

# Dual Binding Capacity of Mucosal Immunoblasts to Mucosal and Synovial Endothelium in Humans: Dissection of the Molecular Mechanisms

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## Summary

Lymphocytes continuously migrate throughout the body in search of antigens. Virgin lymphocytes recirculate freely between the blood and different lymphatic organs, whereas immunoblasts extravasate preferentially into sites similar to those where they initially responded to antigen. Tissue-specific extravasation of lymphocytes is largely controlled by distinct lymphocyte surface receptors that mediate lymphocyte binding to high endothelial venules (HEV). In the present study, the molecular mechanisms determining the specificity of human mucosal (lamina propria) lymphocyte binding to different endothelial recognition systems were analyzed. Mucosal immunoblasts adhered five times better than small mucosal lymphocytes to mucosal HEV. Importantly, mucosal immunoblasts also bound to synovial HEV almost as efficiently as to mucosal HEV, but they did not adhere to peripheral lymph node HEV. To study the impact of different homing-associated molecules in this dual endothelial binding, we used a gut-derived T cell line and freshly isolated mucosal immunoblasts. Both cell types expressed integrins  $\alpha 4$ ,  $\beta 1$ ,  $\beta 7$ , and lymphocyte function associated antigen 1 (LFA-1), and were CD44 positive, but practically L-selectin negative. Binding of mucosal immunoblasts to mucosal HEV was almost completely abolished by pretreatment with anti- $\beta 7$  monoclonal antibodies, but it was independent of  $\alpha 4/\beta 1$  function. In contrast,  $\alpha 4/\beta 1$  partially mediated immunoblast adherence to synovial HEV, whereas  $\alpha 4/\beta 7$  had only a minor role in adherence of blasts at this site. CD44 and LFA-1 contributed to HEV-binding both in mucosa and synovium. Taken together, this is the first report that demonstrates a critical role for  $\alpha 4/\beta 7$  in the binding of gut lymphocytes to mucosal venules in humans. Moreover, a hitherto unknown interaction between mucosal effector cells and synovial endothelial cells was shown to be only partially mediated by the currently known homing receptors. The dual endothelial binding capacity of mucosal blasts may help to explain the pathogenesis of reactive arthritis not uncommonly associated with inflammatory and infectious bowel diseases.

Lymphocyte migration is an essential component of an effective immune defense system allowing each lymphocyte with a unique antigen receptor to travel throughout the body to seek for specific stimulus (1). Virgin lymphocytes recirculate continuously from one anatomically distinct lymphatic organ to another via the lymph and blood. When a lymphocyte encounters its specific antigen in a suitable microenvironment it halts and starts to proliferate and differentiate. Finally, a mature immunoblast leaves the regional lymphatic tissue via lymphatics and enters the circulation. The migratory capacity of this effector cell has changed dramatically. It no longer recirculates between different lymphatic organs, but instead, specifically extravasates into sites similar to that

where it initially became activated (1). For example, a naive lymphocyte that responds to an intestinal antigen at mucosa-associated lymphatic tissues differentiates in Peyer's patches. Thereafter, the antigen-activated immunoblasts enter the blood circulation via the lymphatic system and specifically home back to the mucosal sites (1, 2). In humans, at least four functionally distinct endothelial binding specificities have been defined that direct lymphocyte trafficking to gut-associated lymphatic tissues, inflamed synovia, peripheral lymph nodes, and skin (3-5).

When lymphocytes leave the blood, they first bind to the endothelial lining of vessels and then transmigrate between the endothelial cells into the underlying tissue. Most lym-

phocyte trafficking occurs via specialized endothelial cells of postcapillary venules that are called high endothelial venules (HEV)<sup>1</sup> because of their characteristic morphological appearance (6). Several lymphocyte surface receptors mediate lymphocyte-endothelial cell interactions by binding to their ligands on HEV (7-10). Integrins are heterodimeric molecules that contain one  $\alpha$  chain noncovalently associated with a  $\beta$  chain (11). Integrin  $\alpha 4$  subunit can associate with two distinct  $\beta$  chains,  $\beta 1$  and  $\beta 7$ . In mouse,  $\alpha 4/\beta 7$  heterodimer (formerly called LPAM-1) is the principal homing receptor that mediates tissue-specific binding of lymphocytes to venules in gut-associated lymphatic tissues by interacting with endothelial mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (12-16). The other  $\alpha 4$  containing lymphocyte integrin,  $\alpha 4/\beta 1$ , does not bind to MAdCAM-1, but it can recognize vascular cell adhesion molecule 1 (VCAM-1) which is an inducible endothelial adhesion molecule (16-18). Other known tissue-selective homing receptors are L-selectin which guides lymphocyte trafficking to peripheral lymph nodes (19, 20), and cutaneous lymphocyte antigen (CLA) which supports lymphocyte binding to vessels in inflamed skin (21). Other lymphocyte homing-associated molecules, like CD44 (22) and LFA-1 (CD11a/CD18; 23) are important for lymphocyte-endothelial cell interactions (24-26), but they mainly function in a nontissue-specific manner.

Lymphocyte recirculation pathways have been extensively studied in animal models. In those studies it has been shown that an *in vitro* frozen section assay predicts very well the actual *in vivo* homing capacity of cells (27). In humans, there is only a limited amount of information available on the endothelial binding of mucosal lymphocytes. We have earlier shown that small lymphocytes isolated from the lamina propria of gut bind to HEV in both mucosa and peripheral lymph nodes. In contrast, mucosal immunoblasts do not adhere to peripheral lymph node HEV at all, although they bind extremely well to mucosal HEV (28). However, in humans essentially nothing is known about the molecular mechanisms that mediate the interactions between mucosal lymphocytes and vascular endothelium. We also wanted to analyze binding of mucosal cells to synovial HEV, since several clinically important infectious and inflammatory bowel diseases are not uncommonly followed by reactive arthritis (29, 30), and we reasoned that capacity of mucosal effector cells to home into joints could be a connection between the two manifestations of these diseases. Therefore, in the present work we isolated lamina propria lymphocytes (LPL) from human bowel and studied their endothelial cell recognition specificities. We found that mucosal immunoblasts were able to bind almost equally well to both mucosal and synovial HEV. When the function of each known homing-associated molecule was studied individually, it was evident that immunoblasts used a distinct

set of homing receptors for binding to mucosal and synovial HEV.

