Immunohistologic and Functional Characterization of a Vascular Addressin Involved in Lymphocyte Homing into Peripheral Lymph Nodes

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Abstract. The tissue localization or "homing" of circulating lymphocytes is directed in part by specialized vessels that define sites of lymphocyte exit from the blood. In peripheral lymph nodes, mucosal lymphoid tissues (Peyer's patches and appendix), and sites of chronic inflammation, for example, lymphocytes leave the blood by adhering to and migrating between those endothelial cells lining postcapillary high endothelial venules (HEV). Functional analyses of lymphocyte interactions with HEV have shown the lymphocytes can discriminate between HEV in different tissues, indicating that HEV express tissue-specific determinants or address signals for lymphocyte recognition. We recently described such a tissue-specific "vascular addressin" that is selectively expressed by endothelial cells supporting lymphocyte extravasation into mucosal tissues and that appears to be required for mucosa-specific lymphocyte homing (Streeter, P. R., E. L. Berg, B. N. Rouse, R. F. Bargatze, and E. C. Butcher. 1988. *Nature (Lond.)*. 331:41–46). Here we document the existence and tissue-specific distribution of a distinct HEV differentiation antigen. Defined by monoclonal antibody MECA-79, this antigen is expressed at high levels on the lumenal surface and in the cytoplasm of HEV in peripheral lymph nodes. By contrast, although MECA-79 stains many HEV in the mucosal Peyer's patches, expression in most cases is restricted to the perivascular or ablumenal aspect of these venules. In the small intestine lamina propria, a mucosa-associated site that supports the extravasation of lymphocytes, venules do not stain with MECA-79. Finally, we demonstrate that MECA-79 blocks binding of both normal lymphocytes and a peripheral lymph node–specific lymphoma to peripheral lymph node HEV in vitro and that it also inhibits normal lymphocyte homing to peripheral lymph nodes in vivo without significantly influencing lymphocyte interactions with Peyer's patch HEV in vitro or in vivo. Thus, MECA-79 defines a novel vascular addressin involved in directing lymphocyte homing to peripheral lymph nodes.

TISSUE- or site-specific cell–cell interactions are thought to be important in a variety of biologic systems, including the targetting of neuronal processes, the migration of cells during morphogenesis, and the trafficking or "homing" of lymphocytes to specific tissues or organs in the body. The latter system has proven particularly accessible to experimental analysis, due to the availability of in vitro and in vivo assays of lymphocyte interactions with venules that support and direct the extravasation of lymphocytes from the blood into lymphoid tissues and sites of chronic inflammation. In peripheral lymph nodes and mucosal lymphoid tissues (Peyer's patches and appendix), lymphocytes leave the blood by adhering to and migrating between the morphologically distinct endothelium lining high endothelial venules (HEV[1]; Gowans and Knight, 1964; Marchesi and Gowans, 1964). Lymphocyte entry into distinct lymphoid tissues can exhibit remarkable tissue specificity (Guy-Grand et al., 1974; Cahill et al., 1977; McWilliams et al., 1977; Rose et al., 1978; Hall et al., 1979; Chin and Hay, 1980; Smith et al., 1980; Butcher, 1986), determined at least in part by selective recognition of endothelial cells (Butcher et al., 1980; Chin et al., 1984; Jalkanen et al., 1986*b*; Schmitz et al., 1988). Functional in vitro studies have revealed the existence of several independent lymphocyte high endothelial cell (HEC) recognition systems mediating lymphocyte entry into peripheral lymph nodes, mucosal lymphoid tissues, and into the synovium of inflamed joints (Butcher et al., 1980; Jalkanen et al., 1986*b*). Lymphocyte surface molecules involved in lymphocyte–HEC interactions have been defined (Gallatin et al., 1983; Rasmussen et al., 1985; Chin et al., 1986; Jalkanen et al., 1986*a*; Jalkanen et al., 1987; Hamman et al., 1988; Pals et al., 1988; Wu and Butcher, manuscript in preparation); those that appear to mediate tissue-spe-

1. *Abbreviations used in this paper*: HEC, high endothelial cells; HEV, high endothelial venules.

cific endothelial cell recognition have been termed "homing receptors".

In an effort to identify endothelial cell surface antigens that function as tissue-specific position markers or address signals for recognition by circulating lymphocytes, we produced mAbs against mouse HEC. We have recently described one such "vascular addressin" that is selectively expressed in mucosal tissues and appears to be required for lymphocyte extravasation into mucosal lymphoid organs (Streeter et al., 1988). Here we describe the existence and patterns of expression of an antigenically distinct vascular addressin that is involved in lymphocyte trafficking into nonmucosal peripheral lymph nodes. Like its functional counterpart in mucosal lymphoid tissues, this addressin is expressed in a tissue-selective manner. The mAb raised against this peripheral lymph node addressin blocks the in vitro binding of lymphocytes to peripheral lymph node HEV, and inhibits the homing of lymphocytes to peripheral lymph nodes in vivo.

Materials and Methods

mAb Production

Antigen isolation/preparation: axillary, brachial, inguinal, and mesenteric lymph nodes from 8–12-wk-old BALB/c mice (Institute for Medical Research, San Jose, CA) were pooled in Hanks' balanced salt solution (HBSS), minced, and gently pressed between glass microscope slides to release lymphocytes. The resulting cell suspension was then passed through nitex nylon mesh (Sullivan Co., San Francisco, CA). The stromal elements that remained on top of the mesh were collected, treated for 10 min at 37°C with HBSS containing 0.32 mg collagenase/ml (5 ml/mouse), washed, and again passed through nitex. The stromal elements remaining on the nitex were collected, suspended in HBSS, and used for immunizations.

Immunization and hybridoma production: a Wistar rat (Simonsen Laboratories, Inc., Gilroy, CA) was immunized with collagenase-dispersed stromal elements mixed with precipitated aluminum potassium sulfate (adjuvant/carrier) in a 3:2 ratio, final volume 1 ml, and delivered i.p. 4 d before fusion, the rat was boosted i.p. with stromal cells (from 10 mice) in HBSS. Spleen cells from immune animals were fused (Kohler and Milstein, 1975) with the mouse myeloma Sp2/0 (Schulman et al., 1978), and hybrids were selected in medium containing hypoxanthine, aminopterin, and thymidine. Supernatants from hybrid-containing wells were initially screened on tissue sections by immunofluorescence. The MECA-79 secreting hybridoma was subcloned three times by limiting dilution, and adapted to serum free HB101 medium (New England Nuclear, Boston, MA). The isotype of MECA-79 was determined to be IgM by Ouchterlony analysis.

Rat IgM control mAbs included 2C2, an antibody defining a B cell lineage antigen, B220 (Coffman and Weissman, 1981), a gift from R. Coffman (DNAX Research Institute, Palo Alto, CA); and OZ-42, an antibody that binds a determinant expressed in developing mouse brain, generously supplied by L. Pickford and R. Rouse.

