

Requirement for Sialic Acid on the Endothelial Ligand of a Lymphocyte Homing Receptor

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Abstract. The entry of blood-borne lymphocytes into most secondary lymphoid organs is initiated by a highly specific adhesive interaction with the specialized cuboidal endothelial cells of high endothelial venules (HEV). The adhesive receptors on lymphocytes that dictate interactions with HEV in different lymphoid organs are called homing receptors, signifying their critical role in controlling organ-selective lymphocyte migration. Considerable work has established that the mouse peripheral lymph node homing receptor (pnHR), defined by the mAb MEL-14, functions as a lectin-like adhesive protein. We have previously shown that sialidase treatment of peripheral lymph node (PN) HEV abrogates lymphocyte attachment to

the HEV both in vivo and in vitro. We extend this evidence by demonstrating that *Limax* agglutinin (LA), a sialic acid-specific lectin, when reacted with HEV exposed in cryostat-cut tissue sections, blocks lymphocyte attachment to PN HEV and, unexpectedly, to the HEV of Peyer's patches (PP) as well. Using a recombinant form of the pnHR as a histochemical probe for its cognate adhesive site (HEV-ligand) on PN HEV, we demonstrate that both sialidase and *Limax* agglutinin functionally inactivate this ligand. It is concluded that the requirement for sialic acid is at the level of the pnHR interaction with its HEV ligand. A distinct sialyloligosaccharide may encode the recognition determinant of a PP HEV ligand.

THE process of lymphocyte extravasation into lymph nodes and most other secondary lymphoid organs requires that blood-borne lymphocytes first bind to the endothelium of specialized high walled postcapillary venules known as high endothelial venules (HEV)¹ (reviewed in Berg et al., 1989; Duijvestijn and Hamann, 1989; Rosen, 1989; Stoolman, 1989; Woodruff et al., 1987). The selective interaction of lymphocytes with HEV in different anatomical sites is believed to play a pivotal role in the organ-selective migration or "homing" of lymphocytes within the body (Butcher, 1986). The lymphocyte-associated molecules responsible for organ-selective interaction with HEV are operationally termed "homing" receptors (Gallatin et al., 1986), whereas the cognate adhesion molecules on the endothelium are referred to as "HEV-ligands" or "vascular addressins" (Berg et al., 1989). Acting in concert with homing receptors are accessory molecules, such as LFA-1, which augment lymphocyte adhesion to HEV but do not impart organ selectivity (Hamann et al., 1988; Pals et al., 1988). The peripheral lymph node homing receptor (pnHR) was initially defined in the mouse by the mAb MEL-14 (Gallatin et al., 1983). This antibody reacts with a ≈90-kD surface gly-

coprotein (gp90^{MEL}) on the lymphocyte causing a blockade of lymphocyte migration to peripheral lymph nodes (PN) in vivo and preventing lymphocyte attachment to PN HEV in the Stamper-Woodruff in vitro adherence assay (Stamper and Woodruff, 1976). As further evidence for the homing function of gp90^{MEL}, both the purified molecule (Geoffroy and Rosen, 1989) and a recombinant form (Watson et al., 1990) can interact selectively with PN HEV and block lymphocyte attachment. With respect to its mechanism of action, a substantial body of work has now established that gp90^{MEL} and its human homologue function as calcium-dependent, lectin-like receptors (Stoolman and Rosen, 1983; Stoolman et al., 1984, 1987; Yednock et al., 1987a, b; Brandley et al., 1987; Stoolman and Ebling, 1989; Imai et al., 1990). Central to this evidence are the findings that a limited set of anionic carbohydrates selectively inhibit the adhesive function of the homing receptor. The most active inhibitors include mannose-6-phosphate (M6P), fructose-1-phosphate, a M6P-rich polysaccharide known as PPME, fucose-sulfate-containing polysaccharides (e.g., fucoidin), and the galactose-sulfate glycolipid known as sulfatide (Imai et al., 1990). In addition to these results establishing sugar-selective inhibition, the molecular cloning of gp90^{MEL} (Lasky et al., 1989; Siegelman et al., 1989) has revealed the presence of a calcium-dependent (C-type) lectin motif of 117 amino acid residues at the amino-terminal extracellular domain of the receptor. The epitope for the MEL-14 mAb maps

1. *Abbreviations used in this paper:* BSM, bovine submaxillary mucin; DBA, *Dolichus biflorus* agglutinin; HEV, high endothelial venules; M6P, mannose-6-phosphate; LA, *Limax flavus* agglutinin; PN, peripheral lymph node; pnHR, peripheral lymph node homing receptor.

to this lectin domain (Bowen et al., 1990). This domain precedes an EGF motif, two contiguous complement-regulatory motifs ("short consensus repeats"), a transmembrane domain, and a short cytosolic tail. A similar organization of motifs has been discerned for two other adhesion molecules known as GMP-140 and ELAM-1 (Bevilacqua et al., 1989; Johnston et al., 1989) as well as the human homologue of gp90^{MEL} (Bowen et al., 1989; Camerini et al., 1989; Kishimoto et al., 1990; Siegelman and Weissman, 1989; Tedder et al., 1989, 1990). This newly defined family of cell adhesion receptors has been designated "LEC-CAMs" (Stoolman, 1989) or alternatively "selectins" (Geng et al., 1990). A possible role for the EGF domain in the adhesive function of the pnHR is implied by the recent work of Siegelman et al. (1990). Three candidates (LPAM-1 antigen, gp90^{HERMES}, and HEBF_{PP}) for a Peyer's patch (PP) homing receptor have been identified in rodents and human (Chin et al., 1986; Holzmann et al., 1989; Jalkanen et al., 1986). The first two are not members of the LEC-CAM family, whereas the third requires further biochemical characterization (Chin et al., 1986; Goldstein et al., 1989; Holzmann et al., 1989; Stamenkovic et al., 1989).

The evidence that the pnHR functions as a lectin has led to the examination of PN HEV for the presence of carbohydrate-based recognition determinants. Initial studies demonstrated the periodate sensitivity of the adhesive sites on PN HEV and PP HEV, consistent with an essential role for carbohydrates at both endothelial sites (Rosen et al., 1985; Rosen and Yednock, 1986). Subsequently, it was shown that treatment of HEV in vitro (Rosen et al., 1985) or in vivo (Rosen et al., 1989) with bacterial sialidases selectively abrogates lymphocyte attachment to PN HEV while having no effect on binding to PP HEV. A partial effect of sialidase was observed on mesenteric lymph node HEV, consistent with the evidence that this endothelium expresses a mixture of PN- and PP-like ligands (Butcher et al., 1980; Streeter et al., 1988a). In the present study, we extend the evidence that sialic acid is critical for the adhesive function of PN HEV by showing that the sialic acid-specific lectin, *Limax flavus* agglutinin (LA), functionally inactivate adhesive sites on PN HEV and, surprisingly, on PP HEV as well. Using a recombinant form of pnHR as a histochemical staining reagent for its cognate HEV ligand, we provide direct evidence that the requirement for sialic acid is at the level of the homing receptor interaction.