## Materials and Methods

**Isolation of LPLs.** 11 normal gut specimens were obtained from patients who were operated on for premalignant or malignant gut lesions (polyps and adenocarcinomas) or for reconstructive abdominal surgery. The tissue pieces used in these studies were taken from the macroscopically and microscopically normal area of the removed tissue. LPL were from colon in 3/11 samples, from small bowel (ileum) in 2/11, and in the rest (6/11), both small and large bowel LPL were pooled to provide sufficient number of cells. We have shown earlier that no evident differences exist in the endothelial cell binding properties of LPL isolated from small and large bowel (31). LPL were isolated essentially as previously described (28, 32). In brief, mucosa of the gut was dissected free, and epithelial cells were detached by treatment with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free HBSS containing 5 mM EDTA. After washings, LPL were released from the remaining tissue pieces by an overnight incubation in RPMI 1640, 10% AB-serum, and 20 U/ml collagenase type II (from *Clostridium histolyticum*; Sigma Chemical Co., St. Louis, MO). Mononuclear cells were collected using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Finally, macrophages were removed from the mononuclear cell population by allowing the cells to adhere to plastic for 2 h at 37°C in the presence of serum. The nonadherent lymphocytes were collected and used for further studies. Isolated cells were  $\geq 98\%$  lymphocytes as judged by leukocyte markers. PBL were isolated from venous blood of healthy adults by Ficoll-Hypaque. All cell and tissue sampling protocols used in this study were approved by institutional review boards.

**Mucosal T Cell Lines.** IL-2-dependent T cell lines were established as described earlier (33). Briefly, isolated LPL were cultured in IL-2 (1,000 U/ml recombinant human IL-2; a gift from P. Kar-nani, Orion-Farmos, Turku, Finland) containing RPMI 1640 medium that was supplemented with 4 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 20% human AB-serum, and 1  $\mu\text{g}/\text{ml}$  PHA for 4 wk at 37°C in 5%  $\text{CO}_2$ .

**Antibodies.** Antibodies used in this study are listed in Table 1. mAb HP 2/1 recognizes the  $\alpha 4$  chain of very late activation antigen 4 (VLA-4) (34), and it does not cross-react with the  $\alpha$  chain of intraepithelial lymphocyte integrin ( $\alpha\text{IEL}$ ) (Butcher, E., unpublished observations). mAb HML-1 against  $\alpha\text{IEL}/\beta 7$  recognizes either a combinatorial epitope composed of both chains of this integrin or an epitope that is conformation specific (35). Anti-mouse  $\beta 7$  mAbs were produced by immunizing rats with TK1 cells and screened for their ability to block TK1 cell aggregation induced by an activating anti- $\beta 7$  mAb LS722. The three Fib mAbs recognize nonoverlapping epitopes in  $\beta 7$  chain, and they all inhibit cell adhesion to VCAM-1, fibronectin, MAdCAM-1, and intestinal epithelium and they block lymphocyte homing to Peyer's patches (36). In the present study we show that these antibodies also cross-react with functionally relevant epitopes of human  $\beta 7$ . A polyclonal rabbit antiserum and a rat mAb AIIB2 (37) against  $\beta 1$  were used with similar results. mAb SR 84 against  $\alpha 1$  (38) inhibits binding to laminin and to collagens I, IV, and V (Heino, J., unpublished observations). mAbs AIIB2 and SR 84 were purified from ascites using protein G-Sepharose columns. Leu-8 and Dreg-56 against L-selectin gave identical staining results, and in HEV-binding assays the function-inhibiting Dreg-56 was used (39). All mAbs were used as purified Ig or as serum-free cell culture supernatants. All antibodies, except Leu-8, are against functional epitopes.

<sup>1</sup> Abbreviations used in this paper:  $\alpha\text{IEL}$ ,  $\alpha$ -chain of intraepithelial lymphocyte integrin; HEV, high endothelial venules; LPL, lamina propria lymphocytes; MAdCAM-1, mucosal addressin cell adhesion molecule 1; RAR, relative adherence ratio; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late activation antigen 4.

**HEV-binding Assays.** Endothelial binding of LPL was studied using an in vitro frozen section assay (41). Normal appendices were obtained from elective appendectomies, inflamed synovial membranes from synovectomies (rheumatoid arthritis patients), and peripheral lymph nodes from surgical explorations performed for diagnosis of lymphadenopathy (only nonmalignant nodes were used). Alternatively, mouse peripheral lymph nodes and Peyer's patches were used in these assays. It has been previously shown that binding of cells to HEV in corresponding human and mouse tissues is comparable (33, 42). 8- $\mu$ m frozen sections from tissues were freshly cut, and overlaid with  $3 \times 10^6$  LPL suspended in RPMI 1640 supplemented with 5% AB-serum and 10 mM HEPES, pH 7.2. Under constant rotation, lymphocytes were allowed to bind to endothelial cells in the sections for 30 min at +7°C. Thereafter, the nonadherent cells were gently tilted off, and the adherent cells were fixed to the sections overnight in ice-cold PBS containing 1% glutaraldehyde. At least 4 sections and 100 HEV per sample were analyzed under dark-field illumination. Adherence of small and large lymphocytes was counted separately. Large lymphocytes were defined as cells with a diameter at least 1.3 times (and two-dimensional surface area  $\geq 1.75$  times) that of small lymphocytes. The proportion of large cells was counted microscopically in each sample before the HEV assay, and it was 5% on average. The same percentage was obtained from fluorescence-activated cell sorting analyses (see below). The few monocytes that remained in the starting population occasionally adhered to HEV, but were easily identified by their large diameter and ruffled appearance and excluded from the analyses. Binding of LPL from separate experiments was normalized by using PBL in each experiment as controls. The results are expressed as relative adherence ratios (RAR) where RAR 1.0 defines the adherence of PBL. Thus, for LPL RAR value 1.0 means that they bind as well as PBL, RAR values  $>1.0$  mean that they bind better than PBL, and RAR values  $<1.0$  mean that they bind less efficiently than PBL to the endothelium studied. In preliminary assays, we ruled out the possible effects of collagenase treatment on HEV-binding by showing that fresh PBL and PBL coprocessed with lamina propria cells had no significant differences in their HEV-binding properties.

For inhibition assays, a mucosal T cell line was initially used because of the high number of immunoblasts needed ( $3 \times 10^6$  blasts/tissue section, six sections/antibody, five inhibitory antibodies plus controls, and two target tissues). The phenotypical and functional properties of this T cell line that justify its use are shown in Results. Cells were first treated with saturating concentrations of inhibitory rat mAbs against  $\beta 7$ , inhibitory mouse mAbs against human CD18, VLA-4 $\alpha$ , CD44, and negative controls (3G6, a mouse mAb, and 281.2, a rat mAb) for 30 min at 4°C. Similarly, polyclonal rabbit antiserum against  $\beta 1$  and normal rabbit serum as control were employed in the inhibition assays. Thereafter, cells were applied onto synovial and mucosal (human appendix) tissue sections (without washing away the antibodies), and the HEV-binding assay was then carried out as described above. Counting of the cells adherent to HEV was done from coded samples in a single blinded manner. The results are expressed as percentage of maximal binding where binding of cells in the presence of the appropriate negative control (mAb 3G6, 281.2 or normal rabbit serum) defines 100% binding.

