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Journal

Journal of Cell Biology, 96(3)

ISSN

0021-9525

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Publication Date

1983-03-01

DOI

10.1083/jcb.96.3.722

Peer reviewed

Possible Role for Cell-surface Carbohydrate-binding Molecules in Lymphocyte Recirculation

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ABSTRACT We are investigating the hypothesis that carbohydrate-binding molecules on the cell surface are involved in the recirculation of lymphocytes from the bloodstream into lymphoid organs. This phenomenon requires the specific attachment of circulating lymphocytes to the endothelial cells of postcapillary venules. Using an *in vitro* assay to measure the adhesive interaction between lymphocytes and postcapillary venules, we have found that L-fucose, D-mannose, and the L-fucose-rich, sulfated polysaccharide fucoidin specifically inhibit this binding interaction. L-fucose shows stereo-selective inhibitory activity at concentrations >18 mM while fucoidin produces 50% inhibition at $\sim 1-5 \times 10^{-8}$ M. Fucoidin appears to interact with the lymphocyte, and not the postcapillary venule, to inhibit binding. These data suggest that cell surface carbohydrates (fucose-like) and carbohydrate-binding molecules (cell surface lectins) may contribute to the specific attachment of lymphocytes to postcapillary venules.

Normal lymphocytes circulate repeatedly through the bloodstream, tissues and lymphatic system (1, 2). This phenomenon has been termed "recirculation" and is thought to be critical to the normal function of the immune system, perhaps ensuring the widest possible distribution and exposure to antigen of an individual's repertoire of lymphocyte clones. The portal of entry for bloodstream lymphocytes into tissues appears to be the postcapillary venules. In rodents and humans, the postcapillary venules of lymph nodes and Peyer's patches have an unusual histologic appearance characterized by a single layer of prominent, cuboidal endothelial cells resting on a basement membrane (termed high endothelial venules, HEV).

Histologic studies have documented an intimate association of intravascular lymphocytes with HEV (2-4). Electron microscopic studies suggest that circulating lymphocytes first attach to the luminal surfaces of HEV, then pass through their inter-endothelial junctions and basement membrane into the surrounding nodal parenchyma (4). Thus, the first step in lymphocyte recirculation is the attachment of blood-borne cells to specialized postcapillary venules.

A more detailed examination of this adhesive interaction was made possible by the development of an *in vitro* assay using lymphocyte suspensions and frozen tissue sections. Stamper and Woodruff, and Butcher et al. were able to show that fresh lymphocytes bound avidly and specifically to the HEV of either glutaraldehyde-fixed (5, 6) or air-dried (7, 8) frozen sections of lymph nodes. This binding was found to

correlate with the flux of blood-borne lymphocytes into lymph nodes *in vivo*. For example, thymocytes, which have a diminished capacity to enter peripheral lymphoid organs when compared to cells derived from spleen, lymph nodes, or peripheral blood, show correspondingly less binding to HEV in frozen sections (5, 7). The most convincing demonstration of the correlation between attachment to HEV *in vitro* and entry into lymph nodes *in vivo* is in the work of Butcher et al. (8). These investigators quantified the binding of lymphocytes from different species to the HEV of mouse lymph nodes *in vitro*, and compared this measurement to the flux of these cells into lymph nodes *in vivo*. The binding *in vitro* and the flux *in vivo* diminished in parallel as the evolutionary distance between the mouse and the source of the lymphocytes increased. These data suggest that the binding of lymphocytes to HEV in frozen sections reflects a physiologically significant adhesive interaction.

We have employed a modification of the *in vitro* assay described above to study the nature of the adhesive molecules on the surface of lymphocytes and HEV. In this study we report evidence suggesting that carbohydrate-binding molecules on the cell surface participate in this interaction.

MATERIALS AND METHODS

Chemicals: Monosaccharides were obtained from Sigma Chemical Co. (St. Louis, MO). Fucoidin was purchased from K & K Labs, Plainview, NY and

used without further purification except as indicated. Heparin (H3125), chondroitin sulfate (C3254), dextran sulfate (D7515), hyaluronic acid (H1751), dermatan sulfate (C4259), thyroglobulin (T1126), horseradish peroxidase (P8250), invertase (I4753), glycogen (G9251), mannan (M7504), and bovine serum albumin (A4378) were obtained from Sigma Chemical Co. Heparan sulfate was a kind gift of Dr. A. Linker (Veterans Administration Hospital, Salt Lake City, UT).

Preparation of Frozen Sections: Superficial or deep cervical lymph nodes were dissected from ether-anesthetized rats (Sprague-Dawley, 180–200 g, obtained from Simonsen Laboratories, Gilroy, CA), snap frozen at -160°C (in 2-methyl-butane cooled in liquid nitrogen), and immediately placed in a cryostat for sectioning (IEC Cryostat at -20°C). 10- μm sections were prepared and transferred onto glass slides (Clay Adams Laboratory Systems #A1460, Becton-Dickinson & Co., Rutherford, NJ; or Carlson Scientific Inc., Petone, IL; #00301) kept at room temperature. Sections must be relatively free of corrugations and defects for optimal results. We found that moving the tissue sections from the cutting edge to a highly polished, flat portion of the blade before transfer onto the glass slide increased the yield of such sections. Sections were dried onto the slides at room temperature for 1–3 h, then stored at 4°C until use (in tightly closed slide boxes). Sections prepared and stored in this fashion maintained binding activity for 1–2 wk (storage at room temperature resulted in loss of activity usually within 2–3 d). Before use, the slides were warmed to room temperature in a dessicator (to minimize the formation of condensation on the tissue section). Once removed from 4°C storage, sections were used within 3 h.