Materials and Methods

Materials

Biotin-caproyl lectins (peanut agglutinin [PNA], Con A, *Ricinus communis* agglutinin-I [RCA-I], WGA) were purchased from Biomed Corp. (Foster City, CA). The unconjugated lectins were obtained as follows: Con A, grade IV from Sigma Chemical Co. (St. Louis, MO); *Limax flavus* agglutinin (LA), *Ulex europaeus* I (UEA-I), and WGA from Calbiochem-Behring Corp. Inc. (La Jolla, CA); *Dolichos biflorus* (DBA) and *Limulus polyphemus* agglutinin (limulin) from Worthington Biomedicals (Freehold, NJ); and PNA from Vector Laboratories (Burlingame, CA). The principal hapten sugar-binding specificities of the lectins used in this study are as follows: PNA (β -D-Gal); ConA (α -D-Man, α -D-Glc); RCA-I (β -D-Gal); WGA (β -D-GlcNAc); UEA-I (α -L-Fuc); DBA (α -D-GalNAc); limulin (α -NeuAc); *Limax* agglutinin (α -NeuAc). N-acetyl-neuraminic acid from *Escherichia coli* (Neu5Ac), D-glucuronic acid (GlcUA), bovine submaxillary mucin (BSM), D-galactose, α -methyl D-mannoside, and lactose were from Sigma Chemical Co. *Vibrio cholera* neuraminidase (sialidase) was from Gibco Laborato-

ries (Grand Island, NY). *Clostridium perfringens* neuraminidase or sialidase (Type X, affinity-purified) was from Sigma Chemical Co. All other reagents used were of analytical quality.

In Vitro Adherence Assay

The basic Stamper-Woodruff in vitro adherence assay (Stamper and Woodruff, 1976), which measures lymphocyte attachment to profiles of HEV exposed within cryostat-cut sections of lymphoid organs, was adapted for the present studies. 10- μ m cryostat sections of mouse (ICR, female) peripheral lymph nodes and Peyer's patches were cut and fixed with paraformaldehyde as previously described (Stoolman et al., 1984). The tissue sections were exposed to the individual lectins: 120 μ l at 180 μ g/ml of lectin in TBS (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl₂, pH 7.5) for 30 min at 4°C. (The activity of the lectins was confirmed by hemagglutination assays with appropriate inhibition by hapten monosaccharides or glycoconjugates.) The sections were then fixed in 0.5% glutaraldehyde in sodium cacodylate buffer, (0.1 M, pH 7.3) for 20 min at 4°C followed by treatment with 0.2 M lysine in TBS to block reactive aldehyde groups. Alkaline denaturation consisted of a 45-min exposure of the sections to 0.1 N NaOH at 4°C. After washing, the sections were then used for the in vitro adherence assay with test lymphocytes harvested from mesenteric lymph nodes. The lymphocyte suspension included 300 μ g/ml of BSM to reduce nonspecific binding to the sections. Subsequent steps of the in vitro adherence assay were as described before (Stoolman et al., 1984). Quantification of lymphocyte binding to HEV was done by digital morphometry (Bioquant II; R & M Biometrics, Nashville, TN) as detailed previously (Rosen et al., 1985). The results were computed as the mean number of lymphocytes bound per unit area. At least nine HEV segments per section were analyzed. Four to five independent tissue sections were used for each experimental condition from which means and SEM values were computed. The results are presented as percentages of control samples, which are set to 100%. In one experiment, PP sections were treated with *Vibrio cholera* (5 Gibco units in 50 mM Na acetate, 100 mM NaCl, 4 mM CaCl₂, pH 5.5) for 30 min at 7-10°C before exposure to LA. The lymphocyte suspension used in this experiment was treated with 100 μ g/ml of trypsin (T-8253; Sigma Chemical Co.) for 15 min at 37°C. This treatment eliminated background binding to non-HEV regions of PP, but did not affect the specific binding to PP HEV.

Histochemical Staining with Biotinylated Lectins

Paraformaldehyde-fixed tissue sections of PN and PP, prepared as described above, were exposed to biotinylated-lectins (100 μ l per section at the indicated concentrations) suspended in Dulbecco's PBS containing 5 mg/ml of BSA. After 45 min at 4°C, the sections were washed and incubated with 100 μ l of ABC-HRP reagent (Elite kit; Vector Laboratories, Burlingame, CA) for 30 min at 22°C followed by development of the reaction product with HRP chromagen (Biomed, Foster City, CA) at 22°C. After termination of the reaction with a water rinse, the sections were counterstained with aqueous hematoxylin (Biomed) and mounted with Immu-mount (Shandon Instruments, Inc., Pittsburgh, PA). Specificity of staining was evaluated by exposing each lectin to a competitive saccharide or glycoconjugate before and during the incubation with the tissue section. The following inhibitors were used: PNA (100 mM lactose); Con A (50 mM α -methyl D-mannoside), RCA-I (500 mM D-galactose) and WGA (5 mg/ml BSM). These competitors essentially eliminated staining by the biotinylated lectins (results not shown), thus establishing that the lectins were binding to glycoconjugates to the sections via their sugar-binding functions.

Histochemical Staining with *Limax* Agglutinin

An antibody to LA was prepared by injecting a rabbit at multiple intradermal sites with 100 μ g of LA emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Serum obtained 7 wk after immunization was used. IgG was isolated using Zeta-Chrom 60D1 amine disks (AMF-Cuno Laboratory Products, Meriden, CT) following the manufacturer's specifications. For staining, paraformaldehyde-fixed tissue sections were exposed to LA at 10 μ g per section in PBS for 1 h at 4°C. After washing, the sections were treated with anti-LA IgG, diluted 1:500 in PBS, and incubated for 30 min at 22°C, followed by additional washes, and incubation with biotinylated-goat anti-rabbit IgG (Vector Laboratories), diluted 1:50 in PBS with 5% normal mouse serum. As described in the previous section, reaction product was produced with ABC-HRP and HRP chromagen, and counterstaining was with aqueous hematoxylin. Without the addition of LA, there was no staining of the sections. As additional specificity controls, LA

