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Interactions Between Endothelial Cells and Leukocytes

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We present evidence that specific receptors are utilized by neutrophils to control their interaction with endothelial cells at sites of acute inflammation and that these receptors are related if not identical to lymphocyte "homing receptors" for lymphoid tissue high endothelium. We speculate that such receptors play a fundamental but not exclusive role in controlling the extravasation and tissue localization of all bone marrow-derived nucleated cells. In addition, we emphasize the active role of endothelial cells in the process of lymphocyte migration and leukocyte extravasation. By the expression of as yet unidentified organ-specific determinants for lymphocyte recognition, endothelial cells control the exit of particular lymphocyte subsets into mucosal versus nonmucosal sites, thus helping to determine the unique features of mucosal versus nonmucosal immune responses. Furthermore, we argue that endothelial cells are exquisitely responsive to local immune reactivity and present evidence that specific lymphokines, including γ-interferon, play an important role in inducing postcapillary venules to express differentiated features required for the support of lymphocyte traffic into lymphoid organs and into sites of chronic inflammation. Leukocytes, endothelial cells, and probably other tissue cell classes appear to interact at multiple levels by a variety of mechanisms to regulate the local extravasation of immune effector cells.

Key words: leukocyte-endothelial interaction, cell-cell recognition, inflammation, neutrophil or lymphocyte migration, circulation, traffic, endothelial differentiation, antigens, high endothelial venules, interferon

The entry of leukocytes into the various tissues of the body is precisely regulated, controlled at least in large part at the level of leukocyte interaction with the vascular endothelium. The interaction of neutrophils with cultured endothelial cells in vitro has been studied by numerous investigators. Recent analyses have shown that IL-1 induces synthesis of neutrophil/monocyte adhesion sites in cultured human umbilical vein endothelium [1,2], and in vivo studies support a proposed role for IL-1 in controlling leukocyte-endothelial cell interaction during the acute inflammatory response [3]. Neutrophil-endothelial cell adhesion can be induced in seconds in vivo

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with mediators such as leukotriene B4 [4], however, suggesting that the IL-1-dependent mechanisms (which require protein synthesis [1]) are only one component of the adhesive interaction. The rapidity of adhesion demonstrated in model systems in fact supports a proposed role for neutrophil or endothelial cell "activation" by chemotactic or other factors in the initial adhesion event. Harlan [5] has recently reviewed many of these studies of the cellular and molecular events involved in neutrophil binding to cultured endothelium.

We and others have focused on another leukocyte-endothelial cell interaction, the selective binding of circulating lymphocytes to specialized postcapillary high endothelial venules (HEV) in lymph nodes and Peyer's patches. Lymphocyte-HEV recognition has been extensively studied both in vivo and in an elegant in vitro system developed by Stamper and Woodruff [6] in which rodent [6–8] or human [9] viable lymphocytes bind specifically to HEV in fresh frozen sections of lymph nodes or mucosal lymphoid tissues (see Fig. 1, for example). To outline briefly some of the major features of this interaction, lymphocytes bind to HEV via specific cell surface recognition elements—"homing receptors"—that have now been identified on mouse [10], rat [11, and R. Rasmussen, Y.-H. Chin, and J. Woodruff, personal communication], and human [12] lymphocytes by specific monoclonal antibodies. There are at