Binding Assay: We adapted our method from that of Butcher et al. (6, 7). Lymphocytes were teased from the superficial and deep cervical lymph nodes of two to four Sprague-Dawley rats (8 wk old, 180–200 g), and suspended in buffer at 4°C (Hanks' balanced salt solution containing 1 mg/ml of bovine serum albumin (BSA), pH 7.4, or minimal essential medium in Earle's salts (without sodium bicarbonate) containing 1 mg/ml BSA and buffered with 40 mM Tricine, pH 7.4). Both the Hanks' balanced salts buffer (HBS+) and the Earle's minimal essential medium (MEM+) were isotonic and isoionic relative to normal rat serum (9). The cells were dispersed by rapid, in-out pipetting (~ 100 cycles), and the cell clumps removed by passage through several layers of cheese cloth. The resultant single cell suspension was washed two to three times in 30-ml aliquots of buffer, suspended at a final concentration of 4×10^7 cells/ml, and kept on ice until use. Cell suspensions prepared in this manner were relatively free of phagocytic cells ($<10\%$ as judged by latex particle ingestion) and were $\sim 80\%$ viable by trypan blue exclusion.

Potential inhibitors were added to lymphocyte suspensions ($1-2 \times 10^7$ cells/ml at 4°C) 10–30 min before the start of the binding assay and, unless otherwise stated, were present throughout the subsequent binding incubation. The binding incubations were conducted on glass slides in 1.4 or 2 cm diameter wax or epoxy circles (wax circles were drawn onto slides at room temperature immediately before the start of a binding assay; premade slides, with 1.4-cm diameter holes cut into a thin epoxy film, were obtained from Carlson Scientific Inc.).

The frozen sections were used in the order in which they were cut. Aliquots of untreated lymphocytes (controls) and suspensions containing potential inhibitors were layered on the sections in an arbitrary sequence such that the control and each test substance were represented once in a series. The series was repeated until the desired number of replicates had been generated. This procedure was designed to minimize bias arising from the possible heterogeneity in the binding characteristics of HEV from different regions of the lymph node. In view of the marked quantitative difference in binding that we and others (6) have observed from one lymph node to the next, only sections prepared from a single node were used in any one experiment.

The assay was initiated by layering the 4°C cell suspensions onto the sections (at room temperature). The slides were then placed on a metal tray, supported on packed ice, and agitated on a gyratory shaker at 80 or 100 rpm (G-24, New Brunswick Scientific Co., Inc., Edison, NJ; $\frac{3}{4}$ -in radius of gyration). The temperature of the slides equilibrated at $7-10^{\circ}\text{C}$. After a 50-min incubation, the cell suspensions were decanted and the sections fixed in 3% glutaraldehyde (20 min at 4°C). The slides were then placed in racks, washed in Dulbecco's phosphate-buffered saline (five, one-second immersions), stained with 0.5% toluidine-blue in a 20% ethanol solution (one, 15–60 second immersion), washed in 95% EtOH (one, two-second immersion), and mounted in glycerol. Sections processed in this manner were stored at 4°C until analyzed.

General Characteristics and Quantitation of Lymphocyte

Binding in Vitro: In the absence of inhibitors, lymphocytes attach, primarily, to the HEV and to the glass surrounding the tissue section on the slide. Lymphocytes also adhere to the nodal parenchyma away from the HEV; however, the density of binding is at least 50-fold greater at the HEV than at these other sites. The binding to the HEV was easily distinguished from binding elsewhere by virtue of the characteristic picture of tightly packed, darkly stained lymphocytes overlying the histologically distinctive HEV (Fig. 1). Lymphocytes overlying non-HEV sites do not show the same degree of organization and specificity—

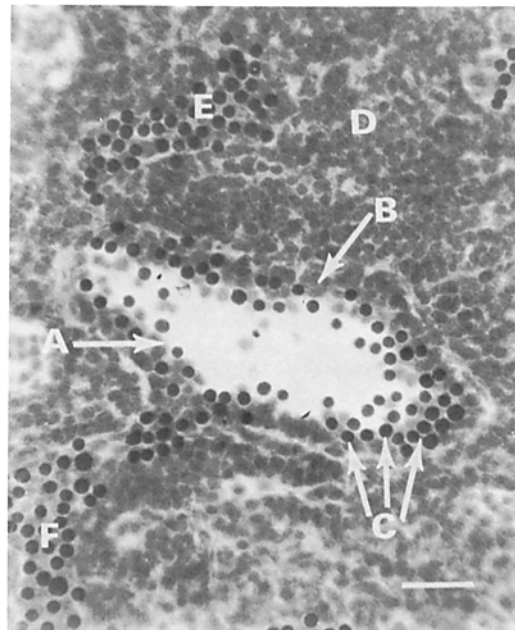


FIGURE 1 View of lymphocyte-HEV binding interaction. A, luminal surface of HEV. B, cuboidal endothelial cells. C, lymphocytes adhering to various endothelial cell surfaces and, possibly, the extracellular matrix. D, diffuse (para-) cortex of the lymph node. E and F, binding to HEV sectioned tangentially. Bar, $45 \mu\text{m}$. $\times 225$.

they are spaced farther apart and generally do not adhere to specific cell types (lymphocytes versus phagocytes) or anatomic structures (germinal centers versus medullary cords or large vessels). In a small percentage of experiments (10–20%), exogenous lymphocytes adhered selectively to sinusoidal cells as well as to the HEV. This binding interaction was most pronounced in lymph nodes with prominent, cellular sinusoids. Our preliminary results (data not shown) indicate that, unlike binding at HEV, binding to sinusoids cannot be inhibited with fucoidin. Only those sections in which binding to HEV could be readily distinguished from binding at non-HEV sites were used in our studies.