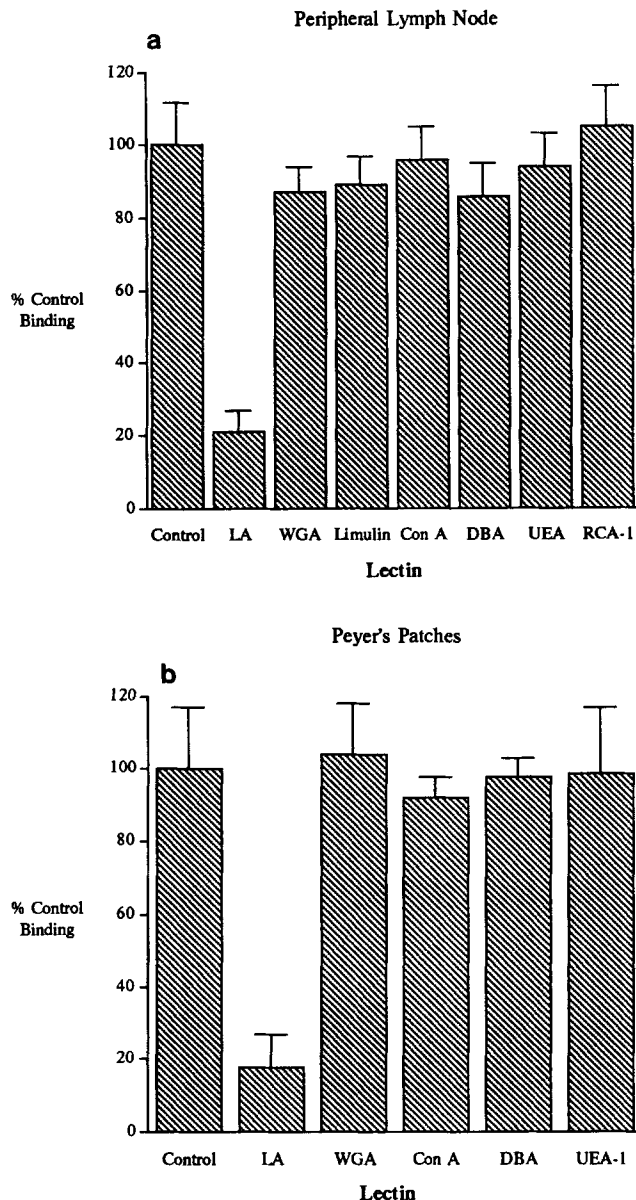


Figure 1. Effects of lectin pretreatment on adhesive function of HEV. Sections of PN and PP were treated with the indicated lectin, and then subjected to glutaraldehyde fixation followed by exposure to alkaline pH to cross-link the lectin to the tissue sections and inactivate unoccupied lectin-binding sites. Lymphocyte attachment to HEV was determined in the in vitro adherence assay and normalized to a percentage of the control level of binding (no added lectin). Absolute levels of binding in control samples were 41 lymphocytes/ $10^4 \mu\text{m}^2$ in PN sections and 20 lymphocytes/ $10^4 \mu\text{m}^2$ in PP sections. (a) PN sections (b) PP sections.

was mixed with 50 mM of sialic acid (Neu5Ac) before and during incubation with the sections, or alternatively, the sections were pretreated with *Vibrio cholera* sialidase (50 Gibco units per section in acetate buffer for 30 min at 7°C) just before LA addition. Both treatments substantially reduced the overall staining of the sections and virtually eliminated the staining of HEV.

Histochemical Staining with the pnHR-IgG Chimera

The chimera was produced and purified as previously described (Watson et al., 1990). For enzyme treatments, paraformaldehyde-fixed sections, prepared as above, were exposed to sialidases (*Vibrio cholera* in 50 mM Na

acetate, 100 mM NaCl, 4 mM CaCl_2 , pH 5.5 or *Clostridium perfringens* in 50 mM Na acetate, 100 mM NaCl, pH 5.0) or to the respective buffers alone for 30 min at 7°C. After washing in PBS, the sections were treated with methanol containing 0.3% H_2O_2 for 20 min on ice. The sections were washed and then exposed to 6 μg per section of chimera in PBS for 20 min at 7°C. Reaction product was developed through sequential application of biotinylated-goat anti-human IgG, ABC-HRP, and HRP chromagen as described above. Counterstaining was with hematoxylin. For a specificity control, some sections were treated with *Vibrio cholera* sialidase plus 100 mM sialic acid (Neu5Ac) before staining with the chimera. For the LA experiments, paraformaldehyde-fixed sections of PN, exposed to H_2O_2 in methanol as above, were treated with the lectin (100 μl per section, 200 $\mu\text{g}/\text{ml}$ in TBS) for 60 min at 4°C. The sections were washed in PBS and reacted with desialylated chimera (3 μg per section) in the presence of 300 $\mu\text{g}/\text{ml}$ of BSM. The remaining steps were as described above. Desialylated chimera was prepared by incubation with agarose-linked *Clostridium* neuraminidase (N-4883; Sigma Chemical Co.). 45 μg of chimera in PBS was incubated with 0.15 IU (international units) of enzyme for 30 min at 37°C. The use of desialylated chimera together with BSM reduced the background staining resulting from binding of the chimera to LA that was associated with the sections.

Results

Effects of Lectins on Lymphocyte Attachment Sites of HEV

Lectins of defined carbohydrate specificity have been widely used as probes for cell surface glycoconjugates (Lis and Sharon, 1986; Nicolson, 1974). The availability of sialic acid-specific lectins provided the opportunity for further study of the involvement of sialyloligosaccharides in the adhesive function of HEV. LA, isolated from the slug *Limax flavus*, is highly specific for sialic acid in many of its naturally occurring forms and linkages (Miller, 1987; Miller et al., 1982; Ravindranath et al., 1985; Schulte et al., 1984; Wagner and Roth, 1988). LA and a series of other lectins, representing a wide range of carbohydrate-binding specificities (see Materials and Methods), were compared for their ability to inhibit the adhesive function of HEV in tissue sections. The multivalency of several of the lectins caused indiscriminate binding of lymphocytes to the tissue sections. It was, therefore, necessary to inactivate the unoccupied binding sites of these lectins, once they had bound to the tissue sections. To achieve this objective, the lectin-treated sections were subjected to a "cross-linking and denaturation" procedure consisting of sequential glutaraldehyde fixation and alkaline denaturation. In control experiments, it was determined that this combination of treatments still permitted highly specific lymphocyte binding to HEV at levels close to those of untreated tissue sections. As shown in Fig. 1, pretreatment of sections with LA (180 $\mu\text{g}/\text{ml}$) markedly reduced lymphocyte attachment to both PN and PP HEV. The other lectins, including two that react with sialyloligosaccharides (limulin and WGA), produced no inhibition. The MEL-14 antibody retained its ability to inhibit lymphocyte-HEV binding after pretreatment of PN sections with WGA or limulin, thus establishing that the adhesive mechanism was not qualitatively altered by these lectins (data not shown). Among the inactive lectins were several (Con A, WGA, and RCA-1) that were able to bind to HEV as determined by histochemical staining (Fig. 2). If PN or PP sections were treated with LA in the presence of 50 mM sialic acid (Neu5Ac), lymphocyte attachment to HEV returned to near control levels, whereas coincubation with glucuronic acid did not abrogate the inhibitory activity of the lectin (Fig. 3).