Additional mAbs used in this investigation included MECA-367, a rat IgG2a that defines a vascular addressin in mucosal lymphoid tissues of the mouse (Streeter et al., 1988), and a class-matched control antibody, Hermes-1, that binds lymphocyte homing receptors in man but does not react with mouse tissues (Jalkanen et al., 1986*a*).

Immunofluorescence Staining

One-color immunofluorescence: supernatants from hybrid-containing wells were initially screened for reactivity with tissue-specific markers on mouse HEV by immunofluorescence staining of acetone fixed frozen sections containing both peripheral lymph nodes and Peyer's patches. Briefly, sections were incubated with culture supernatants, washed, and exposed to a fluoresceinated goat anti-rat Ig (Sigma Chemical Co., St. Louis, MO) diluted 1:40 in PBS containing 5% normal mouse serum.

Two-color immunofluorescence: two color visualization of distinct markers on endothelial cells was accomplished by using a three-stage detection system for rat IgM, and a two-stage detection system for rat IgG. Briefly, after incubation with either experimental (MECA-79 and MECA-367), or class-matched control antibodies (OZ-42 and Hermes-1), sections (or enzyme-dispersed endothelial cells, see below) were washed and incubated in an affinity-isolated biotinylated goat anti-rat IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD; diluted 1:80) in PBS containing 5% normal mouse serum. The sections (or enzyme-dispersed endothelial cells) were then washed, and incubated with PBS containing texas red conjugated avidin (Cappel Laboratories, Malvern, PA; diluted 1:50), an affinity-isolated fluoresceinated goat antibody to rat IgG (Kirkgaard and Perry Laboratories, Inc.; diluted 1:80), a rat monoclonal IgM (either OZ-42 or MECA-79) at a concentration of 100 μg/ml (to block low level reactivity of the anti-rat IgG with section bound rat IgM), and 5% NMS.

Immunoperoxidase Staining

Immunoperoxidase staining of acetone fixed frozen sections of lymphoid and extralymphoid tissues was performed using a standard technique. Briefly, sections were incubated with purified mAb in PBS, washed, and treated with a solution of PBS containing a 1:40 dilution of horseradish peroxidase-conjugated rabbit anti-rat IgG (DAKOPATTS, Copenhagen; this reagent reacts with rat IgM) and 5% NMS. The horseradish peroxidase-conjugated second antibody was detected after exposure to a solution of 0.05M Tris (pH 7.6) containing 0.05% diaminobenzidine and 0.009% H_2O_2 (wt/vol), and the staining was enhanced by incubation in 0.5% copper sulfate in saline. Hematoxylin was used to lightly counterstain sections.

Slight modification of the procedure described above allowed detection of either rat IgM or rat IgG after the intravenous administration of a mixture containing both antibodies. Briefly, IgM was detected using a three-stage system, with an affinity isolated biotinylated second stage goat antibody to rat IgM (Kirkegaard & Perry Laboratories, Inc.; diluted 1:80 in PBS containing 5% NMS), and horseradish peroxidase-labeled streptavidin as the third stage (Kirkegaard & Perry Laboratories, Inc.; diluted 1:80 in PBS). IgG was detected using an affinity-isolated horseradish peroxidase-labeled goat anti-rat IgG (Kirkegaard & Perry Laboratories, Inc.) diluted 1:10 in PBS containing 5% NMS and a rat monoclonal IgM (either OZ-42 or MECA-79) at a concentration of 100 μg/ml (to block reactivity with rat IgM on the section). The horseradish peroxidase-conjugated reagents were detected as described above.

Cells

Normal lymphocytes: mesenteric lymph nodes from 6–12-wk-old BALB/c mice were minced in HBSS and gently pressed between glass microscope slides to release lymphocytes. The cell suspension was then passed through nitex to eliminate stromal elements. The lymphoid cells were then washed (2×) in HBSS, and used in either in vitro lymphocyte-HEV binding assays, or in vivo homing experiments.

Lymphomas: we used two well-characterized lymphomas, TK1 and 38C13 (Butcher et al., 1980; Gallatin et al., 1983), for in vitro HEV binding assays. TK1, stored frozen in liquid nitrogen and used here immediately after thawing, specifically binds HEV in mucosa-associated tissues (Butcher et al., 1980). The lymphoma 38C13, which for these studies was taken directly from an adoptive recipient of the tumor, binds HEV via an alternate, peripheral lymph node-specific recognition system (Gallatin et al., 1983).

Endothelial cells: viable HEC, in single cell suspension, were prepared using a modification of an enzyme-dispersion protocol provided by P. Andrews. Briefly, mesenteric lymph node stromal elements (isolated as described above) were treated for 30 min (37°C) with HBSS containing 0.32 mg of collagenase per ml (5 ml per mouse). Stromal elements were allowed to sediment at 1 g for 10 min, and the supernatant was decanted. After repeating this enzymatic treatment/sedimentation procedure (1×), stromal elements were treated for 30 min (37°C) with HBSS containing 0.32 mg of collagenase and 0.4 mg of dispase per ml (5 ml per mouse). After washing the enzyme-dispersed cells (2×) with HBSS containing 5% newborn calf serum, cells were either stained in suspension for two-color immunofluorescence analysis (as described above), or were used to prepare cytospins that were then acetone fixed (permeabilized) and stained. For cytospins, cells from 5 mice were resuspended in 2 ml of HBSS/5% NCS, and 200 μl aliquots were spun for 5 min at 1,000 rpm.

In Vitro Lymphocyte–HEV Binding Assay

The in vitro assay of lymphocyte binding to HEV in frozen sections has been described (Stamper and Woodruff, 1976; Butcher et al., 1979), but was modified slightly for these studies. Briefly, 12-μm-thick, freshly cut, unfixed tissue sections (Peyer's patches, mesenteric lymph nodes, and peripheral lymph nodes), were pretreated for 30 min at 7°C with 100 μl of

either MECA-79 or 2C2 (an isotype-matched control antibody) diluted to a concentration of 100 μg/ml in RPMI 1640 medium containing 5% newborn calf serum and 20 mM Hepes, pH 7.2 (complete medium), or complete medium alone. After pretreatment, medium was removed from both experimental and control sections, and 5×10^5–3×10^6 lymphocytes or lymphoma cells were applied in 100 μl of complete medium. After mild rotation for 30 min at 7°C, nonadherent cells were decanted, and adherent cells were fixed to tissue sections by emersion in cold PBS containing 1.5% glutaraldehyde. Cell binding to HEV was assessed microscopically under darkfield illumination, and the mean number of bound cells per HEV was determined. Results are presented as percent of media control (±SEM).