All experiments were initially examined blindly. The experiments depicted in Figs. 3–6 were quantified in a single-blind fashion. The binding of lymphocytes to HEV was assessed by examining the cortices of the lymph node sections and calculating the average number of lymphocytes attached to HEV in either low power ($\times 200$) or high power ($\times 400$) microscopic fields (HEV-bound lymphocytes per field). Specifically, contiguous fields were surveyed until the entire surface of the node had been examined. The number of lymphocytes attached to HEV was determined in a representative sample of the cortical fields (ranging from every field to every third field). We adjusted the sample size (number of fields counted) so that at least 200 lymphocytes were scored on each section (if fewer than 200 cells were bound to an entire frozen section, then all cortical fields were counted). Individual data points represent the mean and standard error of the mean (SEM) for measurements on three to five separate frozen sections taken from various regions of the lymph node (see Binding Assay). When calculated in this fashion, SEMs for individual data points ranged from 15% to 40%.

In one experiment (Fig. 9), binding was quantified by determining the average number of lymphocytes bound per HEV (cells bound per HEV). The sampling procedure was identical to that described above, except that the number of discrete HEV (regardless of their size), as well as the number of attached lymphocytes, was determined. At least 200 lymphocytes and 40 HEV were counted on each section.

In the absence of inhibitors, 0.5–5% of the added lymphocytes adhere to the HEV. This percentage varied from node to node but stayed relatively constant from section to section within any one lymph node. As noted previously, other investigators have observed a similar intermodal variation in binding. This phenomenon may reflect, in part, fluctuations in the cross-sectional area of HEV from one node to the next. We have also noted that the density of binding at the HEV, i.e., the number of lymphocytes attached per μm^2 , also varied considerably (data not shown). This measure is independent of the actual number or area of HEV available in any particular frozen section; therefore, this finding suggests that the adhesive bond between lymphocytes and HEV may fluctuate in strength from one lymph node to the next. We used lymph nodes that vary markedly in the nature and degree of their recent immunologic activity. If antigenic stimulation alters the strength of the HEV-lymphocyte binding interaction, then the

fluctuations in the density of lymphocyte binding in vitro may reflect the varied immunologic histories of the lymph nodes and lymphocyte suspensions used in different experiments. This apparent variation in the strength of the lymphocyte-HEV binding interaction did not affect our data since individual experiments were carried out using sections from a single node and single pools of freshly prepared lymphocytes.

Protein Synthesis: The rate of protein synthesis in lymphocytes was determined by measuring the incorporation of [³H]leucine (ICN Pharmaceuticals, Inc., Irvine, CA; Cat. #20032, 120 Ci/mM) into TCA-precipitable counts. Lymphocytes prepared as described above were suspended at 5×10^6 cells per ml in leucine-free buffer containing penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively). Incubations were begun by adding 1 µCi of [³H]leucine to 1-ml aliquots of cells containing the various test substances. After varying periods of incubation at 37°C, the reactions were stopped with cold trichloroacetic acid (final concentration of 10%) and the precipitates were collected by filtration onto GFC filters (Whatman Inc., Paper Div., Clifton, NJ). The filters were washed in TCA and absolute ethanol, added to scintillation vials with 10 ml of Hydrofluor (National Diagnostics, Inc., Advanced Applications Institute Inc., Somerville, NJ), and counted. Nonspecific binding was determined by using zero-time blanks. The rate of [³H]leucine incorporation was found to be linear over at least 2 h. Levels of incorporation were compared after 90 min of incubation (the approximate time of exposure of lymphocytes to the inhibitors in the binding assays).

RESULTS

Effect of Monosaccharides

We examined the effects of a series of neutral monosaccharides on lymphocyte attachment to HEV in vitro (Fig. 2). This panel included sugars that are commonly found in cell surface glycoconjugates and that are known ligands for endogenous mammalian lectins (10). At a concentration of 150 mM, D-

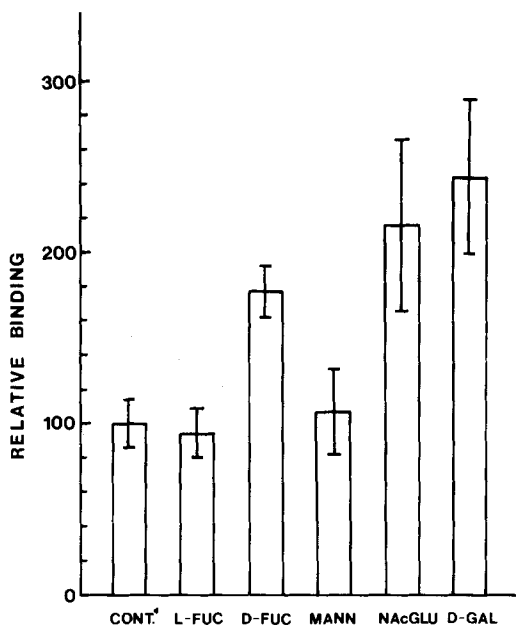


FIGURE 2 Lymphocyte attachment in the presence of monosaccharides (150 mM). 0.2 ml of lymphocyte suspensions, containing various test substances, were incubated over sections in 2-cm circles and gyrated at 80 rpm as specified in Materials and Methods (7–10°C; $1-2 \times 10^7$ cells/ml in HBS+). CONT. (no-added-sugar control); L-FUC (L-fucose); D-FUC (D-fucose); MANN (D-mannose); NAcGLU (N-acetyl-D-glucosamine); D-GAL (D-galactose), RELATIVE BINDING = Binding in presence of inhibitor + binding in control \times 100. Data pooled from multiple independent experiments, total number of replicates (frozen sections)—control (15), L- and D-Fucose (19), D-mannose (11), N-acetyl-D-glucosamine (8), D-galactose (10). Differences between L-fucose and D-fucose, D-mannose and D-fucose statistically significant (by t test) with probabilities less than 0.0005 and 0.005 respectively. D-glucose (data not shown) produced no significant inhibition relative to D-fucose.