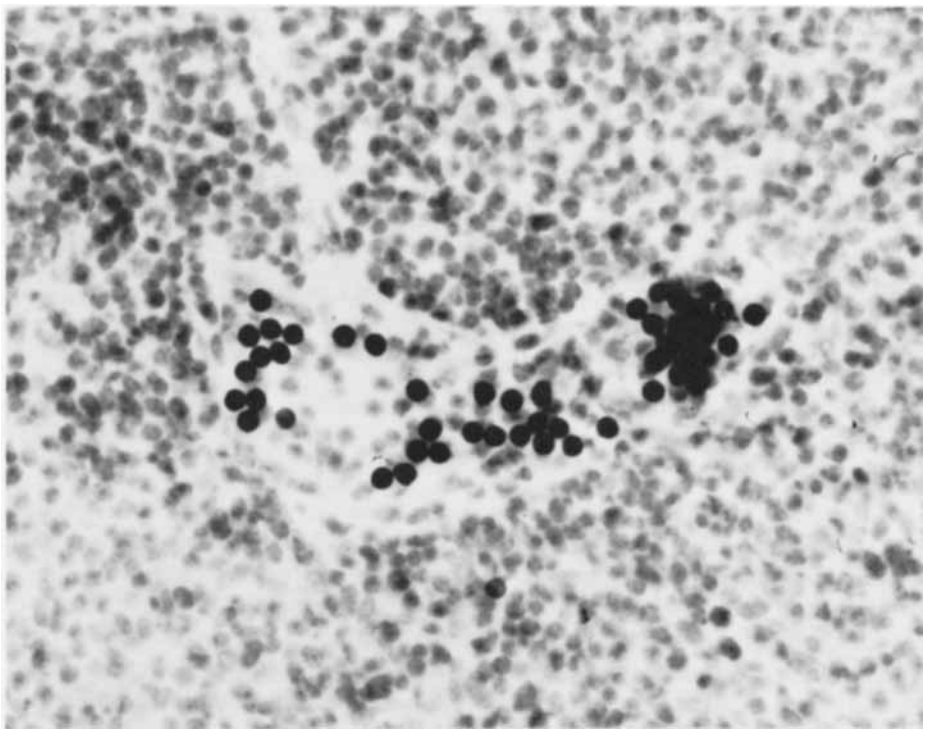

Fig. 1. Selective binding of lymphocytes to an HEV in a frozen section of a mouse lymph node. Lymphocytes were incubated at 7°C for 30 min on fresh frozen sections of a mouse lymph node, and bound cells were fixed to the section with glutaraldehyde in PBS. Adherent cells are easily visualized because they stain more heavily with thionine than the underlying fragmented cells in the tissue section. From Butcher et al [36], with permission.

least two classes of these recognition receptors, one mediating recognition of specific HEV determinants in lymph nodes and probably in the skin, the other directing lymphocyte interaction with mucosal endothelial cells. These receptors are exquisitely regulated during lymphocyte differentiation, controlling not only the overall capacity of lymphocytes to circulate but also directing their migration through particular lymphoid sites or sites of inflammation. The regulation of the traffic of particular effector cell populations by these receptors clearly plays an important role in determining the characteristics of local immune responses. The experimental definition of mouse and human homing receptors, and their importance in the physiology of lymphocyte circulation, have been adequately reviewed [13,14].

With this brief introduction, we will present some recent studies suggesting that lymphocytes and neutrophils, and perhaps all leukocytes, in fact share a common mechanism for endothelial cell recognition. In addition, we will describe the existence of unique antigens associated with the high endothelial venules mediating lymphocyte exit from the blood, and we will discuss studies suggesting that the characteristics of high endothelium can be induced in endothelial cells by factors elaborated during immune responses.

A COMMON LEUKOCYTE RECEPTOR SYSTEM FOR RECOGNITION OF ENDOTHELIUM?

All nucleated cells derived from the bone marrow must at some stage in their life cycles interact with endothelial cells in traveling from the bone marrow through the blood vasculature to their eventual tissue destinations. Marchesi and Gowans [15] addressed this phenomenon in their 1964 study of cellular traffic into lymph nodes, in which they showed that neutrophils and monocytes can be induced to extravasate via HEV when lymph nodes are acutely inflamed. Of course, monocytes and polymorphonuclear leukocytes generally extravasate through *flat-walled* postcapillary venules in inflamed tissues, and, unlike lymphocytes, they exhibit no preference for high endothelium even in inflamed lymph nodes. An additional level of specificity is exhibited in the timing of leukocyte extravasation during tissue inflammatory responses—neutrophils usually precede monocytes, and lymphocytes generally become a significant component of the infiltrate only after 1–3 days. Different leukocytes thus exhibit both temporal and site selectivity in migrating from the blood. What, then, leads to the suggestion that they share a common endothelial cell recognition system?