In Vivo Lymphocyte Homing Assay

In vitro labeling of lymphocytes with ^{51}Cr: normal mesenteric lymph node cells from 6–10-wk-old BALB/c mice were labeled with ^{51}Cr as previously described (Streeter et al., 1988). Briefly, lymphocytes were labeled by incubation of 2.5×10^7 cells/ml for 1.5 h at 37°C, with mixing every 15 min, in DME supplemented with 20 mM Hepes, 5% FCS, and 100 μCi/ml sodium chromate ($Na_2{}^{51}CrO_4$; New England Nuclear). After labeling, the cells were centrifuged through a layer of FCS, and washed twice with HBSS.

In vivo homing: recipient syngeneic mice were injected intravenously with 1 mg of either MECA-79 or control monoclonal IgM antibody OZ-42 diluted in HBSS. After 4 h, 2.5×10^7 chromium-labeled cells were administered intravenously. 2 h later, animals were sacrificed, and lymphocyte localization was determined by quantitating the cpm in the indicated organs. Results are presented as percent of isotype matched control (±SEM).

Results

Production of MECA-79

Collagenase-dispersed mesenteric and peripheral lymph node stromal elements were used as immunogen for the production of mAbs defining tissue-specific endothelial cell molecules involved in lymphocyte homing. Initial screening of hybridoma supernatants by immunofluorescence revealed one well containing antibody (MECA-79) that selectively stained peripheral lymph node HEV.

Expression Patterns of the MECA-79 Antigen

Immunohistologic examination of frozen sections revealed that MECA-79 stained all HEV in peripheral (axillary, brachial, and inguinal) lymph nodes with intense cytoplasmic as well as apparent lumenal and ablumenal cell surface reactivity (Fig. 1 *a*). In contrast, HEV in mucosal Peyer's patches of the small intestine exhibited variable staining. While a subset of Peyer's patch HEV was completely negative, most of the HEV in this tissue stained with MECA-79. However, unlike the staining in peripheral lymph nodes, reactivity was generally localized to the ablumenal aspect of the vessels (for example, see Fig. 1 *c*). On a serial section of this Peyer's patch, MECA-367, an antibody raised against a mucosal vascular addressin (Streeter et al., 1988), intensely stains the full thickness of HEV in this tissue (Fig. 1 *d*). While immunohistologic examination of the reactivity pattern for MECA-79 did not permit definition of the precise subcellular location of the antigen, the staining was consistent with its presence on the ablumenal cell membrane, and/or in association with the surrounding extracellular or cellular components (e.g., basal lamina or pericytes). A small subset of Peyer's patch HEV also bore low levels of cytoplasmic and/or lumenal MECA-79 antigen.

HEV in the intestine-draining mesenteric lymph node bind both peripheral lymph node–specific and Peyer's patch–specific HEV-binding lymphomas, suggesting the dual expression of peripheral lymph node– and mucosa-associated endothelial cell determinants involved in lymphocyte recognition (Butcher et al., 1980). In keeping with these lymphocyte-binding properties, we previously demonstrated that a mucosal vascular addressin is expressed by most HEV in this tissue (Streeter et al., 1988). Immunohistologic staining with MECA-79 revealed that this marker is also present on mesenteric lymph node HEV. The pattern of staining ranged from intense full thickness reactivity comparable to that of peripheral lymph node HEV, to partial staining (Fig. 1 *b*) or, on rare venules, a complete lack of reactivity. Interestingly, the staining intensity of positive HEV in the mesenteric lymph node was generally less than that of peripheral lymph node HEV.

To assess directly the presence or absence of MECA-79 antigen at the lumenal surface of HEV and to facilitate a direct comparison between the expression of this antigen and the mucosal addressin (defined by MECA-367), MECA-79 and MECA-367 were injected intravenously into mice. The recipients were sacrificed 15 min after injection and then gently perfused with 25 ml of HBSS over a 5-min period to remove unbound antibody from the vasculature. Perfusion was performed by injection of HBSS into the left ventricle of the heart, with perfusate removed from the venae cavae. Peyer's patches, mesenteric lymph nodes, and peripheral lymph nodes from these animals were then sectioned, and membrane-associated MECA-79 (an IgM antibody) and MECA-367 (IgG) were visualized by immunoperoxidase staining of serial sections with anti–rat IgM or anti–rat IgG specific reagents. MECA-79 bound at high levels to peripheral lymph node HEV (Fig. 2 *a*), but was absent or only weakly detectable on the lumenal surface of most HEV in Peyer's patches (Figure 2 *c*). Thus little of the MECA-79 reactive antigen associated with Peyer's patch HEV is in direct contact with the blood. Interestingly, when perfusion was carried out under excessive pressure (i.e., 25 ml of HBSS was perfused in roughly 1.0–1.5 min), MECA-79 bound to the ablumenal aspect of Peyer's patch HEV, yielding much the same pattern as seen by direct immunohistology (except for the absence of cytoplasmic staining of HEC). Thus some or all of the ablumenal antigen is associated with extracellular components, pericytes, or the HEC membrane. In contrast to MECA-79, MECA-367 localized at high levels to Peyer's patch HEV (Fig. 2 *f*), but was not detectable on peripheral lymph node HEV (Fig. 2 *d*).

Mesenteric lymph node HEV in the animals receiving mAbs intravenously exhibited pronounced local variation in lumenal expression of both the MECA-367 and MECA-79 defined antigens. While most HEV in this gut-draining lymph node were stained with both antibodies (Figs. 2, *b* and *e*), a subset of HEV or portions of HEV stained exclusively with either MECA-367 or MECA-79. The exclusive expression of one or the other of these antigens by HEV segments was confirmed by two-color immunofluorescence analysis (Figs. 3, *a–d*). Cytoplasmic expression of the MECA-367 and MECA-79 defined antigens by mesenteric lymph node high endothelial cells is explored further below. No HEV staining was observed in recipients of control rat IgM or IgG antibodies.