fucose, D-glucose, D-galactose, and N-acetyl-D-glucosamine enhanced lymphocyte binding relative to the no-added-sugar control, whereas L-fucose and D-mannose had no significant effect. However, lymphocyte attachment in the presence of L-fucose and D-mannose was 50% lower than that in the presence of the other sugars examined.

The mechanism underlying the enhancement of lymphocyte binding to HEV in the presence of a variety of structurally dissimilar monosaccharides is unknown. The effect appeared to be relatively selective since neither the nonspecific binding of lymphocytes to tissue sections away from the HEV, nor the adhesion of lymphocytes to one another in suspension increased under these conditions. It is conceivable that alterations in the membrane structure of either the lymphocyte or the HEV may be involved. Scanning electron micrographs of lymphocytes in suspension have shown an increase in the number and prominence of membrane microvilli in hypertonic solutions (11). Since circulating lymphocytes anchor to the HEV at the tips of their microvilli (4), one might expect that changes induced in these structures by hypertonic sugar solutions might enhance lymphocyte binding in vitro. Regardless of the mechanism, this enhancement suggested that the most appropriate control for judging the inhibitory potency of a particular sugar was an equimolar concentration of other sugars. When viewed in this manner, L-fucose and D-mannose appeared to produce a significant depression in the affinity of lymphocytes for HEV.

In an effort to enhance the suspected inhibitory activities of the simple sugars, we examined the effect of high ionic strength buffers on the attachment of lymphocytes to HEV. Fig. 3 shows that increasing the ionic strength of the incubation buffer, by adding NaCl, results in a dose-dependent reduction in lymphocyte binding. A 40% increase in ionic strength relative to

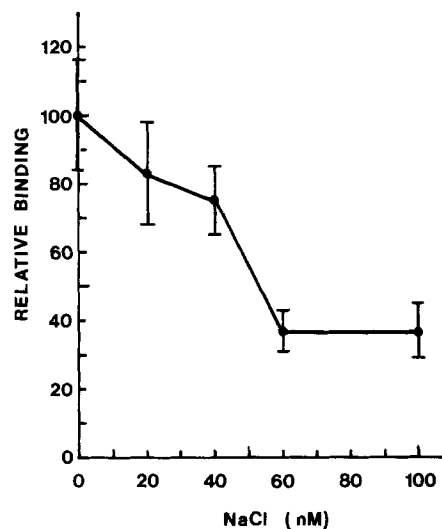


FIGURE 3 Lymphocyte attachment in the presence of NaCl. 0.12 ml of the lymphocyte suspensions, containing various test substances, were incubated over sections in 1.4-cm diameter circles and gyrated at 80 rpm as specified in Materials and Methods (7–10°C; 2×10^7 cells/ml in MEM+). RELATIVE BINDING (ordinate) = (binding in presence of NaCl) + (binding in control) \times 100. Means and SEMs based on four replicates. Degree of inhibition by NaCl varied between experiments and may reflect heterogeneity in the strength of the binding interaction with different lymphocyte suspensions and lymph nodes. The difference between the control (no-added-salt) and 60 mM added NaCl is statistically significant (by t test) with a probability of <0.025 .

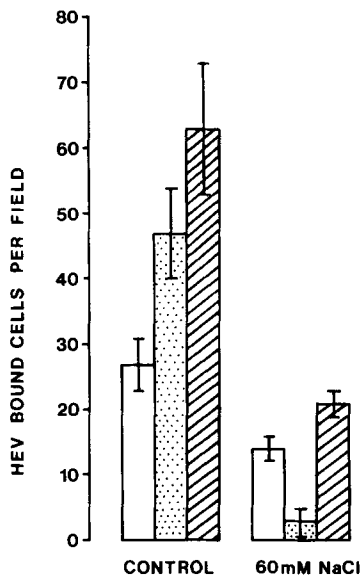


FIGURE 4 Lymphocyte attachment in the presence of L- or D-fucose (150 mM) with and without added NaCl (60 mM). Additional incubation conditions as specified in legend of Fig. 3. Clear bar, no added sugar; stippled bar, L-fucose; cross-hatched bar, D-fucose. Ordinate = HEV bound cells/LOW power cortical field. Means and SEM based on three replicates. The following differences (in the presence of 60 mM added NaCl) are statistically significant (by t test): Control vs. L-fucose, ($p < 0.0005$); control vs. D-fucose, ($p < 0.05$); L-fucose vs. D-fucose, ($p < 0.005$).

normal rat serum (produced by adding 60 mM NaCl) resulted in a 60% decrease in lymphocyte attachment to HEV. Ionic strength rather than osmolarity appeared to be the critical factor in reducing adhesiveness, since hypertonic solutions of D-fucose, D-galactose, and GlcNAc (in which ionic strengths were unchanged) enhanced rather than inhibited lymphocyte attachment (Fig. 2).