To confirm the HEV-binding results with freshly isolated cells, LPL from normal bowels were isolated, treated with inhibitory mAbs, and applied onto the tissue sections (human appendix and synovium). In these experiments, mAbs HML-1 against  $\alpha$ IEL/ $\beta 7$ , Dreg-56 against L-selectin, AIIB2 against  $\beta 1$ , and SR 84 against  $\alpha 1$  were included. Also, a control mAb against a monomorphic

determinant on HLA-A,B,C was used to rule out nonspecific effects of cell-bound mAbs on HEV adherence. In these experiments, only  $10^6$  cells/section and four sections per tissue type could be analyzed because of the limitations of the starting material. Binding of small and large LPL were counted separately as described above.

**FACS<sup>®</sup> Analyses.** Cells were stained for immunofluorescence using a two-step procedure as described earlier (28). Briefly,  $0.5 \times 10^6$  cells were incubated with the first-stage mAbs for 20 min at 4°C. After washings in PBS containing 2% FCS and 1 mM sodium azide, appropriate FITC-conjugated second-stage antibodies were added for 20 min. Thereafter, cells were washed, fixed in PBS containing 1% formaldehyde, and analyzed by a FACScan<sup>®</sup> cytometer (Becton Dickinson & Co., Mountain View, CA). Gates for small lymphocytes and large immunoblasts were defined on the basis of the characteristic light scatter patterns and absence of CD14, as described (31). Routinely, 10,000 events from each sample were collected, and for blasts  $\sim 5,000$  events were also separately acquired using live gating.

## Results

**Mucosal Immunoblasts Bind to Synovial HEV.** LPL were enzymatically isolated from eight normal bowel specimens, and their adherence to three distinct endothelial specificities was studied using an in vitro HEV-binding assay (Fig. 1). Small lymphocytes from normal gut bound almost equally well to both mucosal and synovial HEV, but less efficiently to peripheral lymph node HEV. Immunoblasts from normal gut bound approximately five times better to mucosal HEV than did small lymphocytes. Mucosal immunoblasts also exhibited a remarkable capacity to bind to synovial HEV. In fact, gut immunoblasts adhered almost as efficiently to synovial HEV (RAR 4.9) as to mucosal HEV (RAR 5.0). An example of gut immunoblasts interacting with synovial endothelium is shown in Fig. 2. In contrast, no mucosal immunoblasts adhered to normal peripheral lymph node HEV (Fig. 1). Thus, normal mucosal immunoblasts can interact with endothelium in mucosa and synovium, but not in peripheral lymph nodes.

**Characterization of Functional mAbs against Human  $\beta 7$ .** Since  $\beta 7$  plays a critical role in mucosal homing in mouse (13, 16), it was essential to include this integrin in the functional assays. As shown in Fig. 3 A, each of three different rat mAbs to mouse  $\beta 7$  (Fib 21, 27, and 504) cross-reacted with human PBL. The expression level is in concordance with recent reports showing that resting B cells and a subpopulation of T cells in peripheral blood express  $\beta 7$  (43, 44). In mouse all these anti- $\beta 7$  mAbs detect nonoverlapping epitopes, yet they all inhibit mouse lymph node cell and human PBL binding to mouse MAdCAM-1 (36). To ascertain that these mAbs also block lymphocyte-endothelial cell interaction in humans, HEV assays were performed. As shown in Fig. 3 B, all three mAbs effectively inhibited human PBL binding to human appendix HEV.

**Freshly Isolated and IL-2-dependent Mucosal Immunoblasts Display Similar Adhesive Properties.** Since it is extremely difficult to isolate sufficient numbers of lamina propria immunoblasts from clinical specimens, we generated IL-2-dependent T cell lines from LPL to be used in the initial inhibition experiments. The validity of this approach is based on the following

**Table 1.** *Antibodies Used in this Study*

Antibody			
Name	Type	Antigen	Source
HP 2/1	Mouse IgG1	$\alpha 4$	*(Ref. 34)
Fib 21	Rat IgG2a	$\beta 7$	†(Ref. 36)
Fib 27	Rat IgG2a	$\beta 7$	†(Ref. 36)
Fib 504	Rat IgG2a	$\beta 7$	†(Ref. 36)
B1	Polyclonal rabbit	$\beta 1$	§
AIB2	Rat IgG	$\beta 1$	¶(Ref. 37)
SR 84	Mouse IgG	$\alpha 1$	§(Ref. 38)
Leu-8	Mouse IgG2a	L-selectin	¶
Dreg-56	Mouse IgG2a	L-selectin	†(Ref. 39)
Hermes-3	Mouse IgG2a	CD44	†(Ref. 24)
TS 1/18	Mouse IgG1	CD18	** (Ref. 40)
TS 1/22	Mouse IgG1	CD11a	** (Ref. 40)
HML-1	Mouse IgG2a	$\alpha$ IEL	*(Ref. 46)
W6/32	Mouse IgG2a	HLA A,B,C	**
3G6	Mouse IgG1	Chicken T cells	†
281.2	Rat IgG2a	Mouse syndecan	‡‡

\* Immunotech (Marseille, France).

† Made in our laboratory.

§ Gift from Dr. J. Heino (MediCity Research Laboratory, Turku, Finland).

¶ Gift from Dr. C. Damsky (University of California at San Francisco).

¶ Becton Dickinson & Co.

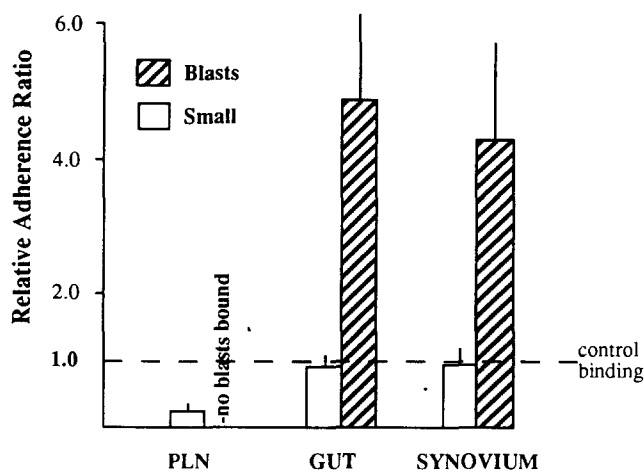
\*\* American Type Culture Collection (Rockville, MD).