In the extralymphoid lamina propria of the small intestine, a mucosa-associated site that supports the extravasation of lymphocytes, all venules were MECA-79$^-$, whether assessed

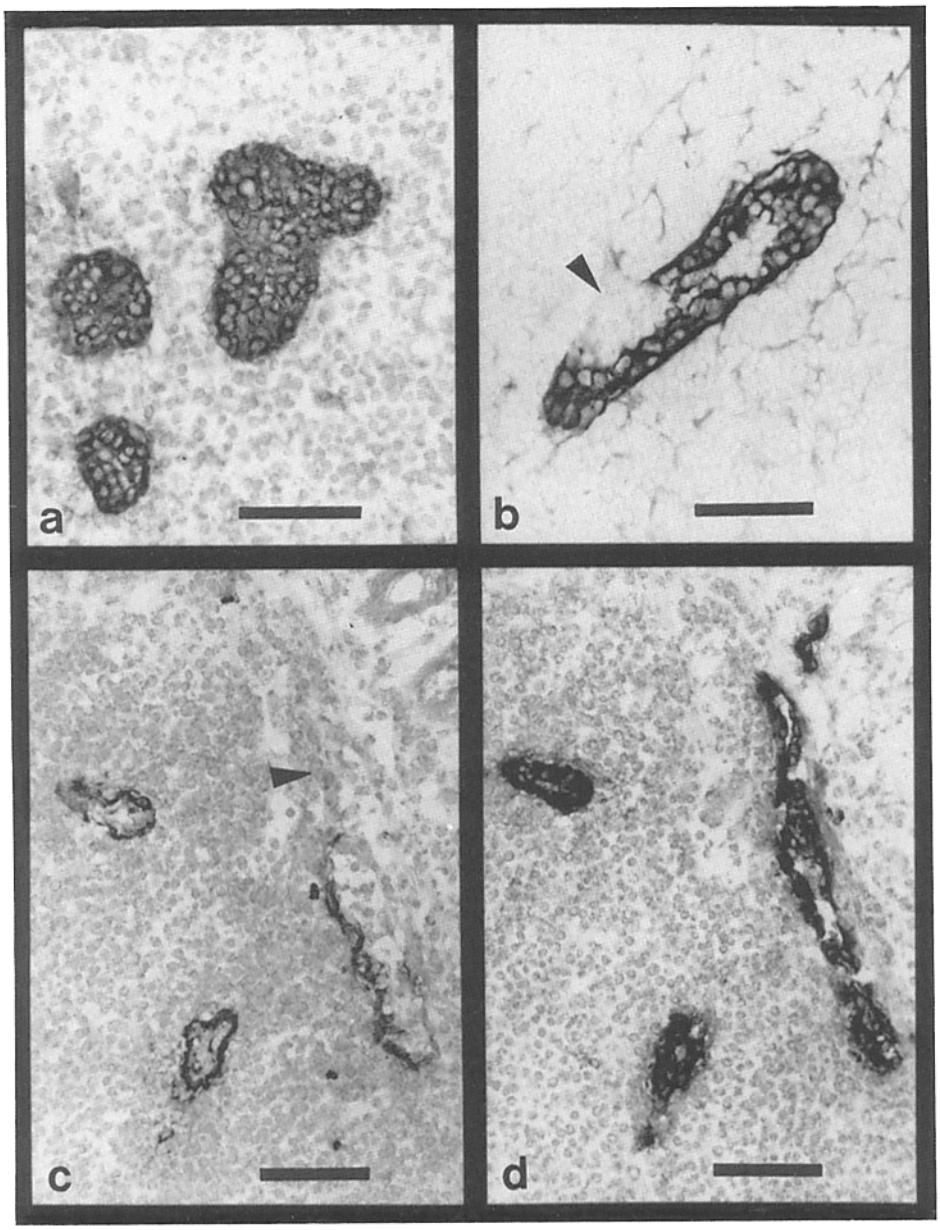

Figure 1. Tissue-selective expression of the MECA-79 antigen. (*a*) Immunohistologic staining of peripheral lymph nodes reveals that MECA-79 stains all HEV with intense cytoplasmic as well as apparent lumenal and ablumenal cell surface reactivity. (*b*) Most HEV in the mesenteric lymph node also stain intensely with MECA-79. However, some HEV or portions of HEV are negative. (*Arrow* indicates a MECA-79$^-$ segment). (*c*) In contrast, in the mucosal Peyer's patches staining is generally localized to the ablumenal aspect of the venules. (*Arrow* indicates a MECA-79 negative segment.) (*d*) On a serial section of the Peyer's patch presented in *c*, the anti-mucosal addressin antibody, MECA-367, intensely stains the full thickness of all HEV. Bar, 50 μm.

by direct immunohistology or in recipients of intravenously administered MECA-79. Indeed, immunohistologic examination of a variety of normal extralymphoid tissues, including skin, skeletal muscle, mammary gland, and brain failed to reveal staining with MECA-79. Table I summarizes the results.

MECA-79 Blocks Lymphocyte Binding to Peripheral Lymph Node HEV

The tissue distribution of the MECA-79 antigen suggested that it might function as a specific recognition element involved in lymphocyte binding to peripheral lymph node HEV. We therefore asked whether MECA-79 could prevent lymphocyte binding to HEV in the Stamper-Woodruff in vitro assay. Frozen sections of peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches were pretreated with a saturating concentration of MECA-79, an equivalent concentration of a control rat IgM antibody, or media alone, and the effect of pretreatment on the binding of normal mesenteric lymph node lymphocytes was determined. As shown in Fig. 4, MECA-79 inhibited lymphocyte binding to peripheral lymph node HEV by 95%. Consistent with the expression of both mucosal and peripheral lymph node endothelial cell specificities in mesenteric lymph nodes, MECA-79 inhibited lymphocyte binding to HEV of this tissue by 54%. In contrast, lymphocyte binding to Peyer's patch HEV was not significantly effected. Pretreatment of tissue sections with the control IgM antibody had no effect on lymphocyte binding to the HEV of any tissue examined. A direct effect of MECA-79 on the lymphocytes was ruled out by pretreatment of either the tissue sections or the lymphocytes followed by extensive washing. Under these conditions,