Fig. 4 demonstrates the effect of increased ionic strength (permissive conditions) on the inhibitory potencies of D- and L-fucose. In this experiment, 60 mM additional NaCl depressed lymphocyte attachment twofold relative to the control (containing neither added salt nor sugar). The addition of 150 mM L-fucose resulted in a further six- to eightfold reduction in binding, while an equal concentration of D-fucose enhanced rather than inhibited attachment relative to the control (containing salt but no added sugar). Thus, increasing the NaCl concentration of the incubation medium, such that its ionic strength exceeded that of normal rat serum by 40%, both weakened the adhesive bond between lymphocytes and HEV and enhanced the inhibitory potency of L-fucose.

The dose-response curves for L- and D-fucose under permissive conditions are shown in Fig. 5. Relative to the no-added-sugar control (Fig. 5a), D-fucose produced a dose-dependent increase in lymphocyte attachment that plateaued at a level 50% above the control ($p < 0.05$ at 37 mM). In contrast, L-fucose caused little if any increase in lymphocyte attachment at low concentrations followed by a significant inhibition in binding at concentrations of 75 and 150 mM ($p < 0.05$). When compared to equal concentrations of D-fucose, L-fucose produced significant inhibition at concentrations of 18 mM (see figure legend for p values) and above (Fig. 5b). D-mannose also produced dose-dependent inhibition but was approximately twofold less potent than L-fucose. Thus, increasing the ionic strength of the incubation medium with NaCl enhanced the potency of L-fucose relative to both no-added-sugar controls and controls containing equal concentrations of D-fucose.

In light of NaCl's synergistic effect on inhibitory monosaccharides, we re-examined the behavior of a series of neutral sugars in high salt (Fig. 6). At a concentration of 150 mM, only L-fucose, D-mannose, and α -methyl-D-mannoside depressed lymphocyte attachment relative to the no-added-sugar control. D-galactose, N-acetyl-D-glucosamine, L-rhamnose, D-fucose,

D-glucose, D-ribose, and N-acetyl-D-galactosamine either enhanced binding or had no significant effect relative to the control.

We were concerned that inhibition under "permissive" conditions might be due to the selective agglutination of lymphocytes in the presence of inhibitory monosaccharides. Therefore, we estimated the degree of agglutination by fixing lymphocytes remaining in suspension after a binding assay (with glutaraldehyde) and determining the number of single cells. The number of single cells depends on both the number of cells forming aggregates and the number of cells that attach to the glass or the tissue section. Since the majority of added lymphocytes remain in suspension at the end of a binding assay (>80%), the number of singles should reflect, primarily, the degree of cell agglutination. We found that L-fucose did not produce a significant decrease in the number of single cells when compared to a no-added-sugar control, D-galactose, or D-fucose. We obtained identical results when lymphocyte agglutination was assessed under the same conditions in the absence of tissue sections. Thus, cellular agglutination does not appear to be a factor in the inhibition of binding produced by specific monosaccharides.

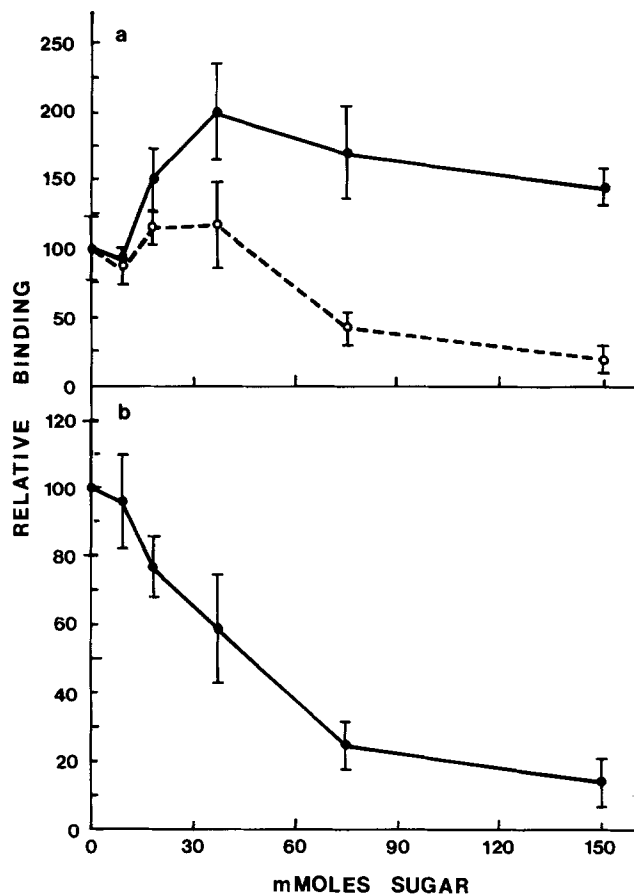


FIGURE 5 Dose-response curves for D- and L-fucose with added NaCl (60 mM). Additional incubation conditions as specified in the legend of Fig. 3. Means and SEMs based on four replicates. (a) Binding relative to no-added-sugar control. Relative binding (ordinate) = (binding in the presence of fucose) + (binding in the control) \times 100. D-fucose, solid line; L-fucose, dashed line. (b) Inhibition of L-fucose relative to D-fucose. RELATIVE BINDING (ordinate) = (binding in the presence of L-fucose) + (binding in the presence of D-fucose) \times 100.