‡‡ Gift from Professor M. Jalkanen (Centre of Biotechnology, Turku, Finland).

observations. First, approximately 2/3 of mucosal immunoblasts are T cells (28, 45). Second, the expression profiles of homing-associated molecules on freshly isolated mucosal immunoblasts and IL-2-dependent mucosal T cell lines were comparable. When analyzed by flow cytometry, both cell types were shown to be  $\alpha 4^+$ ,  $\beta 1^+$ ,  $\beta 7^+$ ,  $CD18^+$ ,  $CD44^+$ , and L-selectin<sup>-</sup>, although some quantitative differences in the intensity of staining did occur (Fig. 4). Third, IL-2-dependent T cells originated from the gut bind well to both mucosal and synovial HEV, but poorly to peripheral lymph node HEV (33), and hence fresh mucosal immunoblasts and mucosal T cell lines seem to be also functionally similar. The selective HEV-binding pattern was also confirmed with the cell line used in the present study (Fig. 5). Although in quantitative terms, the cells of the T cell line bound to a lesser extent than freshly isolated immunoblasts to all endothelial recognition specificities, the gut-derived T cell line displayed exactly the same HEV-binding selectivity (i.e., mucosal  $\approx$  sy-

novial  $\gg$  peripheral) as freshly isolated mucosal blasts. Moreover, binding of the gut-derived T cell line to synovial HEV was shown not to be nonspecifically due to the inflammatory status of the target tissue, since these cells did not bind to any considerable extent even to severely inflamed peripheral nodes (Fig. 5). In this experiment, endothelial cells in the normal peripheral node lacked E-selectin and VCAM-1, and contained only very low levels of intercellular adhesion molecule 1 (all three molecules are induced in endothelium during inflammation), whereas the inflamed node displayed the hallmarks of severe lymphadenitis (data not shown). Thus, IL-2-dependent mucosal T cell lines closely resemble freshly isolated lamina propria immunoblasts, both phenotypically and functionally.

*Mucosal T Cell Line Binds to Mucosal HEV via  $\beta 7$ .* Inhibition HEV assays were performed to analyze the contribution of different homing receptors in mediating the binding of gut-derived T immunoblasts to mucosal HEV. The binding

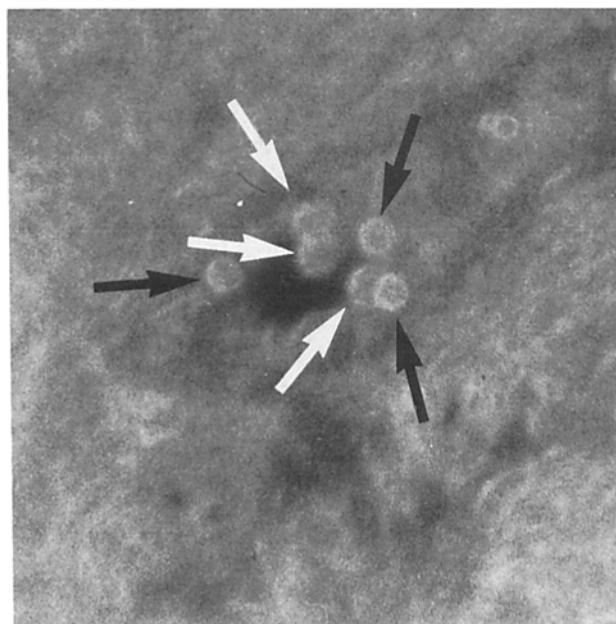


**Figure 1.** Mucosal immunoblasts bind to synovial HEV. Small and large (Blasts) LPL were freshly isolated from normal human gut and their binding to different endothelium specificities (PLN, peripheral lymph node; GUT, appendix/Peyer's patches; and SYNOVIUM) was analyzed using the in vitro frozen section assay. Results from eight specimens are expressed as RAR  $\pm$  SEM (see Materials and Methods). Binding of control PBL (defining RAR 1.0) is indicated by the dashed line.

of the mucosal T cell line to mucosal HEV was inhibited by 80% by a pretreatment with an anti- $\alpha 4$  antibody (Fig. 6 A). Since  $\alpha 4$  can associate with either  $\beta 1$  or  $\beta 7$ , cells were also separately preincubated with antibodies against each  $\beta$  chain. These assays revealed that anti- $\beta 1$  treatment had only marginal inhibitory effect, whereas anti- $\beta 7$  mAb abrogated 80% of binding. Anti-CD44 mAb treatment also dramatically inhibited binding (66%), but anti-LFA-1 mAb diminished the adherence only by 26% (Fig. 6). L-selectin cannot play any role in this interaction since it is absent from the mucosal T cell line (Fig. 4). Thus,  $\alpha 4/\beta 7$  is the principal homing receptor directing binding of human gut-derived T cells to mucosal HEV.

**Mucosa-derived T Cell Line Uses  $\alpha 4/\beta 1$ , CD44, and LFA-1 in Binding to Synovial HEV.** To compare the usage of homing-associated molecules in binding to mucosal and synovial HEV, inhibition HEV assays were performed in which inflamed synovium was used as a target tissue (Fig. 6 B). Anti-VLA-4 $\alpha$  mAb inhibited 28% of binding of the mucosa-derived T cell line to synovial HEV. The contribution of different  $\beta$  chains in the adherence to synovium was completely distinct from that in mucosal binding: anti- $\beta 1$  antibody prevented  $\sim 25\%$  of binding, whereas anti- $\beta 7$  had only marginal, if any, effect. CD44 appeared to be the most important single adhesion molecule involved in binding of mucosal T cell line to synovium, since anti-CD44 mAb (*Hermes-3*) abrogated almost 40% of binding. Anti-CD18 prevented  $\sim 20\%$  of adherence to synovial HEV (Fig. 6 B). These results indicate that CD44, VLA-4, and LFA-1 all contribute to binding of mucosal blasts to synovial HEV, but that  $\alpha 4/\beta 7$  is not used in this interaction.