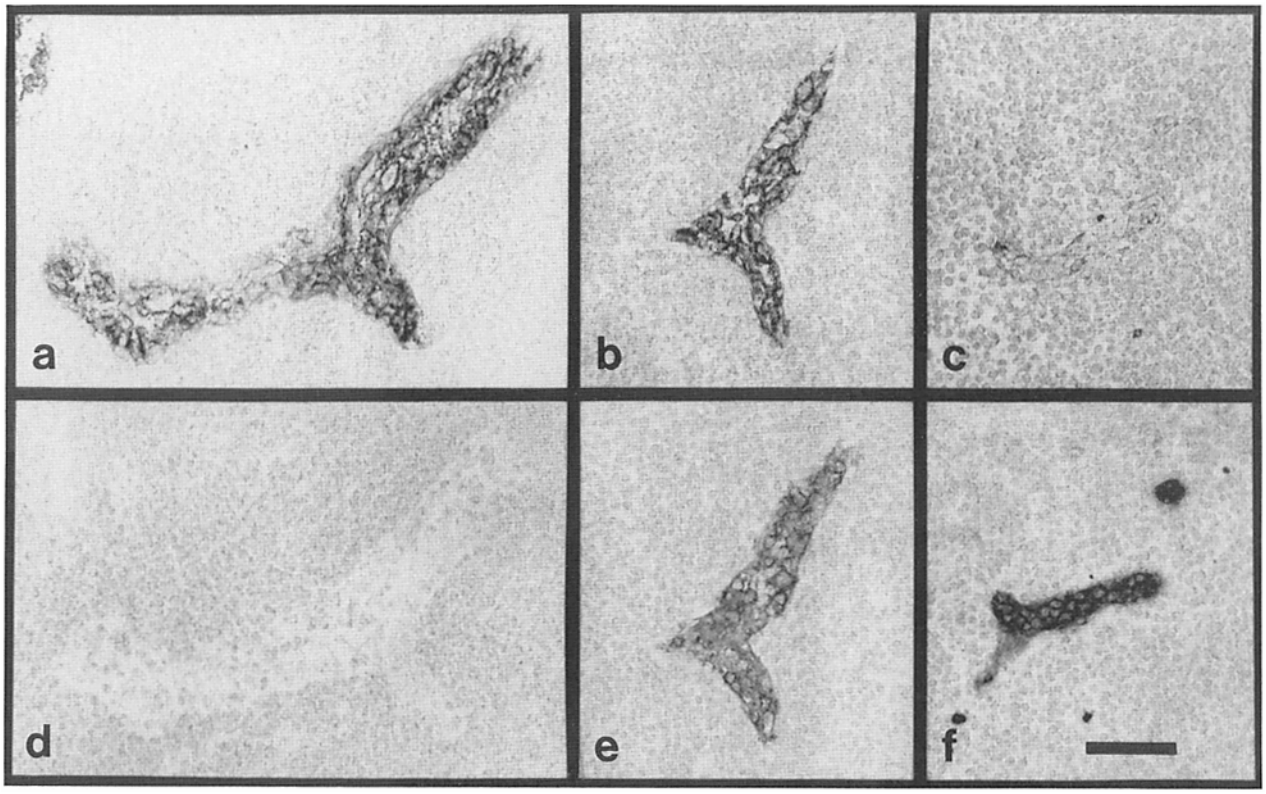

Figure 2. Analysis of the presence of the MECA-79 (*a–c*) and MECA-367 (*d–f*) antigens on the lumenal surface of HEV in peripheral lymph nodes (*a* and *d*) mesenteric lymph nodes (*b* and *e*) and Peyer's patches (*c* and *f*). Briefly, MECA-79 and MECA-367 were administered intravenously into mice. Recipients were sacrificed 15 min after injection and gently perfused with HBSS to remove unbound antibody from the vasculature. The lymphoid tissues of interest were then sectioned and membrane-associated MECA-79 and MECA-367 (IgM and IgG, respectively) were visualized by immunoperoxidase staining of serial sections with anti-rat IgM or anti-rat IgG specific reagents. MECA-79 was detected on the HEV of peripheral lymph nodes (*a*) and mesenteric lymph nodes (*b*), but was absent or only barely detectable at the lumenal cell surface of most HEV in Peyer's patches (*c*). Thus most of the MECA-79 antigen detected by direct immunohistology on Peyer's patch HEV (Fig. 1 *c*) is associated with the ablumenal aspect of the HEV rather than the lumenal cell surface. In contrast to MECA-79, MECA-367 stained HEV in the Peyer's patches (*f*) and in mesenteric lymph nodes (*e*), but not in peripheral lymph node HEV (*d*). *A*, *B*, and *C* are serial sections of *D*, *E*, and *F*, respectively. Bar, 50 μm.

pretreatment of tissue sections yielded results similar to those described above, while pretreatment of lymphocytes had no effect on binding.

Most normal lymphocytes appear to express homing receptors for both peripheral lymph node and mucosal HEV (Butcher, E. C., 1986; Stevens et al., 1982). However, subsets of lymphocytes have been identified that bind to HEV in a highly tissue specific manner, expressing functional homing receptors for only a single HEV class (Butcher et al., 1980; Schmitz et al., 1988). To determine the effect of MECA-79 on HEV binding by tissue-specific cells, we used two well-characterized lymphomas, TK1 and 38C13 (Butcher et al., 1980; Gallatin et al., 1983). MECA-79 effectively blocked the binding of the peripheral lymph node HEV-binding cell line, 38C13, to HEV in peripheral lymph nodes and mesenteric lymph nodes (Fig. 5). Conversely, MECA-79 had no effect on binding of the mucosal HEV-specific cell line, TK1, to either Peyer's patch or mesenteric lymph node HEV. Binding of TK1 cells to mesenteric lymph node HEV occurred on most vessel segments, including those segments staining intensely with MECA-79 (assessed immunohistologically in serial sections). MECA-79 therefore had no detectable effect on lymphocyte binding via mucosa-specific homing receptors, even when binding occurred to MECA-79$^+$ HEV. This excludes a nonspecific inhibitory effect of MECA-79 on cell binding to positive vessels. These findings indicate that the MECA-79 antigen either mediates or associates closely with endothelial cell elements mediating lymphocyte recognition of HEV via the peripheral lymph node–associated recognition system.

MECA-79 Inhibits Lymphocyte Homing In Vivo

Lymphocyte homing studies were used to assess the involvement of the MECA-79 antigen in tissue-specific lymphocyte extravasation under physiologic conditions in vivo. Normal mesenteric lymph node cells were labeled with ^{51}Cr and transfused intravenously into mice that had previously received i.v. injections of either MECA-79 or an isotype-matched control antibody. As predicted by our in vitro analyses, treatment with MECA-79 resulted in a substantial (64%) reduction in lymphocyte extravasation into peripheral lymph