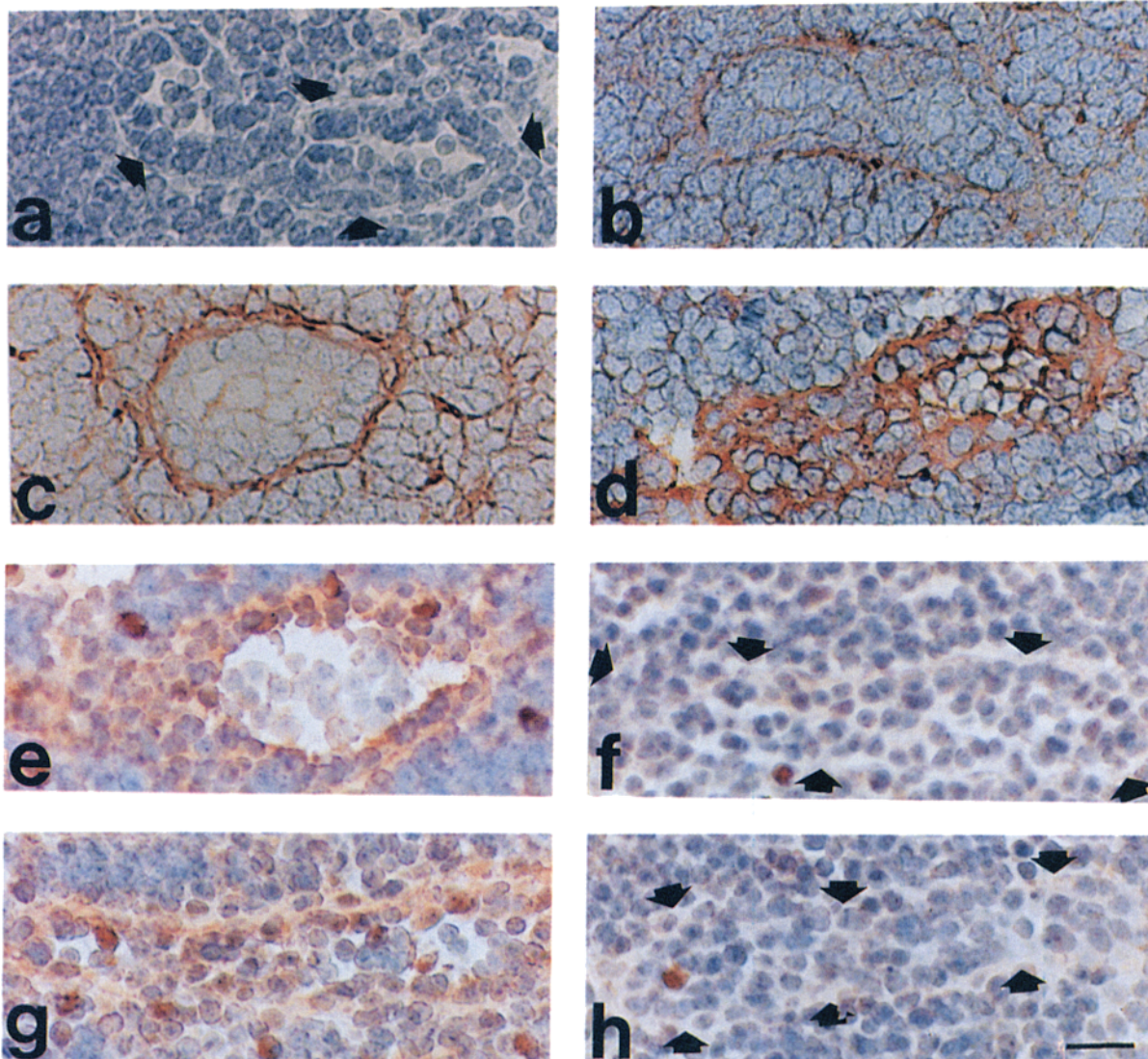


Figure 2. Histochemical staining for binding of lectins to tissue sections. PN sections (*a-f*) or PP sections (*g* and *h*) were reacted with lectins in conjunction with HRP histochemistry as described in Materials and Methods. (*a*) PBS control; (*b*) RCA-1; (*c*) Con A; (*d*) WGA; and (*e-h*) LA. In *f* and *h* the PN and PP sections, respectively, were pretreated with 50 Gibco units of sialidase per section before LA exposure. Almost complete removal of LA-reactive sites was also observed after treatment of both tissues with five units per section. In *a*, *f*, and *h*, arrowheads delimit basement membranes of individual HEV. Bar, 20 μ m.

Lymphocyte binding to HEV was not affected by pretreatment of PN or PP sections with 50 mM sialic acid or glucuronic acid alone (data not shown). Thus, it is concluded that function-blocking effects of LA on HEV were occurring via its sialic acid binding activity rather than through a non-sugar-dependent interaction of the lectin or a contaminant in the lectin preparation.

It seemed plausible that the targets for LA on PN HEV were the same functionally important sialic acid moieties that were sensitive to bacterial sialidases (Rosen et al., 1985, 1989). However, in the case of PP, LA blocked lymphocyte-HEV adhesion despite the fact that sialidases did not inactivate the PP HEV ligand. One possible explanation was that LA sterically inhibited a functional site of the PP HEV ligand through an interaction with physically contiguous, but functionally irrelevant, sialic acid moieties.

To explore this possibility, PP sections were exposed to

sialidase before treatment with LA. This combination of treatments produced a level of inhibition equivalent to the lectin alone (Fig. 4), even though the majority of LA-reactive sites were eliminated by the sialidase pretreatment (Fig. 2). Thus, it appears that the adhesion-blocking activity of LA on PP HEV is directed against sialidase-resistant residues, constituting a minority of the total LA-reactive sites.

Effects of Sialidase and Limax Agglutinin on Binding of a Recombinant Homing Receptor Chimera to HEV

A soluble recombinant form of the p_nHR has been described (Watson et al., 1990). The molecule is a chimera of the extracellular domains of gp90^{MEL} (lectin, EGF and complement-regulatory motifs) and the constant region of human IgG₁. The lectin activity of gp90^{MEL} is retained in this p_nHR-IgG chimera, as demonstrated in an ELISA assay measuring the binding of the chimeric receptor to PPME (a yeast poly-