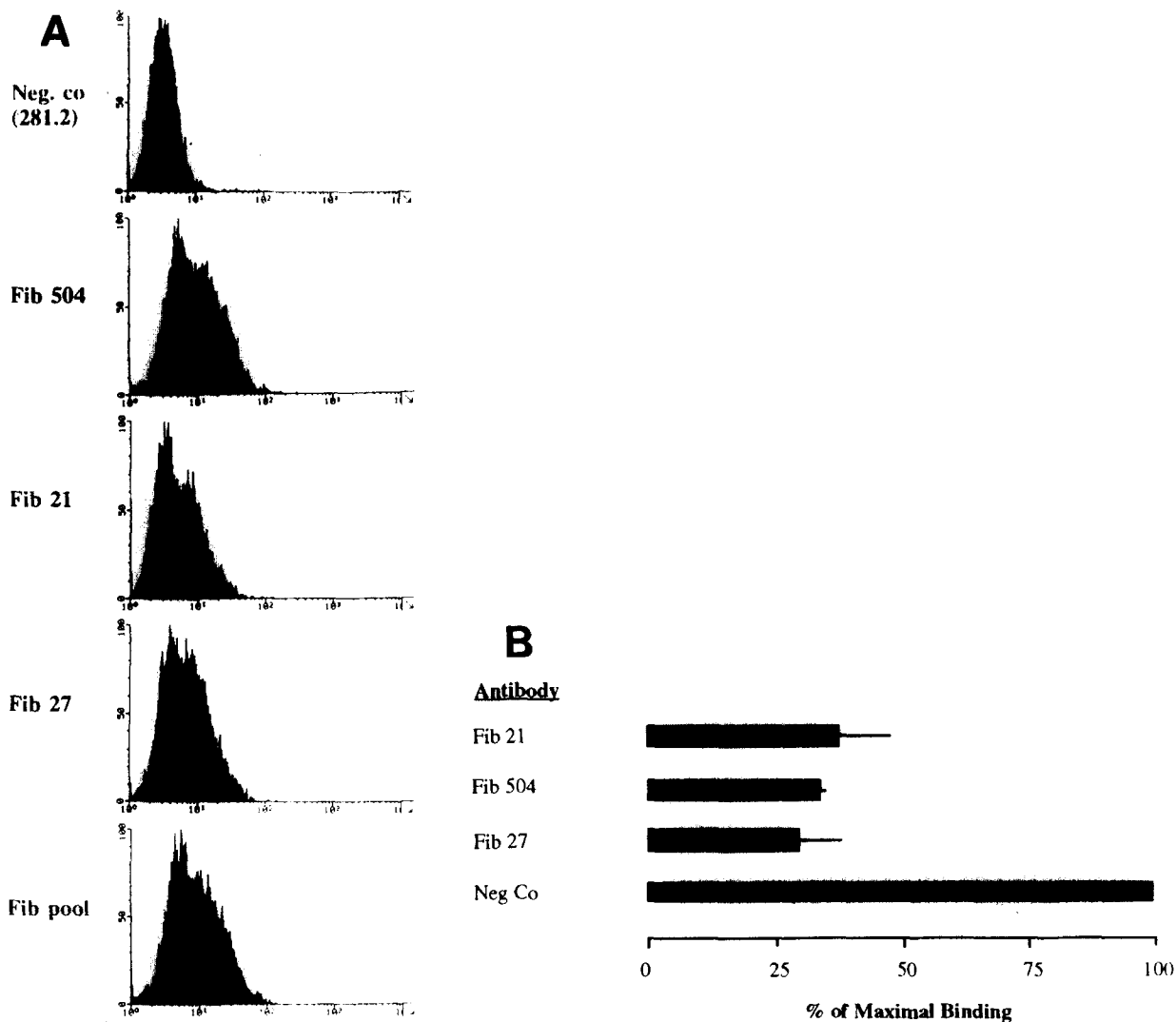
**Binding of Freshly Isolated Mucosal LPL to Mucosal and Synovial HEV.** Since some differences did exist between cultured IL-2-activated T cells and freshly isolated LPL in the



**Figure 2.** Small and large LPL interact with endothelial cells in the synovium. A dark-field micrograph illustrates binding of three mucosal immunoblasts (white arrows) and three small lymphocytes (black arrows) to a synovial HEV. The vessel is delineated by the dark-appearing base-membrane. Original magnification at 160.

expression of adhesion molecules and in absolute binding efficiencies, we wanted to confirm the binding results with freshly isolated LPL. Freshly isolated mucosal immunoblasts also employed  $\alpha 4/\beta 7$  as the principal homing receptor for binding to mucosal endothelium, since mAbs against both subunits of this integrin diminished the binding by  $\sim 75\%$  (Fig. 7 A).  $\alpha 4/\beta 1$  (VLA-4) was not involved in this interaction, since an anti- $\beta 1$  mAb had no effect on the HEV binding. Moreover,  $\alpha IEL/\beta 7$ , an integrin characteristic to intraepithelial T cells and also expressed on a subpopulation of LPL (46) did not mediate adherence of mucosal immunoblasts to mucosal HEV, because immunoblasts preincubated with an anti- $\alpha IEL$  mAb bound to HEV as well as mock-treated cells. CD44 and LFA-1 contributed significantly to the mucosal HEV binding. L-selectin, which is expressed at a very low level on freshly isolated blasts, has no significant effect on mucosal HEV binding in this assay system (Fig. 7 A). Thus, binding of freshly isolated mucosal immunoblasts to mucosal HEV closely resembles that of the mucosal T cell line, and is mainly mediated by  $\alpha 4/\beta 7$  integrin.

Contribution of  $\alpha 4$ -containing integrins to synovial binding of freshly isolated mucosal immunoblasts was less than to synovial adherence of the mucosal T cell line (Fig. 7 B). In all three independent assays, anti- $\beta 1$  caused slightly more inhibition than anti- $\beta 7$ . Anti-CD44 preincubation abrogated  $>50\%$  of synovial binding and anti-CD18 treatment also inhibited binding by 20%. L-selectin and  $\alpha IEL/\beta 7$  did not contribute to synovial binding of mucosal immunoblasts.  $\alpha 1$  which has been found at elevated levels on synovial fluid lymphocytes of rheumatoid arthritis patients (47) did not play



**Figure 3.** Characterization of anti- $\beta 7$  mAbs. (A) Cross-reactivity of three rat anti-mouse  $\beta 7$  mAbs with human  $\beta 7$ . Human PBL were stained for immunofluorescence with Fib 21, 27, 504, and a pool (all three Fibs together) and analyzed using a FACS<sup>®</sup>. x-axis is the fluorescence intensity in a log-scale and y-axis is the relative number of cells. (B) Anti-mouse  $\beta 7$  mAbs inhibit human PBL binding to mucosal endothelium. PBL were preincubated with different anti- $\beta 7$  mAbs and thereafter applied onto frozen sections of human appendices, and the bound cells were counted. Results (mean  $\pm$  SEM of two experiments) are shown as a percentage of maximal binding that is defined as the number of bound cells in a negative control antibody (mAb 281.2) treated samples.

a role in synovial binding. These data indicate that freshly isolated mucosal immunoblasts and the T cell line use analogous mechanisms to bind to synovial HEV. CD44 seems to be the only adhesion molecule studied that is significantly involved in this adherence (Fig. 7 B), but this interaction is apparently largely mediated by other, currently unknown molecules (see Discussion).