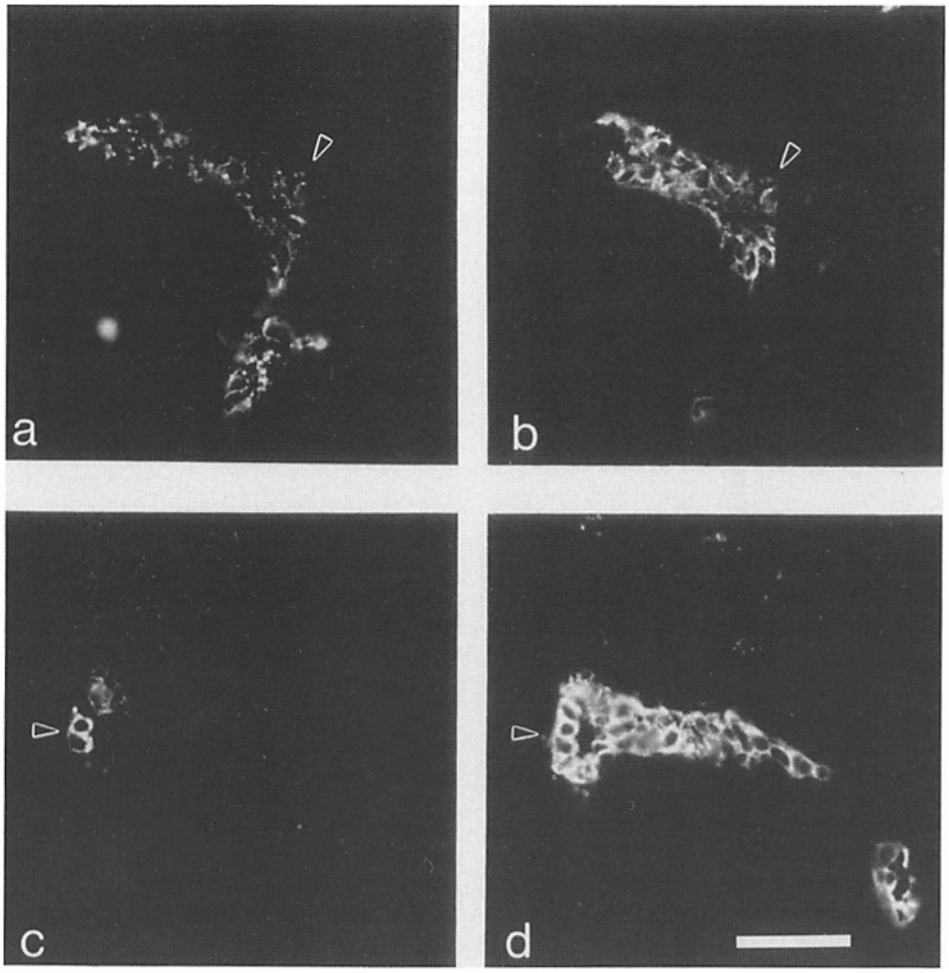

Figure 3. Exclusive expression of the MECA-79 or MECA-367 antigens by some HEV segments in the MLN, demonstrated by two-color immunofluorescence histology. Two selected areas of mesenteric lymph node tissue are shown, one (*a* and *b*) illustrating HEV segments positive for MECA-79 (*a*) but not for MECA-367 (*b*); the other (*c* and *d*) demonstrating HEC staining with MECA-367 (*d*) but not MECA-79 (*c*). The arrows identify reference cells or HEV segments within the sections that stain with both antibodies. Bar, 50 μm.

nodes, a partial (35%) blockade in lymphocyte localization to mesenteric lymph nodes, and no significant effect on homing to Peyer's patches (Fig. 6).

The tissue-selective expression of the MECA-79 antigen in combination with its functional involvement in tissue-specific lymphocyte HEV interactions both in vitro and in vivo qualify it as a "vascular addressin" (Streeter et al., 1988).

Table I. Tissue-specific Distribution of the MECA-79 Antigen (Peripheral Lymph Node Addressin) and the MECA-367 Antigen (Mucosal Addressin)

Tissue	Relative staining intensity with mAb	
	MECA-79	MECA-367
Peripheral lymph nodes	+++	–
Mesenteric lymph nodes	++—+++*	++—+++*
Peyer's patches	+‡	+++
Lamina propria of the gut	–	++§
Lactating mammary gland	–	++§
Normal skin	–	–
Skeletal muscle	–	–
Brain	–	–

MECA-79 and MECA-367 exhibit distinct distributions in lymphoid and extralymphoid tissues. The staining intensity in the different tissues ranges from undetectable levels (–) to high levels (+++).

* In the mesenteric lymph node, some HEV or HEV segments are negative.

‡ Staining of Peyer's patch HEV with MECA-79 is generally restricted to the ablumenal aspect of HEC. Some vessels express barely detectable levels of MECA-79 on their lumenal cell surface.

§ MECA-367 stains small vessels in both the lamina propria of the gut and the lactating mammary gland. These small vessels are morphologically distinct from the HEV that stain in organized lymphoid tissues.

Individual HEC Can Express Both Peripheral Lymph Node and Mucosal Addressins Simultaneously

We next asked whether expression of peripheral lymph node and mucosal addressins by HEC in mesenteric lymph nodes was mutually exclusive, or whether both addressins could be coexpressed by individual cells. Immunohistologic examination of mesenteric lymph node serial sections, described above, had suggested that both the mucosal addressin (defined by MECA-367) and the peripheral lymph node addressin (the MECA-79 antigen) might be coexpressed cytoplasmically by HEC in this tissue. Our two-color immunohistologic staining of tissues from animals that received mAbs intravenously (described above), suggested that individual HEC could also express both addressins at the cell surface simultaneously. To examine these questions further, a single-cell suspension of enzyme-dispersed mesenteric lymph node HECs was prepared, and endothelial cells (either acetone fixed cytospins or viable cells in suspension) were stained with MECA-367 and MECA-79 (or either antibody alone vs. control antibodies, as described in Materials and Methods). In keeping with the direct immunohistology and the two-color staining of perfused tissues, the mucosal and periph-

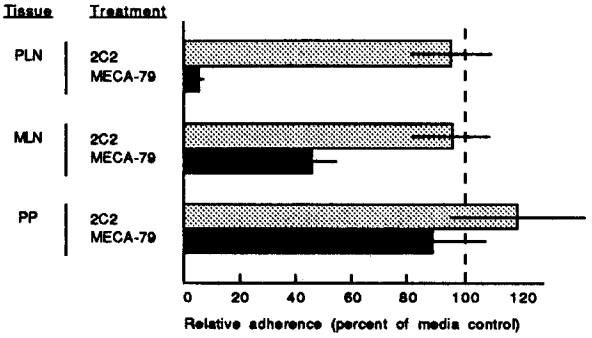

Figure 4. MECA-79 selectively inhibits the in vitro binding of lymphocytes to peripheral lymph node HEV. MECA-79 blocks the binding of normal lymphocytes to mesenteric lymph node and peripheral lymph node HEV, without significantly influencing lymphocyte binding to mucosal (Peyer's patch) HEV. The partial reduction in lymphocyte binding to mesenteric lymph node HEV is consistent with their expression of both peripheral lymph node and mucosal addressins. The isotype matched control antibody, 2C2, has no effect on lymphocyte binding. *PLN*, peripheral lymph node; *MLN*, mesenteric lymph node; and *PP*, Peyer's patches. Results are presented as percent of media control (±SEM).