Neutrophils and Lymphocytes Employ Similar Receptors for Endothelium

The interaction of neutrophils with endothelial cells assumed a special interest for us when we found that, in both the mouse and human systems, neutrophils stain intensely with antibodies against lymphocyte "homing receptors" for HEV. Mouse neutrophils stain as brightly as lymphocytes with MEL-14, a rat monoclonal that selectively blocks mouse lymphocyte binding to lymph node but not to Peyer's patch HEV and thus appears to define a unique determinant associated with the lymph node HEV receptor [10]. Human neutrophils stain with Hermes-1, a rat monoclonal antibody that appears to define a common epitope shared by human lymphocyte surface receptors for lymph node and for mucosal HEV [12,16]. The staining of neutrophils with these antibodies forced us to hypothesize that neutrophils use surface receptors similar or identical to lymphocyte surface homing receptors in interacting

with endothelial cells at sites of inflammation. Based on this hypothesis, we made several predictions:

1. The MEL-14/Hermes-1-defined neutrophil antigens should be biochemically similar to the lymphocyte homing receptors. In fact, in preliminary studies the immunoprecipitated receptors from neutrophils migrated just detectably more slowly in SDS-PAGE than their lymphocyte counterparts, confirming the similarity of the lymphocyte and neutrophil antigens and making it unlikely that Hermes-1 and MEL-14 are simply cross reacting with unrelated molecules on the neutrophils.

2. Neutrophils should bind in vitro to endothelial cells. In fact, many groups have studied neutrophil binding to endothelial cells previously, generally employing cultured endothelial cells. The physiologic relevance of such experiments has been questioned, however, Thus we elected to utilize the in vitro frozen section assay of binding to HEV. Because 1) there is now overwhelming evidence demonstrating the correspondence of in vivo homing with lymphocyte binding to HEV in the frozen section assay [6,7,9,13,14] and 2) it is clear that neutrophils *can* utilize HEV to extravasate into inflamed lymphoid tissues [15, personal observations], we asked whether neutrophils could bind to HEV in lymph node frozen sections. In fact, we have been able to demonstrate that human peripheral blood neutrophils, as well as mouse bone marrow neutrophils identified by chloroacetate esterase staining or by specific staining with the neutrophil-specific antibody RB2-8C5 (a generous gift of R. Coffman, DNAX Research Institute, Palo Alto, California), bind quite well to HEV in our standard in vitro assay system.

3. If this neutrophil-HEV interaction is in fact mediated by homing receptor-related molecules, then MEL-14 should block neutrophil-endothelial cell binding. Indeed, mouse bone marrow neutrophils precoated with MEL-14 no longer bind to lymph node HEV. Precoating with a class-matched antibody against the leukocyte common antigen T200, which is more abundant on neutrophils than the MEL-14 antigen, has no effect. Even more interesting is the observation that MEL-14 fails to inhibit neutrophil binding to Peyer's patch (mucosal lymphoid) HEV; this suggests that, as has been shown for lymphocytes [10,17], neutrophils must use an antigenically and functionally distinct receptor system (not recognized by MEL-14) to interact with mucosal high endothelium.

It could be argued, of course, that this interaction with specialized high endothelial venules is not in fact typical of neutrophil-endothelial cell interaction in most sites of acute inflammation. It was thus crucial to ask whether MEL-14 could also block neutrophil-endothelial cell interaction in vivo, that is, whether MEL-14 could selectively inhibit the migration of precoated neutrophils from the vascular compartment into an induced nonlymphoid site of acute inflammation. Inflammation was induced by implanting a collagen-based sponge, soaked in supernatant from a culture of Escherichia coli, under the skin of anesthesized mice. Three hours later, during the peak of neutrophil extravasation, fluorescent-labeled [18,19] neutrophils were injected intravenously and allowed to migrate to the inflammatory site for a period of 30–60 min. The ability of sample neutrophil preparations to migrate from the vascular compartment into the inflammatory site was assessed as the ratio of neutrophils arriving in the sponge implant to the concentration of neutrophils in the vascular compartment. Precoating of sample neutrophils with MEL-14, but not with class-matched anti-T200 antibodies, resulted in 50–70% inhibition of neutrophil extravasation in different experiments. Taken together, these studies clearly argue that a

molecule similar or identical to the lymphocyte homing receptor is employed by neutrophils to interact with endothelial cells at sites of inflammation.