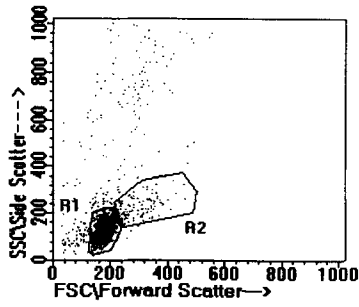
From these same experiments we also evaluated binding of freshly isolated small LPL to mucosal and synovial HEV (Fig. 8). In general, small LPL used the same adhesion molecules as immunoblasts for HEV binding. Thus, binding to mucosal HEV was mainly supported by  $\alpha 4/\beta 7$  (Fig. 8 A),

and involvement of CD44 and VLA-4 was observed in binding to synovial HEV (Fig. 8 B).

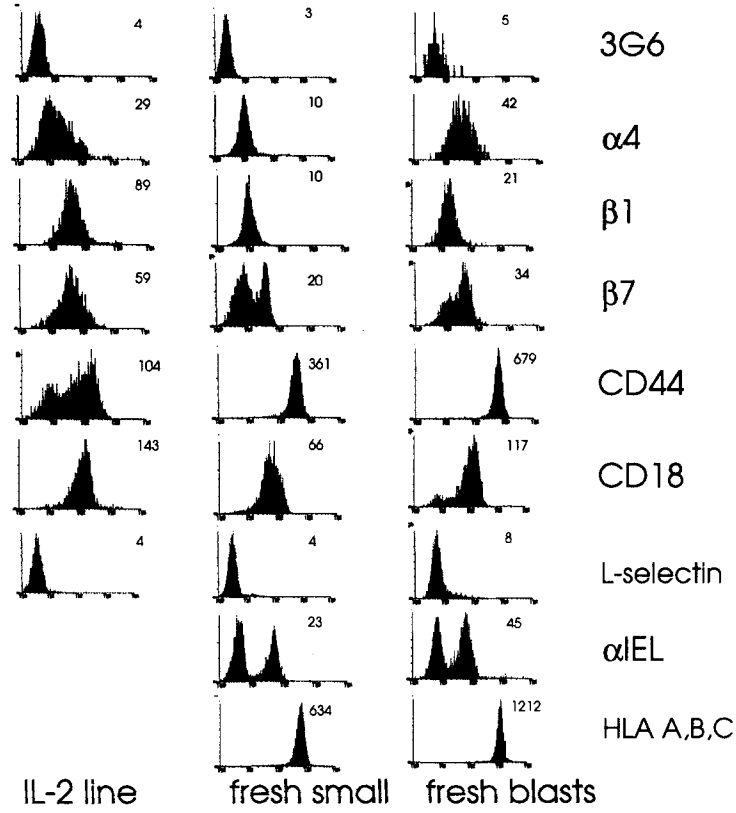
### Discussion

In the present work, the binding of human gut lymphocytes to vascular endothelium was analyzed to study the recirculation pathways of mucosal cells. By using freshly isolated lamina propria immunoblasts and an IL-2-activated mucosal T cell line we found that mucosal immunoblasts bind extremely well to mucosal endothelium, but not at all to peripheral lymph node HEV. Moreover, we showed that blast

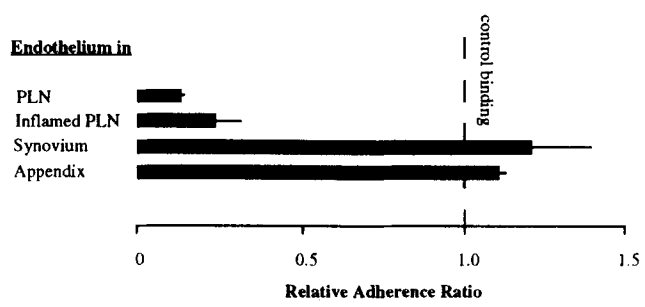
**A**



**B**



**Figure 4.** Expression of homing-associated molecules on mucosal lymphocytes. The IL-2-activated mucosal T-cell line (*IL-2 line*), freshly isolated small LPL (*fresh small*), and freshly isolated lamina propria immunoblasts (*fresh blasts*) were stained for immunofluorescence and analyzed using a FACScan®. (A) Regions used to define small LPL (*R1*) and immunoblasts (*R2*) in freshly isolated samples. (B) Histograms showing the adhesion molecule profiles. *x*-axis is the fluorescence intensity on a log scale, and *y*-axis is the relative number of cells. Mean fluorescence intensities are given in the upper right corner of each panel. With all these cell types staining with anti-CD11a and anti-CD18 mAbs gave identical histograms. (3G6) A negative control (mAb 281.2 and normal rabbit serum gave comparable histograms).

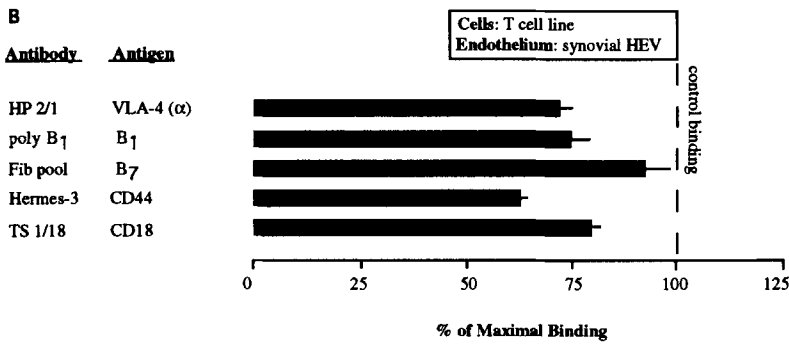
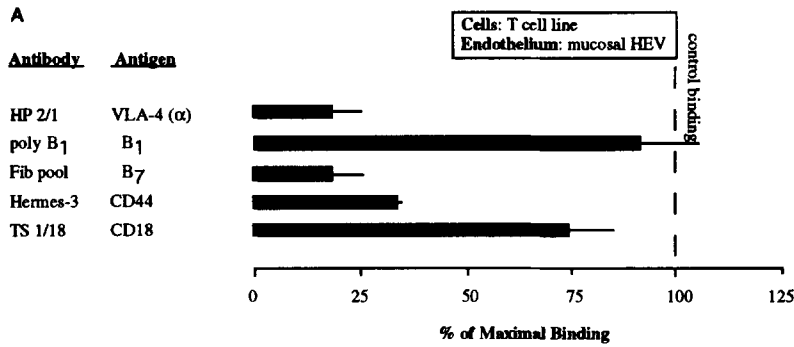