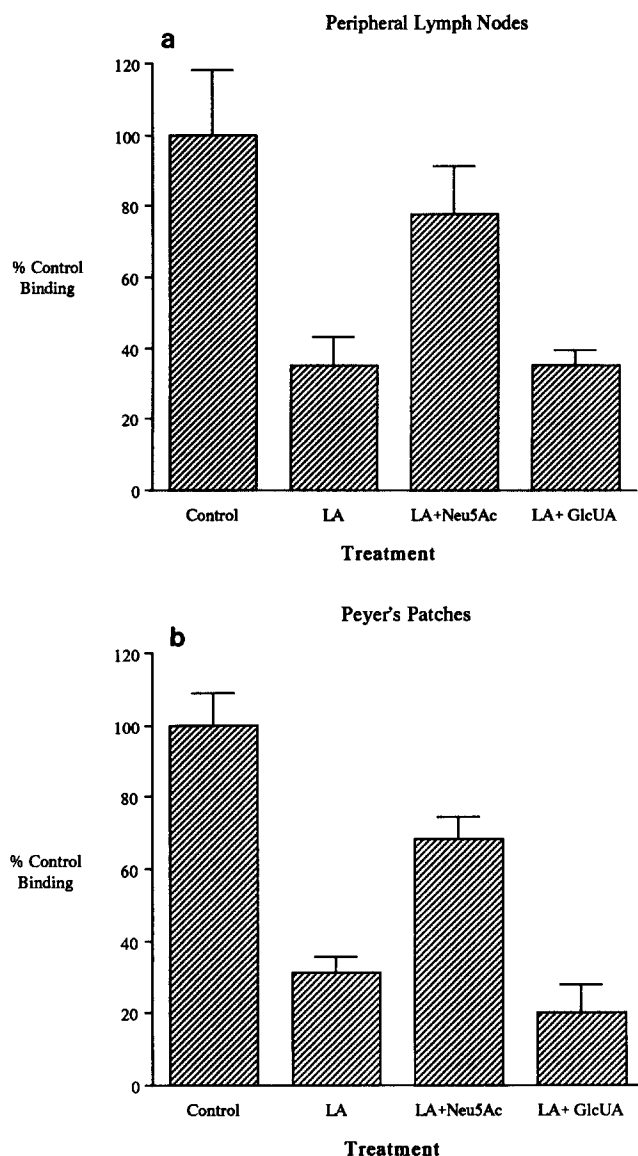


Figure 3. Effects of sugars on blockage of HEV adhesive sites by *Limax agglutinin*. LA was reacted with sections of (a) PN or (b) PP in the presence of sialic acid (25 mM Neu5Ac), glucuronic acid (25 mM GlcUA), or no added sugar. The sections were processed and tested for lymphocyte adherence to HEV as before. Binding to HEV was normalized to a percentage of the control level of binding (no LA). Absolute levels of lymphocyte binding in control samples were 36 lymphocytes/ $10^4 \mu\text{m}^2$ in PN sections and 23 lymphocytes/ $10^4 \mu\text{m}^2$ in PP sections.

saccharide containing high levels of mannose-6-phosphate) (Imai et al., 1990; Watson et al., 1990). This pnHR-IgG chimera, used in combination with a biotinylated-goat anti-human IgG and ABC-HRP, serves as a histochemical reagent for staining of HEV in PN (Watson et al., 1990). In correspondence with the organ selectivity of pnHR, staining of PP HEV is absent or is much reduced compared with that of PN HEV. Moreover, the staining of PN HEV is selectively blocked by MEL-14 mAb, by calcium chelation, or by the specific carbohydrates (e.g., fucoidin) that block lymphocyte attachment to PN HEV. These results established conclusively that the chimeric receptor detects the cognate HEV-ligand

of pnHR. We have, therefore, used the chimera to investigate if the effects of sialidase and LA on lymphocyte attachment to PN HEV could be attributed to direct effects on the PN HEV ligand. As shown in Fig. 5, treatment of PN sections with *Vibrio cholera* sialidase eliminated staining of HEV. Excess free sialic acid is known to inhibit the activity of this sialidase and was found to prevent the reduction in pnHR-IgG staining of PN HEV caused by sialidase (Fig. 5). Affinity-purified *Clostridium perfringens* sialidase, also previously shown to prevent lymphocyte attachment to PN HEV (Rosen et al., 1985), similarly eliminated staining of PN HEV by the chimera (Fig. 5). Finally, in correspondence with the effect of LA on lymphocyte attachment to PN HEV, pretreatment of tissue sections with this lectin prevented the specific staining of PN HEV by the chimera (Fig. 5).

Discussion

Our previous work, using highly specific bacterial sialidases, established a role for sialic acid moieties on PN HEV in the attachment of lymphocytes to these specialized blood vessels. Lymphocyte binding to HEV is thought to involve the concerted action of (at least) one organ-selective homing receptor in conjunction with general accessory adhesion molecule, such as LFA-1. Since there is the potential for multiple adhesive ligands in this system, it was not clear whether sialic acid was required for the activity of the HEV-ligand of the homing receptor or an accessory adhesion molecule. The finding that the sialidase effects on HEV varied with the anatomical site of the lymphoid organ argued that the target of the enzyme on PN HEV was the HEV ligand itself rather than an accessory adhesion molecule. An accessory adhesion molecule, such as a ligand for LFA-1, would be predicted to be invariant among HEV at different sites. Here, we demonstrate directly that sialic acid is required for the interaction

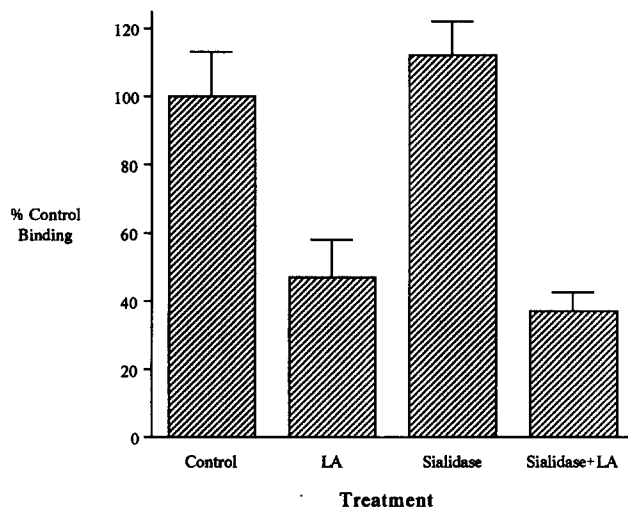


Figure 4. Effects of sialidase pretreatment of PP sections on blockage of HEV adhesive sites by *Limax agglutinin*. PP sections were pretreated with sialidase or with digestion buffer alone and then exposed to LA or to buffer. The sections were processed and tested for lymphocyte binding to HEV as described. Binding to HEV was normalized to a percentage of the control level of binding (no sialidase treatment, no LA). The absolute level of lymphocyte binding in control samples was 13.2 lymphocytes/ $10^4 \mu\text{m}^2$.