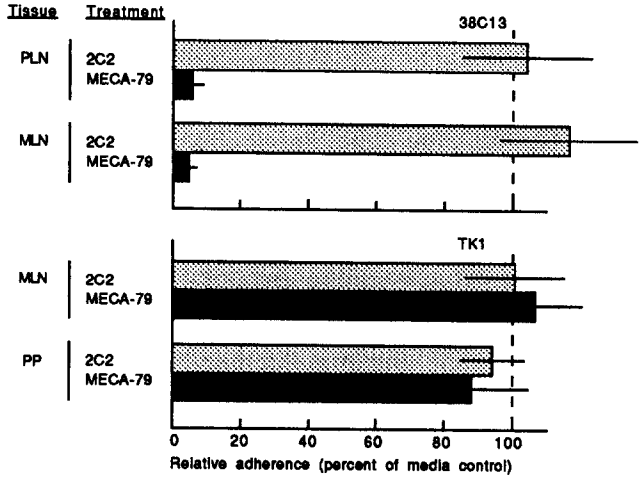

Figure 5. MECA-79 selectively inhibits peripheral lymph node-homing receptor-mediated lymphoma binding to HEV. MECA-79 blocks the binding of a peripheral lymph node-specific lymphoma (38C13) to peripheral lymph node and mesenteric lymph node HEV, but has no effect on the binding of TK1, a lymphoma that binds to HEV in mesenteric lymph nodes and Peyer's patches via mucosal-homing receptors. Cell binding was not influenced by the class-matched control antibody, 2C2. Results are presented as percent of media control (±SEM).

eral lymph node addressins appeared to be present on both the cell surface and in the cytoplasm of most mesenteric lymph node HEC, and as predicted above, a small fraction of cells stained exclusively with either MECA-79 or MECA-367. The results are summarized in Table II. Isotype-matched control antibodies were negative.

Discussion

We have described a mAb, MECA-79, that recognizes an antigen that is selectively expressed on the lumenal cell surface of vessels associated with lymphocyte extravasation into peripheral lymph nodes and that appears to be required for adhesion of normal and neoplastic lymphocytes to the HEV of these lymphoid organs. MECA-79 inhibits the binding of normal lymphocytes to peripheral lymph node HEV, and has no significant effect on lymphocyte binding to HEV in mucosa-associated lymphoid organs, the Peyer's patches. MECA-79 blocks 38C13, a lymphoma that selectively expresses peripheral lymph node-homing receptors, from binding to peripheral lymph node or to mesenteric lymph node HEV. In contrast, the antibody has no effect on the ability of TK1, a mucosal HEV-specific lymphoma, to bind to HEV in Peyer's patches or in mesenteric lymph nodes even when the HEV bound are MECA-79$^+$, thus ruling out a nonspecific effect of the antibody on cell adhesion to MECA-79$^+$ HEV. Finally, the i.v. administration of MECA-79 dramatically reduces the extravasation of normal blood-borne lymphocytes into peripheral lymph nodes in vivo. These data indicate that the antigen defined by MECA-79 is involved in the tissue-selective recognition of HEV by normal lymphocytes and transformed lymphoid cells, and is required for the homing of normal lymphocytes to peripheral lymph nodes in vivo. This vascular addressin must either function as a specific endothelial cell surface ligand for peripheral lymph node-homing receptors or be associated both physically and functionally with such a ligand. Proof of its role in lymphocyte interactions with endothelium will require molecular characterization, including the demonstration that as an isolated molecule it is capable of binding either lymphocytes or lymphocyte homing receptors with appropriate specificity.

A particularly interesting aspect of the expression of the MECA-79 antigen is that, although it is either absent or present at only low levels on the lumenal surface of most HEV in Peyer's patches, it is nonetheless expressed at the ablumenal aspect of many vessels in this site. While the level of resolution obtained in the current study does not permit definitive identification of the subcellular position of the ad-

Table II. Cell Surface and Cytoplasmic Expression of Addressins by Enzyme-dispersed Mesenteric Lymph Node High Endothelial Cells

	Percentage of HEC bearing peripheral lymph node and/or mucosal addressin	
Addressin expressed	Cell surface	Cytoplasmic
Peripheral lymph node only (MECA-79$^+$; MECA-367$^-$)	17	7
Mucosal only (MECA-79$^-$; MECA-367$^+$)	10	6
Both (MECA-79$^+$; MECA-367$^+$)	73	87

Immunohistologic examination of mesenteric lymph node serial sections suggested that the peripheral lymph node addressin and the mucosal addressin might be coexpressed by HEC in this tissue. To address this possibility, we used two-color immunofluorescence analysis to assess expression of these addressins both on the cell surface and within the cytoplasm of enzyme-dispersed mesenteric lymph node HEC (see Materials and Methods for details). While most of the isolated HEC appeared to express both peripheral lymph node and mucosal addressins on the cell surface as well as cytoplasmically, a small subset of cells exclusively express either the peripheral lymph node or the mucosal addressin.

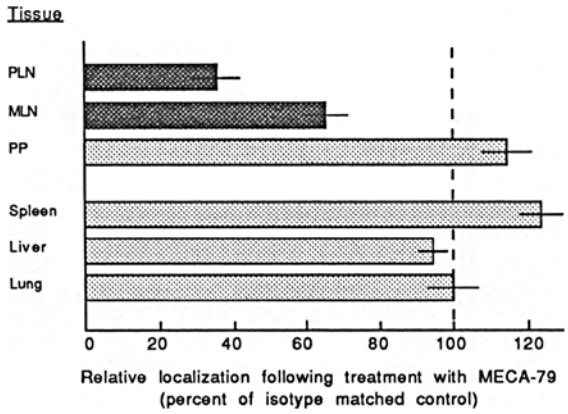

Figure 6. Inhibition of lymphocyte homing by MECA-79. MECA-79 was administered intravenously. 4 h later ^{51}Cr-labeled mesenteric lymph node cells were injected intravenously and 2 h later, recipients were sacrificed. Cell localization was determined by quantitating the cpm in the indicated tissues. Relative localization in MECA-79–treated recipients is expressed as a percent of that observed in recipients of an isotype-matched control antibody, OZ-42 (±SEM), and represents data pooled from two experiments with similar results. Three to four animals were included in each treatment group in these experiments. Lung tissue was harvested in only one of the two experiments. Intravenous injection of MECA-79 resulted in a substantial reduction in lymphocyte homing to peripheral lymph nodes, a partial reduction in lymphocyte extravasation into mesenteric lymph nodes and had no significant effect on homing to Peyer's patches.

dressin, the antigen could be present in either the ablumenal plasma membrane of HEC, or in association with pericytes and the surrounding extracellular material. This finding raises the possibility that the tissue-selective function of this molecule in lymphocyte trafficking may be regulated not only at the level of synthesis and expression, but also by selective control of translocation to the (lumenal vs. ablumenal) cell surface. Alternatively, localization to the lumenal surface may be determined by specific adhesive interactions with other endothelial cell products, perhaps residing in the carbohydrate-rich glycocalyx that is a prominent feature of HEC and that may be the initial point of lymphocyte–HEC contact (Anderson and Anderson, 1976).

The pattern of expression of the MECA-79–defined addressin contrasts with that of the mucosal vascular addressin, defined by mAb MECA-367, which is expressed at high levels on Peyer's patch HEV and is essentially absent from HEV in peripheral lymph nodes. The mucosal vascular addressin has been identified as a protein with a molecular mass of 58–66 kD. Use of MECA-79 to isolate the peripheral lymph node addressin for biochemical characterization has proven difficult, perhaps implying a relatively low affinity of this IgM antibody for its antigen.