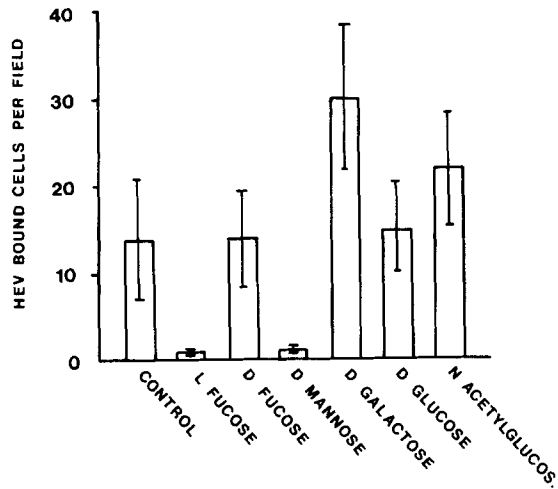


FIGURE 6 Lymphocyte attachment in the presence of a variety of monosaccharides (150 mM) with additional 60 mM NaCl ("permissive" condition). Additional incubation conditions as specified in the legend of Fig. 3. Ordinate = HEV bound cells/LOW power cortical field. Means and SEMs based on four replicates. N-acetyl-D-galactosamine, L-rhamnose, and D-ribose (data not shown) produced no inhibition relative to the control. Degree of accentuation by D-fucose varied between experiments and may reflect heterogeneity in strength of binding interaction with different lymphocyte suspensions and lymph nodes.

In light of the known inhibitory effect of D-mannose on cell division (12) and L-fucose on cell division and DNA synthesis (13) in cultured cells, we examined rat lymphocytes for evidence of sugar-induced toxicity under "permissive" conditions. Pre- and post-assay measurements showed 75–80% of cells capable of excluding trypan blue regardless of the nature of the added sugar. Furthermore, the rate of protein synthesis, as judged by [³H]leucine incorporation, was depressed to the same degree by all sugars whether they inhibited binding or not (Table IA). Therefore, L-fucose and D-mannose do not appear to be selectively toxic for lymphocytes in short-term experiments.

The reason for the generally reduced synthetic activity under "permissive" conditions, with or without the various monosaccharides, is not known. However, the fact that equal concentrations of structurally diverse monosaccharides resulted in equivalent degrees of inhibition suggests that hypertonicity may have had a nonspecific effect on lymphocyte metabolism. Such a phenomenon has been described in other cells (14).

Effect of Glycoconjugates

Our sugar inhibition results suggested that a carbohydrate-binding molecule, with fucose/mannose specificity, might be involved in the adhesive interaction between lymphocytes and HEV. Frequently, the affinity of lectins for polysaccharides greatly exceeds their affinity for monosaccharides. Such enhanced binding is due either to a valency effect, in which multiple sugar residues in the polysaccharide can bind to multiple sites on the lectin (15, 16), or to the presence of specific oligosaccharide structures in the polysaccharide with high affinity for the lectin (17).

We, therefore, examined the effects of a variety of complex polysaccharides, of defined composition, on the attachment of lymphocytes to HEV (Fig. 7). Fucoidin, a 100,000-dalton, sulfated polysaccharide consisting predominantly of α 1,2- and

α 1,3-linked L-fucose (18), proved to be a potent inhibitor (at 5 μ g/ml). A series of structurally dissimilar, charged polysaccharides (19) with equal or greater charge densities (heparin, dermatan sulfate, dextran sulfate), and equal or greater size (hyaluronic acid) showed no significant inhibitory activity at fivefold higher concentrations. In addition, thyroglobulin, yeast mannan, glycogen, horseradish peroxidase, and yeast invertase were inactive at 1 mg/ml.

Fucoidin was significantly more potent than the active monosaccharides with a one-half maximal inhibitory activity, depending on the experiment, of $1-5 \times 10^{-8}$ M (Fig. 8). Gel filtration analysis (Sephacryl 300, Pharmacia Inc., Piscataway, NJ) of the commercial fucoidin preparation revealed a minor (<10% by weight) protein component. However, the protein-free polysaccharide fractions were potent inhibitors of the lymphocyte-HEV binding interaction, indicating that the protein was not essential for inhibitory activity (data not shown).

To determine the target of fucoidin's action, we preincubated either frozen sections or the lymphocyte suspensions with 25 μ g/ml of fucoidin, washed them, and then compared them to untreated controls for binding activity. As shown in Fig. 9, only when lymphocytes were pretreated with fucoidin was there residual inhibitory activity. Thus, fucoidin acts on the lymphocytes to reduce their affinity for HEV.

We observed that fucoidin enhanced the clumping of lymphocytes in suspension, in contrast to the monosaccharide inhibitors; however, this phenomenon did not appear to correlate with the degree of inhibitory activity. Specifically, the percentage of agglutinated cells ranged from 20 to 50% at fucoidin concentrations that decreased lymphocyte attachment by 70–100%. Therefore, the random agglutination of a portion

TABLE I
Effect of Monosaccharides and Polysaccharides on the Rate of Protein Synthesis in Lymphocyte Suspensions

Additive *	A
	% Maximal protein synthesis [‡]
None (control)§	100 ± 5
L-Fucose	
150 mM	14 ± 1.5
75 mM	42 ± 3.3
D-Fucose	
150 mM	16 ± 2.7
75 mM	42 ± 3.2
D-Mannose	
150 mM	17 ± 3.3
D-Galactose	
150 mM	15 ± 0.04
Additive	B
	% Maximal protein synthesis [‡]
None (control)	100 ± 6
Fucoidin	66 ± 5
Heparin	69 ± 5
Dextran sulfate (8 kdaltons)	62 ± 6
Chondroitin sulfate	85 ± 5