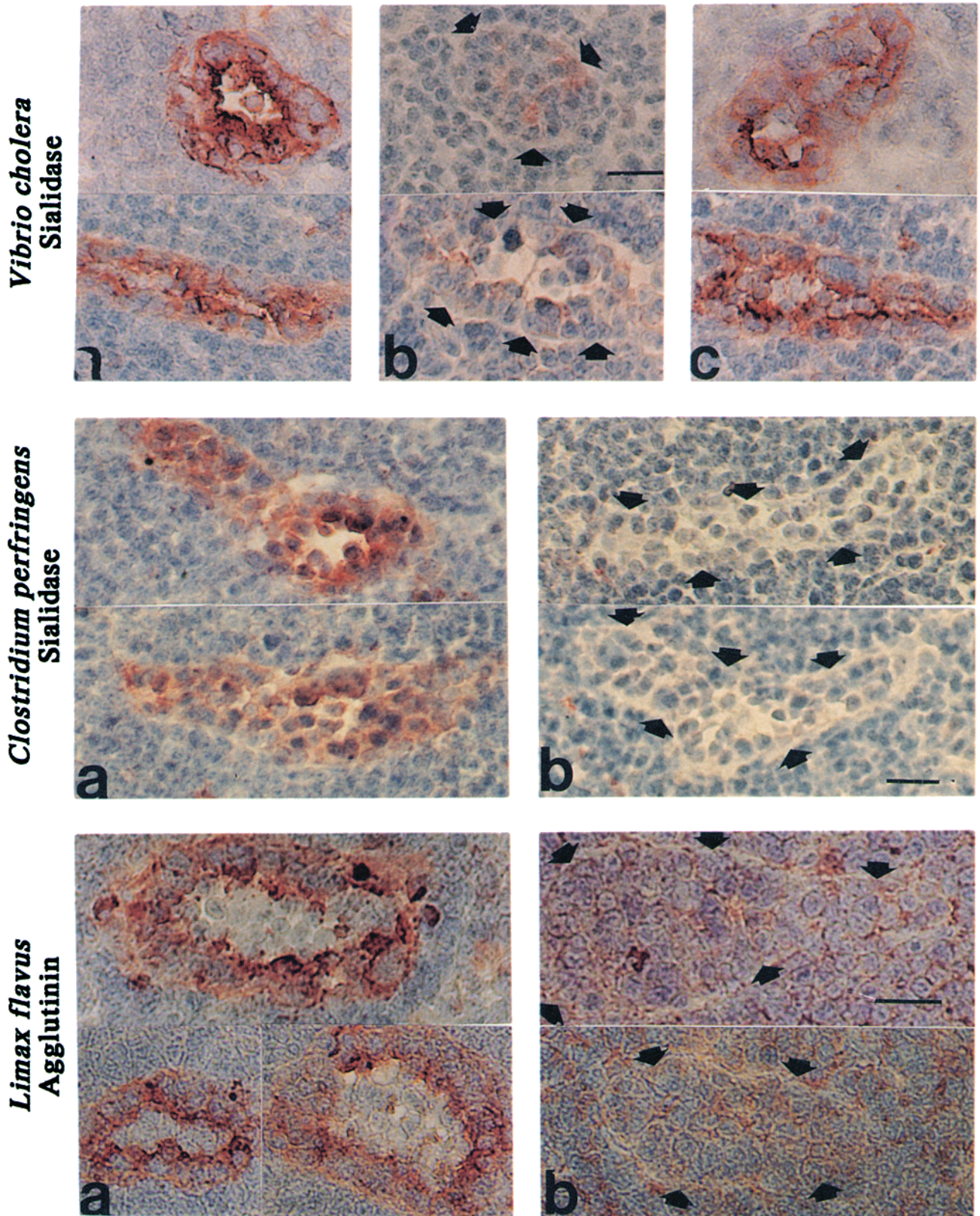


Figure 5. Effects of sialidases and *Limax* agglutinin on staining of PN HEV with pnHR-IgG chimera. (*Top*) Sections of PN were treated with (a) digestion buffer; (b) *Vibrio cholera* sialidase; or (c) *Vibrio cholera* sialidase plus free sialic acid (100 mM). In the middle panel, sections were treated with (a) control digestion buffer or (b) *Clostridium perfringens* sialidase. (*Bottom*) Sections were treated with (a) control buffer or (b) LA. After the above treatments, sections were stained with pnHR-IgG chimera or with desialylated chimera (LA experiment) in conjunction with HRP histochemistry to reveal the remaining HEV ligand. Arrows denote basement membranes of HEV in those sections where staining is greatly reduced. Bars, 20 μ m.

of pnHR with its cognate HEV ligand by showing that sialidase prevents staining of PN HEV by a soluble recombinant form of pnHR. This conclusion is strengthened by the observation that the sialic acid-specific lectin, LA, prevents both lymphocyte binding to PN HEV and staining of PN HEV by the chimera. These effects are mediated by the sialic acid binding site on LA, as evidenced by the blocking effect of sialic acid on LA's inhibition of lymphocyte attachment to HEV. The selectivity of LA is indicated by the finding that a series of other lectins, several of which react with HEV, produced no effect on lymphocyte attachment to HEV. It was surprising that two other sialic-acid binding lectins, WGA and limulin, did not affect lymphocyte attachment to PN-HEV. WGA's lack of activity may be attributable to its relatively restricted range in binding sialyloligosaccharides. For example, WGA does not bind to N-glycolyl sialic acid, while LA recognizes this form of sialic acid as well as the more prevalent N-acetyl form (Bhavanandan and Katlic, 1979; Ravindranath et al., 1985). Furthermore, WGA binds to sialylated neo-lacto glycosphingolipids but fails to recognize brain gangliosides (Molin et al., 1986), while LA binds to both glycolipid classes (True, D. D., B. A. Macher, and S. D. Rosen, in preparation). Perhaps, a crucial sialyloligosaccharide required for lymphocyte binding to PN-HEV is not recognized by WGA. The inactivity of limulin is more difficult to rationalize, since it demonstrates a binding specificity for sialic acid which overlaps significantly with LA (Ravindranath et al., 1985). It is possible that limulin binding to PN HEV is weak, and the lectin is eluted prior to the addition of lymphocytes in the *in vitro* adhesion assay.

Conceivably, the critical sialidase- and LA-susceptible sialic acid moieties of PN HEV reside on endothelial molecules that are physically associated with, and yet distinct from the HEV ligand. For example, it is known that the interaction of complement factor H with C3b on target membranes is positively modulated by sialic acid moieties present on membrane gangliosides (Michalek et al., 1989a,b). Since LA is of relatively low molecular mass, i.e., a dimer of 22-kD subunits (Miller et al., 1982), the critical sialylated sites, if separate from the HEV ligand, must be sterically very close to it. If, as is more likely, the essential sialic acid residues are actually part of the HEV ligand, two distinct roles can be envisioned. One is an indirect role in which sialic acid moieties modulate the orientation or conformation of a distal recognition site of the ligand. Alternatively, sialic acid moieties may directly contribute to an HEV ligand determinant that is engaged by the pnHR. Supporting this latter possibility is the fact that the lectin domain of this receptor contains a large number of lysine residues (Lasky et al., 1989; Bowen et al., 1989b), 16 in the case of the mouse molecule and 12 for the human. By contrast, other C-type lectin domains (e.g., asialoglycoprotein receptors and mannose-binding receptors) contain significantly fewer lysines (an average of five for the family) and have only been found to recognize neutral sugars (Drickamer, 1988). The ligand for the pnHR is not, however, a simple form of sialic acid, since Neu5Ac, either as a free sugar or in the form of sialyllactose, is not an effective inhibitor of the lectin activity of isolated gp90^{MEL} (Imai et al., 1990). Since sialic acid occurs naturally in a multitude of different forms and in many linkages to a variety of sugars (Schauer, 1982; 1985), the strong possibility remains that a distinctive sialyloligosac-

charide may define the recognition determinant of the HEV ligand. The ability of M6P, FIP, and fucose-sulfate or galactose-sulfate containing glycoconjugates to interact with pnHR may reflect a structural mimicry between these substances and the postulated sialyloligosaccharide. The exact role of sialic acid in the function of the PN HEV ligand, whether as a modulatory element or as part of a recognition determinant, awaits the isolation and structural analysis of the ligand. Candidates for the molecule already exist among the complex of proteins recognized by MECA-79, a mAb that selectively stains PN HEV and blocks lymphocyte attachment (Streeter et al., 1988b; Berg et al., 1989; Butcher, 1990).