**Figure 5.** Mucosa-derived T cell line binds to mucosal and synovial HEV, but not to peripheral lymph node HEV. IL-2-activated T cells were applied onto sections of noninflamed human appendices, inflamed synovial membranes, noninflamed peripheral lymph nodes and inflamed peripheral nodes. Binding to HEV was analyzed and compared with that of normal PBL (used as a control). Binding of PBL to each tissue types defines the

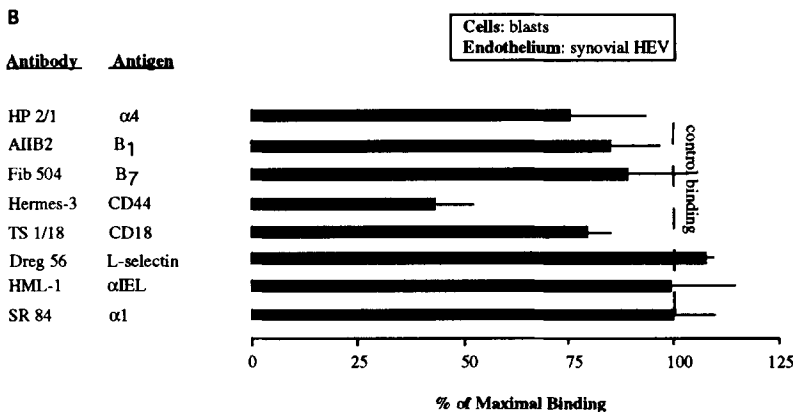
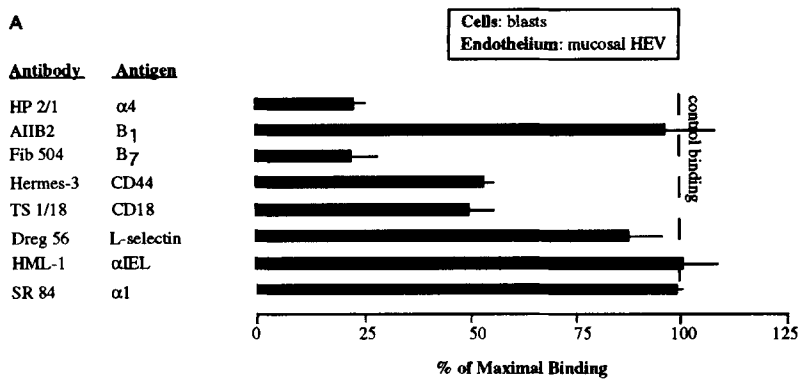
cells from normal human gut have an inherent capacity to interact efficiently with synovial HEV. We also dissected the molecular mechanisms that regulate this dual endothelial binding of mucosal immunoblasts.

Binding of mucosal immunoblasts to mucosal HEV was mediated by  $\alpha 4/\beta 7$  and, to a lesser extent, by CD44 and CD18. The tissue specificity of this interaction is probably conferred in large part by  $\alpha 4/\beta 7$ , because CD44 and CD18 are involved in HEV binding in a nontissue-specific fashion (see below). Since pretreatment with both anti- $\alpha 4$  and anti- $\beta 7$  mAbs yielded virtually indistinguishable inhibition per-

RAR-value 1.0. Results from two independent assays are expressed as RAR  $\pm$  SEM. Note that the inflammatory status of peripheral nodes has only marginal effects on the efficacy of mucosal T cell line adherence.

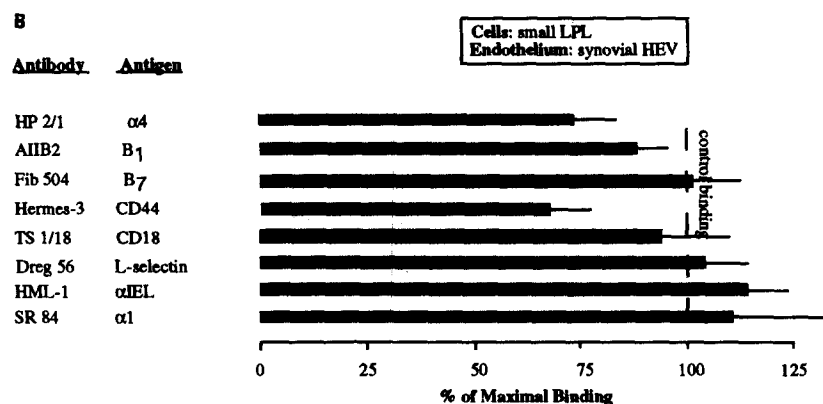
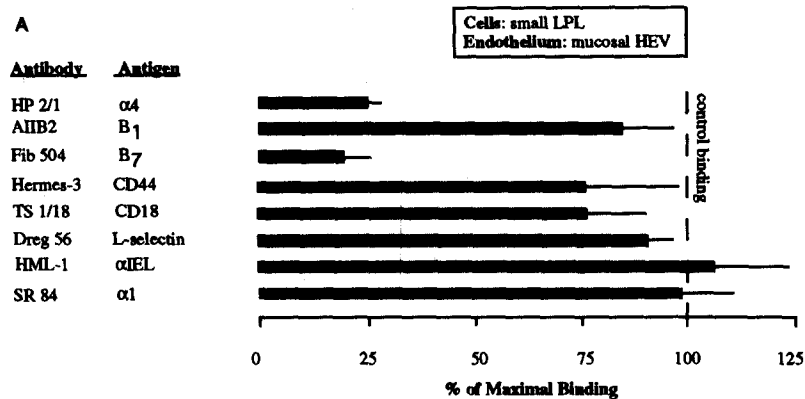


**Figure 6.**  $\alpha$ 4/ $\beta$ 7 is the principal integrin mediating binding of mucosal T cell line to mucosal HEV whereas VLA-4, CD44, and LFA-1 all contribute to the binding to synovial HEV. IL-2-activated mucosal T cells were preincubated with inhibitory antibodies against  $\alpha$ 4,  $\beta$ 1,  $\beta$ 7, CD44, CD18, or with negative controls (3G6, 281.2, and normal rabbit serum). Thereafter, binding of cells (A) to mucosal HEV (human appendix) and (B) to HEV in inflamed synovium was analyzed. Results of two independent experiments are expressed as a percentage of maximal binding  $\pm$  SEM, in which the number of adherent cells in the presence of the appropriate negative control antibody defines 100% binding.