* All solutions contained leucine-free, MEM (pH 7.4) supplemented with 1 mg/ml BSA, 60 mM additional NaCl, penicillin, and streptomycin (see Materials and Methods)

‡ (Counts in presence of additive) + (precipitable counts in control) × 100 (see Materials and Methods)

§ 60 mM NaCl depressed protein synthesis ~40% relative to no-added salt control

|| All solutions contained leucine-free, MEM (pH 7.4) supplemented with 1 mg/ml BSA, penicillin, and streptomycin and 25 μ g/ μ l of the specified polysaccharide (see Materials and Methods)

of the lymphocyte suspension cannot explain fucoidin's effect. However, given the heterogeneity of the lymphocyte suspensions we employ, it is conceivable that fucoidin selectively agglutinated those cells capable of binding to HEV.

Fucoidin does not appear to be selectively toxic for lymphocytes since pre- and post-incubation measurements of trypan blue exclusion were identical regardless of the nature of the added polysaccharide. Furthermore, Table 1B shows that the rate of protein synthesis in the presence of fucoidin did not differ significantly from that in the presence of equal concen-

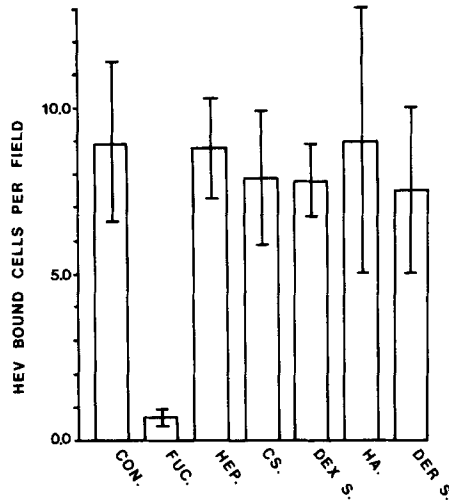


FIGURE 7 Lymphocyte attachment in the presence of various polysaccharides. Additional incubation conditions as specified in the legend of Fig. 2. CON. (no-added-sugar control); FUC. (fucoidin); HEP. (heparin); CS. (chondroitin sulfate); DEX S. (dextran sulfate); HA. (hyaluronic acid); DER S. (dermatan sulfate). Fucoidin at 5 $\mu\text{g}/\text{ml}$, all others at 25 $\mu\text{g}/\text{ml}$. Ordinant = HEV bound cells per HIGH power field. Means and SEMs based on four replicates.

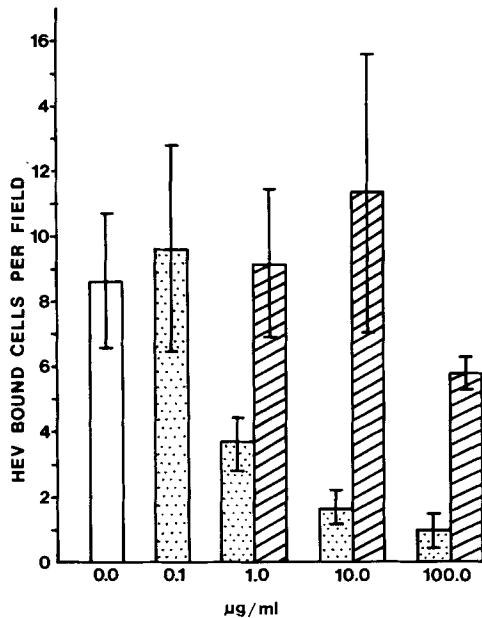


FIGURE 8 Dose-response curves for fucoidin and heparin. Additional incubation conditions as specified in the legend of Fig. 2. Ordinant = HEV bound cells per HIGH power field. Means and SEMs based on three replicates. Control, clear bar. Fucoidin, stipled bar. Heparin, cross-hatched bar.

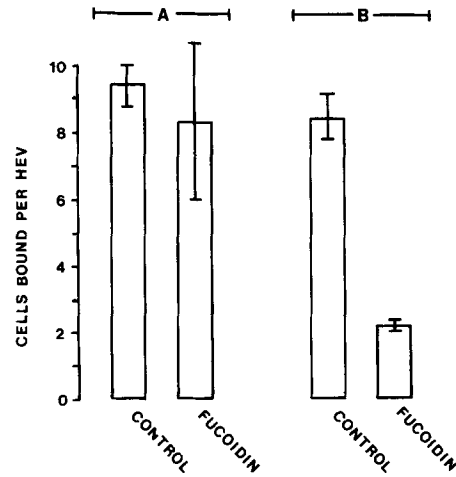


FIGURE 9 Persistent inhibition of binding after preincubation of lymphocytes with fucoidin. Ordinant: see Materials and Methods. Means and SEMs based on four replicates. (A) Frozen sections preincubated with fucoidin (25 $\mu\text{g}/\text{ml}$ at 7–10°C for 30 min), washed (500 ml buffer, 1 dip) and then binding activity compared to frozen sections treated identically except that fucoidin was not present during preincubation. Binding assay as specified in the legend of Fig. 2. (B) Lymphocyte suspension preincubated with fucoidin (25 $\mu\text{g}/\text{ml}$ at 4°C for 30 min), washed by centrifuging through 15 ml of buffer (120-fold dilution of fucoidin) and then binding activity compared to lymphocyte suspension treated identically except that fucoidin was not present during preincubation. Binding assay as specified in the legend of Fig. 2.

trations of several polysaccharides that had no effect on lymphocyte adhesion. Taken together, these data suggest that fucoidin's inhibitory potency cannot be explained simply on the basis of its charge, its molecular size, or a toxic effect. We favor the hypothesis that fucoidin interacts with a fucose-specific lectin on the lymphocyte surface that participates in the attachment of these cells to HEV in vitro.