Previously, we reported that sialidases from *V. cholera* and *C. perfringens* do not inactivate lymphocyte attachment sites on PP-HEV, and in unpublished experiments, *Arthrobacter ureafaciens* sialidase is similarly inactive. These enzymes represent a broad range of activities against sialic acids bearing N-acetyl or glycolyl substitutions in α 2-3 or α 2-6 linkages to various sugars. Thus, it was surprising that LA inhibited adhesive sites on PP HEV to the same extent as those on PN HEV. As was the case for the PN, preincubation of LA with free Neu5Ac blocked its inhibitory effects on PP sites. Therefore, the sialic acid-binding activity of LA would appear to be directly implicated in its function-blocking activity on PP sites. Yet, PP HEV treated with sialidase and lacking the majority of their LA-reactive sites, remain susceptible to inhibition by LA. One explanation of these apparently conflicting observations is that sialidase-resistant sialic acid residues, comprising a small percentage of the total sialylated structures, are the targets for the blocking activity of LA on PP HEV. Among the many known modifications of sialic acid, several forms are resistant to bacterial sialidases (Schauer, 1982). An intriguing possibility, compatible with the known capacity of sialyloligosaccharides to encode highly specific ligands for antibody recognition (Magnani, 1987) and for microbial adhesive receptors (Paulson, 1985; Sharon and Lis, 1989; Wiley and Skehel, 1987), is that distinct sialyloligosaccharides constitute the organ-specific recognition determinants of the HEV-ligands on both PN and PP. Implicit in this speculation is the prediction of a lectin-like homing receptor involved in lymphocyte attachment to Peyer's patch HEV.

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References

- Berg, E. L., L. A. Goldstein, M. A. Jutila, M. Nakache, L. J. Picker, P. R. Streeter, N. W. Wu, D. Zhou, and E. C. Butcher. 1989. Homing receptors and vascular addressins: cell adhesion molecules that direct lymphocyte traffic. *Immunol. Rev.* 108:5-18.
- Bevilacqua, M. P., S. Spengeling, M. A. Gimbrone, Jr., and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science (Wash. DC)*. 243:1160-1165.
- Bhavanandan, V. P., and A. W. Katlic. 1979. The interaction of wheat germ agglutinin with sialoglycoproteins. *J. Biol. Chem.* 254:4000-4008.
- Bowen, B. R., T. Nguyen, and L. A. Lasky. 1989. Characterization of a human homologue of the murine peripheral lymph node homing receptor. *J. Cell Biol.* 109:421-427.