These considerations raise an obvious question: If neutrophils and lymphocytes both use a MEL-14/Hermes-1-defined receptor system to interact with endothelial cells, then why don't lymphocytes migrate along with neutrophils into sites of acute inflammation, and, conversely, why do neutrophils fail to migrate into uninflamed lymph nodes? In that MEL-14 and Hermes-1 react with both lymphocyte and neutrophil receptors, these receptors must be very closely related and thus might recognize the same determinants on endothelial cells. To explain the specificity of endothelial cell interaction in the context of this model, we must postulate the existence of a "second signal"—separate factors operating on the lymphocyte or neutrophil in the vascular lumen to enhance binding or to "trigger" use of receptors at appropriate sites. Such hypothetical factors could be soluble or could in fact operate by binding to the endothelial cell surface, interacting there with the specific leukocyte partner. In the case of neutrophils, traditional activators or chemotactic agents might play an important role. Alternatively, the endothelial cells themselves could be activated to secrete preexisting pools of neutrophil-triggering factors. Similar *lymphocyte-specific* factors might be produced in lymph nodes and at sites of chronic inflammation, and in this regard it is intriguing that Paul Andrews and his coworkers [20–22 and personal communication] have described a sulphated macromolecule produced by high endothelial cells that copurifies (in preliminary size and ion exchange fractionations) with a factor(s) able to enhance lymphocyte binding to HEV substantially in the in vitro assay.

Another possibility is that the lymphocyte and neutrophil receptors, although antigenically and structurally related, in fact have different recognition domains and thus interact with unique neutrophil- or lymphocyte-specific endothelial cell determinants that are differentially expressed in lymph nodes vs inflammatory sites. In this model, binding of neutrophils to HEV in the in vitro frozen section assay could be explained by exposure of an internal pool of neutrophil binding sites during sectioning of the endothelial cells or by conformational activation of preexisting cell surface neutrophil-specific determinants during tissue processing. The recent observation by Rosen and his colleagues (see below) that mannose-6-phosphate specifically inhibits lymphocyte receptors for lymph node HEV suggests the question of whether the specificity of neutrophil and lymphocyte receptors is similar—if the mannose-6-P inhibition proves to be lymphocyte-specific, neutrophils presumably express a distinct recognition specificity.

Other studies suggest that another class of neutrophil membrane glycoproteins also play an important role in neutrophil-endothelial interactions. Antibodies against certain epitopes of a family of leukocyte surface glycoproteins, termed the *LFA-1/Mo1 complex,* inhibit various leukocyte interactions including cytotoxic T-cell/target-cell binding, neutrophil adherence to various substrates, and lymphocyte homotypic aggregation [23, others reviewed in 24–26]. Patients whose leukocytes lack this complex apparently exhibit normal lymphocyte circulation but suffer from a deficiency in *neutrophil* localization to sites of tissue insult. The LFA-1/Mo1-deficient neutrophils display a deficiency in many functions requiring adhesive interactions, including self-aggregation, binding to plastic or glass, and chemotaxis [24–26]. Patients' neutrophils adhere normally to cultured endothelial cells, though, and are thought to marginate normally in vivo as well [25,27]; however, they fail to display

the enhancement of binding exhibited by normal neutrophils after activation [28]. Interestingly, antibodies against mouse LFA-1 cause partial (if variable) inhibition of lymphocyte binding to HEV, as well (Alf Hamann, A.D., and E.C.B., unpublished observations). Involvement of the LFA-1/Mo1 complex in multiple cellular interactions suggests that it plays an important but nonspecific role in enhancing adhesive events. Thus we suggest that the leukocyte-endothelial receptor system defined by MEL-14 mediates the specific *recognition* of endothelial cells by lymphocytes and neutrophils but might recruit accessory adhesive mechanisms (possibly including the LFA-1 complex) in cementing the interaction. The selective deficiency of neutrophil function in LFA-1/Mo1-deficient patients might reflect the involvement of this complex in the many cell-cell and cell-substrate interactions (in addition to endothelial cell binding) that are required for neutrophil chemotaxis through connective tissues in vivo.