DISCUSSION

Membrane-associated carbohydrate-binding molecules (membrane lectins) appear to be involved in a variety of physiologically significant adhesive interactions (20, 21, 22). The attachment of certain bacteria and viruses to their host cells appears to involve lectins (23, 24). The symbiotic relationship between legumes and nitrogen-fixing bacteria is facilitated by the binding of bacterial polysaccharides to lectins on the root hairs of the plant host (25). The developmentally regulated, species-specific intercellular adhesion of the cellular slime molds *Dictyostelium discoideum* and *Polysthondylium pallidum* appears to involve membrane-bound lectins with affinity for galactose and galactose-containing glycoproteins (26–29). Finally, lectins with specificities for fucans have been implicated in the cohesion of teratocarcinoma stem cells (30, 31), and in the adhesion of sperm to egg in the algae *Fucus* (32), in sea urchins (33), and in guinea pigs (34).

Our present studies indicate that lectins on the lymphocyte surface may be involved in the attachment of lymphocytes to HEV in vitro and thus contribute to lymphocyte recirculation in vivo. Specifically, we find that the sulfated, L-fucose-rich polysaccharide fucoidin and the structurally related monosaccharides L-fucose, D-mannose, and α -methyl-D-mannoside (these monosaccharides have an axial 2-hydroxyl and an equa-

torial 4-hydroxyl) can inhibit the attachment of lymphocytes to HEV in vitro. Furthermore, we have shown that fucoidin inhibits binding by acting on the lymphocyte and not the HEV. Finally, since inhibition occurs at incubation temperatures of 7–10°C (temperatures at which the uptake of sugars and membrane fluidity are, presumably, reduced), we suspect that the inhibitors exert their effects at the cell surface. These findings are compatible with the hypothesis that lectins on the lymphocyte surface interact with complementary receptors on the HEV containing fucose, mannose, or related carbohydrates (most likely as part of protein or lipid-bound oligosaccharides). According to this view, the substances with inhibitory activity would compete with the endogenous receptor for binding sites on the lymphocyte surface, thereby weakening the adhesive interaction between lymphocytes and HEV. Alternatively, the inhibitors might produce alterations in the cell shape or affect intracellular metabolic processes that secondarily result in decreased adhesiveness. Although we have no direct evidence that fucoidin, L-fucose, and D-mannose, compete for the same binding site on the lymphocyte surface, there are several examples of cell-surface carbohydrate-binding molecules with combined L-fucose/D-mannose (16) or L-fucose/fucoidin specificities (34).

High ionic strength buffers both inhibit the attachment of lymphocytes to HEV directly and enhance the potency of carbohydrate inhibitors. The fact that high ionic strength alone inhibits the adhesive interaction does not diminish the likelihood that a lectin-carbohydrate interaction is involved. Salt solutions can elute specifically bound carbohydrates from lectin affinity columns, suggesting that ionic bonds or salt-sensitive tertiary structures contribute to the stability of some lectin-carbohydrate interactions (35). Alternative explanations for the salt effect include: (a) a salt-sensitive adhesive mechanism that is distinct from that involving the putative lectin; (b) metabolic inhibition that secondarily affects adhesion; or (c) the quantitative loss of ionically bound adhesive molecules by salt elution. The synergistic effect between NaCl and the specific monosaccharide inhibitors may indicate that, as the strength of the salt-sensitive bonds diminish, the attraction due to lectin-carbohydrate binding persists and provides the major force holding the lymphocytes in place. Regardless of the precise mechanism, the hyperionic buffers utilized in our studies dramatically and selectively enhance the inhibitory potencies of L-fucose and D-mannose.

The sulfated, L-fucose-rich polysaccharide fucoidin shows far greater potency on a molar basis than the monosaccharide inhibitors. Although its high content of sulfate residues and overall size may contribute to its potency, other structural characteristics must also be important since structurally dissimilar polysaccharides, with comparable charge densities and molecular weights, failed to cause significant inhibition. The synergistic effect of NaCl and L-fucose may indicate that both lectin-carbohydrate and ionic interactions are involved in the attachment of lymphocytes to HEV. If this view is correct, then fucoidin's potency may reflect its ability to participate in both types of interactions.

Nearly twenty years have passed since Gesner and Ginsberg (36) first suggested that cell-surface carbohydrates play a role in the homing of lymphocytes. Our data are compatible with the hypothesis that a carbohydrate-binding molecule on the lymphocyte surface interacts with a fucose- or mannose-containing receptor on the postcapillary venule. Since the magnitude of lymphocyte attachment in vitro correlates with the flux of circulating cells into lymph nodes in vivo, we propose that

a lectin-carbohydrate binding interaction is involved in the first stage of lymphocyte recirculation.

We are grateful for the expert technical assistance of Mr. Mark Singer. We also thank Drs. Laura Grabel, Charles Glabe, Laurence Marton, and Edward Smuckler for helpful discussions and support. Finally, we are indebted to Ms. Debra Drake and Mr. Dave Akers for their assistance in the preparation of the figures.

This work was supported by National Institutes of Health Grant GM23547. S. D. Rosen is a recipient of a Research Career Development Award (GM 00322).

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Received for publication 27 July 1982, and in revised form 12 November 1982.

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