Chem.* 40:1318–1325.

Kent, P. W., C. J. Coles, J. R. Cooper, and N. R. Mian. 1978. Sulfate ester groups as potential informational regulators in glycoproteins. *In* Carbohydrate Sulfates. R. G. Schweiger, editor. American Chemical Society, Washington, DC. 29–43.

Kolb, J. P., P. C. Quan, M. P. Poupon, and C. Desaymard. 1981. Carrageenan stimulates populations of mouse B cells mostly nonoverlapping with those stimulated with LPS or dextran sulfate. *Cell. Immunol.* 57:348–360.

Lang, L., M. Reitman, J. Tang, R. M. Roberts, and S. Kornfeld. 1984. Lysosomal enzyme phosphorylation: recognition of a protein-dependent determinant allows specific phosphorylation of oligosaccharides present on lysosomal enzymes. *J. Biol. Chem.* 259:14663–14671.

Largent, B. L., K. M. Walton, C. A. Hoppe, Y. C. Lee, and R. L. Schnaar. 1984. Carbohydrate-specific adhesion of alveolar macrophages to mannose-derivatized surfaces. *J. Biol. Chem.* 259:1764–1769.

Ledeen, R. W., and R. K. Yu. 1982. Gangliosides: structure, isolation, and analysis. *Methods Enzymol.* 83:139–191.

Lee, Y. C. 1972. Analysis of sugars by automated liquid chromatography. *Methods Enzymol.* 28:63–73.

Lee, Y. C. 1978. Synthesis of some cluster glycosides suitable for attachment to proteins or solid matrices. *Carbohydr. Res.* 67:509–514.

Lee, Y. C., C. P. Stowell, and M. J. Krantz. 1976. 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. *Biochemistry.* 18:3959–3963.

McClay, D. R., G. M. Wessel, and R. B. Marchase. 1981. Intercellular recognition: quantitation of initial binding events. *Proc. Natl. Acad. Sci. USA.* 88:4975–4979.

McKelvy, J. F., and Y. C. Lee. 1969. Microheterogeneity of the carbohydrate group of *Aspergillus oryzae* α-amylase. *Arch. Biochem. Biophys.* 132: 99–110.

Medcalf, D. G., and B. Larsen. 1977. Fucose-containing polysaccharides in the brown algae *Ascophyllum nodosum* and *Fucus vesiculosus*. *Carbohydr. Res.* 59:531–537.

Parish, C. R., and J. M. Snowden. 1985. Lymphocytes express a diverse array of specific receptors for sulfated polysaccharides. *Cell Immunol.* 91:201–214.

Pittsley, J. E., P. R. Watson, and R. J. Dimler. 1961. Characterization and properties of the phosphomannan from *Hansenula holstii* NRRL Y-2448. *Arch. Biochem. Biophys.* 92:343–350.

Pless, D. D., Y. C. Lee, S. Roseman, and R. L. Schnaar. 1983. Specific cell adhesion to immobilized glycoproteins demonstrated using new reagents for protein and glycoprotein immobilization. *J. Biol. Chem.* 258:2340–2349.

Raja, R. H., R. D. LeBoeuf, G. W. Stone, and P. H. Weigel. 1984. Preparation of alkylamine and 125I-radiolabeled derivatives of hyaluronic acid uniquely modified at the reducing end. *Anal. Biochem.* 139:168–177.

Rosen, S. D., M. S. Singer, T. A. Yednock, and L. M. Stoolman. 1985. Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. *Science (Wash. DC).* 228:1005–1007.

Schnaar, R. L. 1984. Immobilized glycoconjugates for cell recognition studies. *Anal. Biochem.* 143:1–13.

Schnaar, R. L., P. H. Weigel, M. S. Kuhlenschmidt, Y. C. Lee, and S. Roseman. 1978. Adhesion of chicken hepatocytes to polyacrylamide gels derivatized with N-acetylglucosamine. *J. Biol. Chem.* 253:7940–7951.

Slodki, M. E. 1962. Phosphate linkages in phosphomannans from yeast. *Biochim. Biophys. Acta.* 57:525–533.

Slodki, M. E., R. M. Ward, and J. A. Boundy. 1973. Concanavalin A as a probe of phosphomannan molecular structure. *Biochim. Biophys. Acta.* 304:449–456.

Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell. Biochem.* 18:67–85.

Stamper, H. B., Jr., and J. J. Woodruff. 1977. An in vitro model of lymphocyte homing. I. Characterization interaction between thoracic duct lymphocytes and high-endothelial venules of lymph nodes. *J. Immunol.* 119:772–780.

Stoolman, L. M., and S. D. Rosen. 1983. Possible role for cell-surface carbohydrate-binding molecules in lymphocyte recirculation. *J. Cell Biol.* 96: 722–729.

Stoolman, L. M., T. S. Tenforde, and S. D. Rosen. 1984. Phosphomannosyl receptors may participate in the adhesive interaction between lymphocytes and high endothelial venules. *J. Cell Biol.* 99:1535–1540.

Takasaki, S., and A. Kobata. 1978. Microdetermination of sugar composition by radioisotope labeling. *Methods Enzymol.* 50:50–54.

Weigel, P. H., M. Naoi, S. Roseman, and Y. C. Lee. 1979. Preparation of 6-aminohexyl D-aldopyranosides. *Carbohydr. Res.* 70:83–91.

Woodruff, J. J., I. M. Katz, L. E. Lucas, and H. B. Stamper, Jr. 1977. An in vitro model of lymphocyte homing. II. Membrane and cytoplasmic events involved in lymphocyte adherence to high-endothelial venules of lymph nodes. *J. Immunol.* 119:1603–1610.

Yednock, T. A., E. C. Butcher, L. M. Stoolman, and S. D. Rosen. 1985. Lymphocyte homing receptor: Relationship between the MEL-14 antigen and a cell surface lectin. *J. Cell Biol.* 101:233a. (Abstr.)

Yednock, T. A., L. M. Stoolman, and S. D. Rosen. 1984. Demonstration of a lymphocyte cell-surface mannose 6-phosphate binding activity and correlation with recirculation potential. *J. Cell Biol.* 99:68*a*. (Abstr.)