- Bowen, B., C. Fennie, and L. A. Lasky. 1990. The Mel-14 antibody binds to the lectin domain of the murine peripheral lymph node homing receptor. *J. Cell Biol.* 110:147-153.
- Brandley, B. K., T. S. Ross, and R. L. Schnaar. 1987. Multiple carbohydrate-binding receptors on lymphocytes revealed by adhesion to immobilized polysaccharides. *J. Cell Biol.* 105:991-97.
- Butcher, E. C. 1986. The regulation of lymphocyte traffic. *Curr. Top. Micro. Immunol.* 128:85-122.
- Butcher, E. C. 1990. Cellular and molecular mechanisms that direct leukocyte traffic. *Am. J. Pathol.* 136:3-12.
- 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.
- Camerini, D., S. P. James, I. Stamenkovic, and B. Seed. 1989. Leu-8/TQ1 is the human equivalent of the Mel-14 lymph node homing receptor. *Nature (Lond.)* 342:78-82.
- Chin, Y. H., R. Rasmussen, J. J. Woodruff, and T. G. Easton. 1986. A monoclonal anti-HEBFp with specificity for lymphocyte surface molecules mediating adhesion to Peyer's patch high endothelium of the rat. *J. Immunol.* 136:2556-2561.
- Drickamer, K. 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* 263:9557-9560.
- Duijvestijn, A., and A. Hamann. 1989. Mechanisms and regulation of lymphocyte migration. *Immunol. Today.* 10:23-28.
- Gallatin, M., T. St. John, M. Siegelman, R. Reichert, E. Butcher, and I. Weissman. 1986. Lymphocyte homing receptors. *Cell.* 44:673-680.
- Gallatin, W. M., I. L. Weissman, and E. C. Butcher. 1983. A cell surface molecule involved in organ-specific homing of lymphocytes. *Nature (Lond.)* 303:30-34.
- Geng, J. G., M. P. Bevilacqua, K. L. Moore, T. M. McIntyre, S. M. Prescott, J. M. Kim, G. A. Bliss, G. A. Zimmerman, and R. P. McEver. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature (Lond.)* 343:757-760.
- Geoffroy, J. S., and S. D. Rosen. 1989. Demonstration that a lectin-like receptor (gp90^{MEL}) directly mediates adhesion of lymphocytes to high endothelial venules of lymph nodes. *J. Cell Biol.* 109:2463-2469.
- Goldstein, L. A., D. Zhou, L. J. Picker, C. N. Minty, R. F. Bargatze, J. F. Ding, and E. C. Butcher. 1989. A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell.* 56:1063-1072.
- Hamann, A., D. Jablonski-Westrich, A. Duijvestijn, E. C. Butcher, H. Baisch, R. Harder, and H.-G. Thiele. 1988. Evidence for an accessory role of LFA-1 in lymphocyte-high endothelium interaction during homing. *J. Immunol.* 140:693-699.
- Holzmann, B., B. W. McIntyre, and I. L. Weissman. 1989. Identification of a murine Peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an alpha chain homologous to human VLA-4. *Cell.* 56:37-46.
- Imai, Y., D. D. True, M. S. Singer, and S. D. Rosen. 1990. Direct demonstration of the lectin activity of gp90^{MEL}, a lymphocyte homing receptor. *J. Cell Biol.* 110:1225-1232.
- Jalkanen, S., R. Bargatze, L. Herron, and E. Butcher. 1986. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *Eur. J. Immunol.* 16:1195-1202.
- Johnston, G. I., R. G. Cook, and R. P. McEver. 1989. Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. *Cell.* 56:1033-1044.
- Kishimoto, T. K., M. A. Jutila, and E. C. Butcher. 1990. Identification of a human peripheral lymph node homing receptor: A rapidly down-regulated adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 87:2244-2248.
- Lasky, L. A., M. S. Singer, T. A. Yednock, D. Dowbenko, C. Fennie, H. Rodriguez, T. Nguyen, S. Stachel, and S. D. Rosen. 1989. Cloning of a lymphocyte homing receptor reveals a lectin domain. *Cell.* 56:1045-1055.
- Lis, H., and N. Sharon. 1986. Lectins as molecules and as tools. *Annu. Rev. Biochem.* 55:35-67.
- Magnani, J. L. 1987. Mouse and rat monoclonal antibodies directed against carbohydrates. *Methods Enzymol.* 138:484-491.
- Michalek, M. T., E. G. Bremer, and C. Mold. 1988a. Effect of gangliosides on activation of the alternative pathway of human complement. *J. Immunol.* 140:1581-1587.
- Michalek, M. T., C. Mold, and E. G. Bremer. 1988b. Inhibition of the alternative pathway of human complement by structural analogues of sialic acid. *J. Immunol.* 140:1588-1594.
- Miller, R. 1987. Properties of a sialic acid-specific lectin from the slug *Limax flavus*. *Methods Enzymol.* 138:527-536.
- Miller, R. L., J. F. Collawn, and W. W. Fish. 1982. Purification and macro-molecular properties of a sialic acid-specific lectin from the slug *Limax flavus*. *J. Biol. Chem.* 257:7574-7580.
- Molin, K., P. Fredman, and L. Svennerholm. 1986. Binding specificities of the lectins PNA, WGA, and UEA-1 to polyvinylchloride-adsorbed glycosphingolipids. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 205:51-55.
- Nicolson, G. L. 1974. The interaction of lectins with animal cell surfaces. *Int. Rev. Cytol.* 39:89-190.
- Pals, S. T., A. den Otter, F. Miedema, P. Kabel, G. D. Keizer, R. J. Scheper, and C. J. L. M. Meijer. 1988. Evidence that leukocyte function-associated antigen-1 is involved in recirculation and homing of human lymphocytes via high endothelial venules. *J. Immunol.* 140:1851-1853.
- Paulson, J. C. 1985. Interaction of animal viruses with cell surface receptors. *In The Receptors.* Vol. 2. P. M. Conn, editor. Academic Press, Orlando, FL. 131-219.
- Ravindranath, M., H. Higa, E. Cooper, and J. Paulson. 1985. Purification and characterization of an O-acetylsialic acid-specific lectin from marine crab *Cancer antennarius*. *J. Biol. Chem.* 260:8850-8856.
- Rosen, S. D. 1989. Lymphocyte homing: progress and prospects. *Curr. Op. Cell Biol.* 1:913-919.
- Rosen, S. D., and T. A. Yednock. 1986. Lymphocyte attachment to high endothelial venules during recirculation: a possible role for carbohydrates as recognition determinants. *Mol. Cell. Biochem.* 72:153-164.
- 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* 228:1005-1007.
- Rosen, S. D., S.-I. Chi, D. D. True, M. S. Singer, and T. A. Yednock. 1989. Intravenously injected sialidase inactivates attachment sites for lymphocytes on high endothelial venules. *J. Immunol.* 142:1895-1902.
- Schauer, R. 1982. Chemistry, metabolism, and biological functions of sialic acids. *Adv. Carbohydr. Chem. Biochem.* 40:131-233.
- Schauer, R. 1985. Sialic acids and their role as biological masks. *Trends Biochem. Sci.* 10:357-360.
- Schulte, B., S. Spicer, and R. Miller. 1984. Histochemical localization of sialoglycoconjugates with a sialic acid-specific lectin from the slug *Limax flavus*. *Histochem. J.* 16:1125-1132.
- Sharon, N., and H. Lis. 1989. Lectins as cell recognition molecules. *Science (Wash. DC)* 246:227-234.
- Siegelman, M. H., and I. L. Weissman. 1989. Human homologue of mouse lymph node homing receptor: Evolutionary conservation at tandem cell interaction domains. *Proc. Natl. Acad. Sci. USA.* 86:5562-5566.
- Siegelman, M. H., M. Van de Rijn, and I. L. Weissman. 1989. Mouse lymph node homing receptor cDNA clone encodes a glycoprotein revealing tandem interaction domains. *Science (Wash. DC)* 243:1165-1172.
- Siegelman, M. H., I. C. Cheng, I. L. Weissman, and E. K. Wakeland. 1990. The mouse lymph node homing receptor is identical with the lymphocyte cell surface marker Ly-22: role of the EGF domain in endothelial binding. *Cell.* 61:611-622.
- Stamenkovic, I., M. Amiot, J. M. Pesando, and B. Seed. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell.* 56:1057-1062.
- Stamper, H. B., and J. J. Woodruff. 1976. Lymphocyte homing into lymph nodes: *in vitro* demonstration of the selective affinities of recirculating lymphocytes for high-endothelial venules. *J. Exp. Med.* 144:828-833.
- Stoolman, L. M. 1989. Adhesion molecules controlling lymphocyte migration. *Cell.* 56:907-910.
- 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., and H. Ebling. 1989. Adhesion molecules of cultured hematopoietic malignancies. A calcium-dependent lectin is the principle mediator of binding to the high endothelial venule of lymph nodes. *J. Clin. Invest.* 84:1196-1205.
- Stoolman, L. M., T. S. Tenforde, and S. D. Rosen. 1984. Phosphomannosyl receptors may participate in the adhesive interactions between lymphocytes and high endothelial venules. *J. Cell Biol.* 99:1535-1540.
- Stoolman, L. M., T. A. Yednock, and S. D. Rosen. 1987. Homing receptors on human and rodent lymphocytes-evidence for a conserved carbohydrate-binding specificity. *Blood.* 70:1842-1850.
- Streeter, P. R., E. L. Berg, B. T. N. Rouse, R. F. Bargatze, and E. C. Butcher. 1988a. A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature (Lond.)* 331:41-46.
- Streeter, P. R., B. T. N. Rouse, and E. C. Butcher. 1988b. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol.* 107:1853-1862.
- Tedder, T. F., C. M. Isaacs, T. J. Ernst, G. D. Demetri, D. A. Adler, and C. M. Distech. 1989. Isolation and chromosomal localization of cDNAs encoding a novel human lymphocyte cell surface molecule, LAM-1. Homology with the mouse lymphocyte homing receptor and other human adhesion proteins. *J. Exp. Med.* 170:123-133.
- Tedder, T. F., A. C. Penta, H. B. Levine, and A. S. Freedman. 1990. Expression of the human leukocyte adhesion molecule, LAM1. Identity with the TQ1 and Leu-8 differentiation antigens. *J. Immunol.* 144:532-540.
- Wagner, P., and J. Roth. 1988. Occurrence and distribution of sialic acid residues in developing rat glomerulus: investigations with the *Limax flavus* and the wheat germ agglutinin. *Eur. J. Cell Biol.* 47:259-269.
- Watson, S. R., Y. Imai, C. Fennie, J. S. Geoffroy, S. D. Rosen, and L. A. Lasky. 1990. A homing receptor-IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules. *J. Cell Biol.* 110:2221-2229.
- Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56:365-94.
- Woodruff, J. J., L. M. Clarke, and Y. H. Chin. 1987. Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. *Annu. Rev. Immunol.* 5:201-222.
- Yednock, T. A., E. C. Butcher, L. M. Stoolman, and S. D. Rosen. 1987a. Receptors involved in lymphocyte homing: relationship between a carbohydrate-binding receptor and the MEL-14 antigen. *J. Cell Biol.* 104:725-731.
- Yednock, T. A., L. M. Stoolman, and S. D. Rosen. 1987b. Phosphomannosyl-derivatized beads detect a receptor involved in lymphocyte homing. *J. Cell Biol.* 104:713-723.