Receptor Expression by Other Leukocytes

Although we have focused principally on neutrophils, similar receptors might operate to regulate the extravasation of other leukocyte types, eg, monocytes, basophils, eosinophils, mast cells, natural killer (NK) cells, and perhaps even dendritic cells and related antigen-presenting cells. In this regard, it is pertinent to mention 1) that the mast cell tumor P815 expresses specific functional recognition for Peyer's patch HEV in the mouse [10], 2) that some monocyte cell lines in the mouse and peripheral blood monocytes in the mouse and human stain intensely with antihoming receptor antibodies [D.L., personal observation], 3) and that, in collaboration with Craig Reynolds from the Frederick Cancer Research Institute, we have observed binding of human and rat large granular lymphocytes (presumptive NK cells) to HEV in vitro and staining of human large granular lymphocytes with Hermes-1. Thus the leukocyte surface molecules defined by MEL-14 in the mouse system, and by Hermes-1 in the human, might mediate a common mechanism of leukocyte-endothelial cell interaction serving to control leukocyte extravasation in appropriate tissue sites, either in lymphoid organs in the case of lymphocytes or in particular inflammatory lesions following specific triggering or activation of other leukocyte types.

THE NATURE OF HIGH ENDOTHELIUM

In the second part of this paper, we will focus on the endothelial cell side of leukocyte-endothelial cell interactions and particularly on the high endothelium in lymph nodes and Peyer's patches. Certain clonal transformed lymphoid cell lines (as well as certain normal mouse lymphocyte subsets) can be shown to discriminate almost totally between the HEV in lymph nodes and those in mucosal lymphoid organs (Peyer's patches in mice, appendix in human [29,30]). Thus HEV in lymph nodes and in the mucosae-associated lymphoid organs must bear unique recognition determinants for migrating lymphocytes.

Probable Role of Carbohydrates in Lymphocyte-HEV Interaction

The most provocative studies relating to the nature of these determinants have been carried out by Steve Rosen and his colleagues Ted Yednock and Lloyd Stoolman at UCSF [31,32, personal communication]. They began with the hypothesis that lymphocyte-HEV recognition is mediated by mammalian lectins. They screened a

large number of monosaccharides and phosphorylated monosaccharides for their capacity to interfere with lymphocyte binding to lymph node HEV in the rat and mouse and found that mannose-6-phosphate at millimolar concentrations, as well as very low levels of a mannose-6-phosphate-rich mannan from Hansenula holstii, blocks lymphocyte binding to HEV. In addition, the mannan, when bound to fluorescent beads, was found to bind in a mannose-6-phosphate-inhibitable manner to mature peripheral B and T cells but not to thymocytes (which lack homing receptors for HEV). Together these initial findings suggested that mannose-6-phosphate residues interfere directly with the lymphocyte homing receptor for lymph node HEV. In confirmation of this, when lymphocytes were precoated with MEL-14, binding of the mannan was inhibited [T. Yednock et al, in preparation]. Other antilymphocyte antibodies tested had no effect.

These results clearly suggest that the MEL-14-defined homing receptor can function as a lectin with specificity for mannose-6-phosphate. Thus lymphocyte receptors for lymph node and Peyer's patch HEV might be mammalian lectins whose specific ligands would therefore be particular oligosaccharides expressed on lymph node or mucosal HEV. The formal possibility remains, however, that the interaction of mannose-6-phosphate with the MEL-14-defined receptor represents a specific but unphysiological "cross reactivity" of the recognition domain; that is, mannose-6-phosphate might interact selectively with the homing receptor yet nonetheless be unrelated to the physiologic ligand on endothelial cells. Thus it will be essential to pursue more direct approaches to identifying the native ligand on HEV.

Regulation of the Differentiated Features of High Endothelium

Defining the molecular nature of the endothelial cell ligand, of course, would still represent only a partial explanation for what makes an HEV an HEV. It will be important, for example, to determine whether HEV represent a distinct, specialized lineage of endothelial cells or rather whether they are induced from otherwise normal, flat endothelial cells by specific signals from the surrounding micro- or macroenvironment. In fact, there is evidence to suggest that both the specific endothelial surface determinants for lymphocyte recognition and the overall morphological and functional features of HEV are induced by external factors.

The external regulation of organ-specific endothelial cell determinants is suggested by the observation that the mesenteric node, in contrast to all other lymph nodes examined, contains HEV that are capable of binding both lymph node HEV-specific and mucosal (Peyer's patch) HEV-specific lymphoid cells [28]. Thus mesenteric node HEV appear to express both mucosal and peripheral-type endothelial cell determinants. The mesenteric node is also unique in that it is the only lymph node to receive its afferent lymph supply entirely from a mucosal surface, the intestines. Thus it seems attractive to suggest that specific humoral or cellular factors derived from the mucosal surface are responsible for induction of mucosal specificity in endothelial cells. As an aside, it is intriguing that one can identify segments of HEV within mesenteric node frozen sections that bind exclusively lymph node HEV- or Peyer's patch HEV-specific tumor cells [E.C. Butcher and I.L. Weissman, unpublished observations]. Such specificity is maintained on serial sections, suggesting that the induction of specific determinants is clonally or at least microregionally determined. In that the endothelial cell mechanisms for organ-specific lymphocyte localization appear to be in place at birth in mice, it seems reasonable to postulate that factors

involved in controlling the selection of specific determinants are not immune-related but rather are derived from the ectodermally versus endodermally derived epithelial surfaces themselves.