The mesenteric lymph node, which receives its lymphatic drainage from the intestines, is unique among lymph nodes examined in that it contains HEV that express moderate levels of both peripheral and mucosal addressins. The present studies demonstrate that, although some mesenteric lymph node HEC bear only the antigens defined by MECA-79 or MECA-367, most express intracellular and cell surface addressins of both peripheral lymph node and mucosal types. External factors, perhaps arriving via lymph, may provide information regarding the position or tissue association of lymphoid tissues, and act on HEC to regulate addressin expression. The simultaneous expression of peripheral lymph node and mucosal addressins by individual endothelial cells within the mesenteric lymph node suggests that the endothelial cell response to such signals is not all or none. Instead, each of the addressins may be independently induced and regulated. The exclusive expression of either mucosal or peripheral lymph node addressin by a subset of HEC in the mesenteric lymph node, and indeed by whole HEV or large segments of HEV, may imply either differential responsiveness of HEC clones to mucosal (or peripheral lymph node) addressin-inducing factors or differential exposure of HEV segments to such factors.

In addition to tissue-specific signals, immune response–associated factors are also likely to be involved in regulating the expression of vascular addressins and other features of high endothelial cell differentiation. Hendriks and Estermans (1983) demonstrated that surgical interruption of the afferent lymph supply into rat peripheral lymph nodes causes HEV to lose their high-walled phenotype, appearing as flat-walled, nonfunctional vessels after two to three weeks. The characteristic high-walled phenotype was reinduced after the injection of antigen directly into the lymph node, suggesting that specific factors regulating HEC differentiation may be elaborated during immune responses. Indeed, it is likely that conventional cytokines play a role in inducing or maintaining the HEC phenotype. We have shown that a marker of high endothelial cell differentiation is induced on cultured capillary endothelial cells after treatment with interferon-gamma (Duijvestijn et al., 1986). Cytokines have also been shown to influence the adhesiveness of cultured endothelial cells for leukocytes (Bevilacqua et al., 1985; Cavender et al., 1986). These considerations suggest that the HEC phenotype, probably including expression of particular addressins, is determined by local environmental influences acting on maleable endothelial cells in the adult. It remains formally possible, however, that the specificity of the addressin expressed is determined by tissue-specific influences on endothelial cells during fetal development, and that these specificities are maintained in the adult.

Tissue-specific endothelial cell differentiation has been reported in other systems, as well. For example, Auerbach et al. (1987) and Alby and Auerbach (1984) presented evidence that the adhesive capacity of certain tumor cells for capillary endothelial cells from different sites correlates with their organ-selective metastatic patterns. Similarly, Nicolson et al., (1988) found that liver- vs. lung-colonizing sublines of the lymphosarcoma RAW117 preferentially bind endothelial cell monolayers derived from liver or lung, respectively. Risau et al. (1986) recently described a tissue-specific molecule that is found selectively on brain endothelium in the mouse, and demonstrated that its induction in embryonic tissue correlates with the development of the blood-brain barrier. Local cellular or microenvironmental influences also appear to be critically important in at least some examples of tissue-specific endothelial cell differentiation. For example, Janzer and Raff (1987) dramatically illustrated that astrocytes injected into the cornea induce local vessels to express a diffusion barrier similar to that of the blood/brain barrier.

Stoolman et al. (1984), Rosen et al. (1985), and Yednock et

al., (1987*a*, *b*) have provided evidence that endothelial cell surface carbohydrate components may play an important role in lymphocyte recognition of peripheral lymph node HEV. They found that both mannose-6-phosphate (Stoolman et al., 1984) and the mannose-6-phosphate-rich phosphomannan ester core structure (PPME) from *Hansenula holstii* (Yednock et al., 1987*b*) block the binding of lymphocytes to both rat and mouse peripheral lymph node HEV. Furthermore, PPME binds to peripheral lymph node HEV-binding lymphocytes or lymphoid cell lines, and this interaction is specifically inhibited by the anti-peripheral lymph node-homing receptor mAb, MEL-14 (Yednock et al., 1987*a*), suggesting that the peripheral lymph node-homing receptor functions as a mammalian lectin in binding tissue-specific carbohydrate (or carbohydrate-protein combined) determinant(s) on HEV. This interpretation is supported further by the observation that sialidase treatment inhibits lymphocyte binding to peripheral lymph node but not to mucosal HEV (Rosen et al., 1985). Thus, it will be important to examine the contribution of carbohydrate to the structure and function of the vascular addressins we have defined.

Our findings confirm the existence of two antigenically distinct and independently regulated vascular addressins, one supporting lymphocyte extravasation into mucosal lymphoid tissues (Streeter et al., 1988), and the other into peripheral lymph nodes. The existence of these two tissue-specific endothelial cell molecules has been postulated for several years, based not only on the tissue-selective homing properties of distinct lymphocyte subsets, but also on tissue-selective inhibitory effects of mAbs against lymphocyte "homing receptors" (Gallatin et al., 1983; Rasmussen et al., 1985; Chin et al., 1986; Jalkanen et al., 1987). Functional studies have implied the existence of at least one additional vascular addressin involved in lymphocyte homing to inflamed joints (Jalkanen et al., 1986*b*). Antigenic identification of this inflammation- or joint-specific endothelial cell addressin, and additional addressins that may be expressed in other tissues will be an important area for further investigation. In light of evidence that lymphocyte homing receptors of different specificities are closely related (Jalkanen et al., 1987), it is possible that endothelial cell antigens involved in lymphocyte-endothelial cell interactions will also prove to be very similar, perhaps representing a family of evolutionarily and structurally related molecules defining the position or tissue association of vessels throughout the body.

In conclusion, we have described the immunohistologic identification and tissue distribution of an endothelial cell differentiation antigen involved in lymphocyte recognition of peripheral lymph node HEV in the mouse. Examination of the nature and regulation of this peripheral lymph node addressin, and the mucosal addressin described previously (Streeter et al., 1988), should not only enhance our understanding of the mechanisms controlling lymphocyte entry into diverse tissues, but may also provide insight into the molecular and cellular processes directing cell-cell interactions and cellular positioning in other systems.

We thank R. Young for technical support; J. E. Streeter for preparing the illustrations; P. Verlota for assistance with Photography; and E. Berg, R. Bargatze, M. Jutila, R. Young, and L. Picker for their critical review of the manuscript.

P. R. Streeter was supported by a fellowship from the Bank of America Giannini Foundation and is currently a Senior Fellow of the California Division of the American Cancer Society (No. S-67-87). E. C. Butcher is an Established Investigator of the American Heart Association. This study was supported by National Institutes of Health grant GM 37734.

Received for publication 13 May 1988, and in revised form 22 July 1988.

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