On the other hand, several considerations suggest that the morphologic and overall functional characteristics of high endothelium, including the ability to support lymphocyte exit from the blood, are induced by factors associated with local immune responses and/or chronic inflammatory stimuli. For example, vessels with the characteristic morphologic appearance of HEV, which appear histologically to be transmitting lymphocytes from the blood, are frequently identifiable in sites of chronic tissue inflammation in humans [see, eg, 33], and it has been shown that HEV-like vessels can be induced in sites of chronic inflammation in the guinea pig as well [34]. Hendriks and Eestermans [35] have approached this question from the other side. They showed that, following surgical interruption of the afferent lymph supply into rat lymph nodes, HEV lose their plump endothelium and their capacity to support lymphocyte extravasation, reverting to flat-walled, nonfunctional venules over the course of 2–3 weeks. Furthermore, the loss of functional HEV was reversed by injecting antigen directly into the lymph node, suggesting that specific factors inducing or maintaining "HEV-ness" are in fact elaborated during immune responses.

Although these observations clearly support the proposal that high endothelial cells merely represent an inducible and reversible stage of differentiation of conventional endothelial cells, it is still possible that the apparent de novo appearance of HEV in sites of inflammation results instead from the seeding of the local microvasculature by cells of a separate high endothelial cell lineage from other sites or from a quiescent local precursor. To rule out this possibility, it would be necessary to start with a uniform population of endothelial cells from a non-HEV-containing source and then induce in these cells specific high endothelial cell characteristics using inflammatory or other mediators. In an initial experiment along these lines, described below, we have employed specific antibodies as markers of HEV cell differentiation. Before presenting this experiment, the derivation of the antibodies employed must be described.

High Endothelial Cell Antigens Defined by Monoclonal Antibodies

To develop tools to understand better the differentiated state of high endothelium, and of course with the hope of identifying probes for functional HEV determinants, we immunized rats with lymphocyte-depleted mouse lymph node stroma. Immunoperoxidase staining of lymph node frozen sections was employed for screening, and antibodies reacting with endothelial cells were selected. Three of these were chosen for further characterization. Antibody MECA-20 reacts with all endothelial cells in both large and small vessels. MECA-217 stains the endothelium of large (elastic) arteries in all tissues examined but among small vessels it appears selectively in lymphoid tissues, staining high endothelial venules as well as a subset of other small vessels in lymph nodes and in Peyer's patches (possibly flat-walled, postcapillary venules) but not small vessel endothelial cells in nonlymphoid tissues. The most interesting antibody, however, is MECA-325, which demonstrates exquisite specificity for high endothelial cells themselves (see Fig. 2).

What is the significance of these antibodies? Given the remarkable specificity of MECA-325 (and to a lesser extent MECA-217) for lymphoid organ venules, one cannot avoid speculating that they are in some way involved in the specialized function

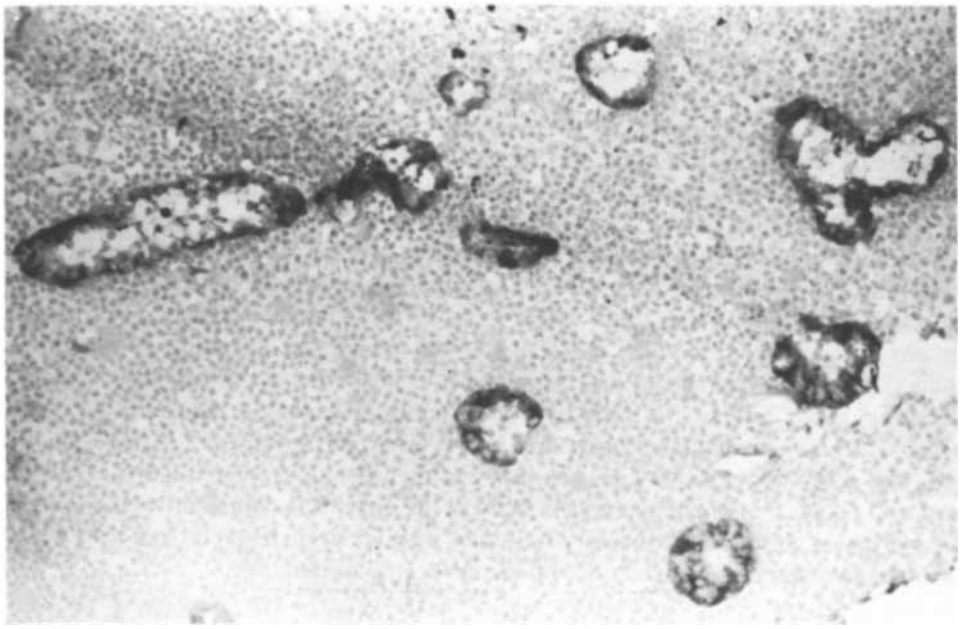

Fig. 2. Immunoperoxidase staining of a mouse lymph node section with monoclonal MECA-325 illustrating the specificity of this antibody for high endothelial venules. The section is weakly counterstained with hematoxylin.

of these vessels, ie, mediating lymphocyte exit from the blood. We have no evidence as yet that this is the case, however. These antibodies do not block lymphocyte binding to HEV in vitro. At the very least, these antibodies will provide powerful tools for identifying and isolating HEV cells from collagenase/dispase-digested lymph node stroma, thus allowing in vitro characterization of growth requirements of HEV, of their cell surface proteins, of their active role in lymphocyte-HEV interaction, and of the susceptibility of HEV cell surface recognition determinants for lymphocytes to specific proteases, glycosidases, etc. Finally, because of the nearly absolute specificity of these antibodies (particularly 325) for lymphoid postcapillary venules, it is clear that the antigenic determinants they define represent powerful and precise markers of high endothelial cell differentiation. They have thus allowed us to address the issue raised above, asking whether these specific HEV-associated antigens can be induced in endothelial cells from nonlymphoid sources.

In collaboration with Alain Schreiber, Syntex Corporation, cultured small vessel endothelium from mouse lung was incubated for 3 days with a panel of lymphokines and growth factors. γ-Interferon specifically induced high levels of the HEV-associated antigens MECA-325 and MECA-217 without effecting the constitutive expression of MECA-20, and immunofluorescence microscopy revealed that essentially all cells in the culture were induced. IL-1, IL-2, epidermal growth factor, and endothelial cell growth factors had no effect on antigen expression. This experiment provides the first direct evidence that at least some of the specialized features of differentiated high endothelium can be induced in nonlymphoid endothelial cells by lymphokines. M. Bevilacqua et al [1] and others [2] have reported that IL-1 is able to induce a massive

increase in the binding of certain mononuclear and polymorphonuclear cells to cultured human umbilical vein endothelium. Thus not only γ-interferon but IL-1 and possibly other lymphokines and tissue factors might each control specific differentiated features of endothelial cells regulating leukocyte interactions in lymphoid tissues or in sites of acute or chronic inflammation.

Thus we are beginning to define and dissect a specific interrelationship between local tissue damage or local immune responses and the capacity of regional vessels to support leukocyte extravasation from the blood. Acute tissue damage might release IL-1 (which can be produced by many cell types), resulting in endothelial cell differentiation to enhance neutrophil extravasation. In the case of chronic inflammation, one can imagine a cycle of events beginning with local antigenic insult, the resulting response of a very few lymphocytes and macrophages that are always present in any tissue, the generation of leukocyte-derived factors including γ-interferon, induction of high endothelial phenotype and function in local postcapillary venules, resulting in an increase in leukocyte extravasation leading to enhanced immune responsiveness, lymphokine generation, HEV induction, lymphocyte extravasation, etc. The continued positive feedback between the immune response and the maintenance of enhanced lymphocyte traffic through the induced endothelium would lead to a progressive chronic inflammatory infiltrate, with the cycle being broken only when the antigenic stimulus or agent driving the cycle was cleared.

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