**Figure 7.** Binding of freshly isolated immunoblasts from normal bowel to HEV. Binding of freshly isolated gut immunoblasts to (A) mucosal HEV (human appendix) and (B) synovial HEV is shown. Inhibition HEV assays were performed exactly as described in the legend for Fig. 6, except that a mAb against  $\beta$ 1 was used and an irrelevant binding anti-HLA-ABC mAb was used as a control (defining 100% binding). In addition, mAbs against  $\alpha$ IEL,  $\alpha$ 1, and L-selectin were included. Results are from three independent experiments (LPL from different individuals) and are presented as mean  $\pm$  SEM.





**Figure 8.** Binding of freshly isolated small LPL from normal bowel to HEV. Binding of freshly isolated gut LPL to (A) mucosal HEV and to (B) synovial HEV was analyzed. These data are derived from the same experiments as shown in Fig. 7, but binding of small LPL was counted.

centages, and anti- $\beta 1$  had no significant effect, all the functionally active  $\alpha 4$  that mediates adherence to mucosal HEV is in the form of  $\alpha 4/\beta 7$ . Theoretically, it is also possible that anti- $\beta 7$  mAbs send a negative signal to the cell, which turns off other mucosa-specific adhesion systems. In mouse,  $\alpha 4/\beta 7$  has been shown to be the principal homing receptor that directs binding of mesenteric lymph node lymphocytes and lymphoid cell lines to gut-associated lymphatic tissues in vitro and in vivo (12, 13, 16, 48). Our findings extend these observations to mucosal effector cells of lamina propria, and for the first time show that  $\alpha 4/\beta 7$  plays a critical role in mucosal homing in humans.

$\beta 7$  can also be associated with  $\alpha$ IEL (35, 49).  $\alpha$ IEL/ $\beta 7$  heterodimer has been reported to be expressed in intraepithelial lymphocytes of gut, and it mediates binding of this lymphocyte subpopulation to epithelial cells (50).  $\alpha$ IEL is also present on a subpopulation of LPL (46). However, our HEV-binding experiments revealed that  $\alpha$ IEL/ $\beta 7$  expressed on lamina propria immunoblasts did not participate in endothelial binding (actually,  $\alpha$ IEL/ $\beta 7$  is not a receptor for MAdCAM-1, Butcher, E. C., unpublished data). Although integrins may not function maximally at 7°C, anti-LFA-1 and anti- $\beta 7$  mAbs do clearly inhibit binding in HEV assays (12, 16, 25, 26, and our present results). A possibility remains, of course, that all the functions of  $\alpha$ IEL integrin may not be completely blocked by HML-1 mAb. However, in the currently employed

binding assays, only pairing of  $\beta 7$  with  $\alpha 4$  yields a functionally active mucosal homing receptor.

In contrast,  $\beta 7$  was not involved to any significant extent in the binding of human mucosal immunoblasts to synovial HEV. It is interesting to note that the other  $\alpha 4$ -containing integrin,  $\alpha 4/\beta 1$  (VLA-4), contributes to synovial adherence of the mucosal T cell line and freshly isolated mucosal immunoblasts. Thus, the two different  $\alpha 4$ -containing integrins are differentially used in the binding of mucosal immunoblasts to mucosal and synovial HEV. However, VLA-4 cannot explain the tissue specificity of synovial HEV binding, because it played only a relative minor role in binding and it mediates adhesion of lymphocytes in several endothelial recognition systems (51–53).

mAb Hermes-3 to CD44 significantly inhibited binding of mucosal lymphocytes to both mucosal and synovial HEV. Earlier, CD44 has been shown to be involved in PBL binding to HEV in gut-associated lymphatic tissues, peripheral lymph nodes, and synovium (24). However, mAb Hermes-3 against CD44 only inhibited binding of PBL to mucosal HEV, but not to synovial HEV (24). Thus, mucosal lymphocytes and nonactivated PBL can apparently use CD44 differently (different epitopes, signaling events, and/or activation stage) when binding to synovial HEV. Moreover, recent data suggest that in lymphocyte adhesion to HEV, CD44 may function as a facilitating molecule rather than a specificity con-

ferrer (16). Thus, on mucosal LPL both CD44 and LFA-1 apparently function as nontissue-specific adhesion molecules.

Small LPL expressed most adhesion molecules at lower level than immunoblasts. However, small lymphocytes basically used the same homing receptors as immunoblasts in HEV binding. Thus, binding to mucosa took place mainly via  $\alpha 4/\beta 7$ , whereas CD44 and VLA-4 conferred adherence to synovial vessels. However, CD18 only played a very minor role in binding of small LPL to synovium. Overall, rather similar use of adhesion molecules by small LPL and immunoblasts most likely reflects the fact that most small LPL in gut are memory-type cells (45) that have same kind of recirculation pathways as immunoblasts (1).

The currently known homing-associated molecules of mucosal lymphocytes do not fully account for the synovial adherence, especially if CD44 mainly functions as an activator of other adhesion receptors. Also in other studies, only partial inhibition of PBL and monocyte and synovial lymphocyte binding to synovial endothelium by mAbs against CD44, L-selectin, VLA-4, and LFA-1 has been reported (51, 54–56). Thus, novel homing molecules may remain to be discovered that mediate binding of different lymphocytes to synovial HEV. Alternatively, the specificity of synovial binding may be conferred by a unique combination of sequential adhesion and activation steps (7, 10).

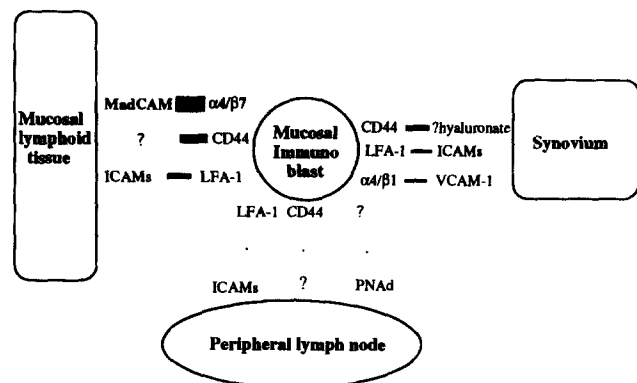
The critical role of  $\alpha 4/\beta 7$  in mucosal HEV binding indicates that it uses MAdCAM-1, a mucosal addressin, as a counter-receptor. Although  $\alpha 4/\beta 7$  can also bind to VCAM-1 and fibronectin (16, 57, 58), only MAdCAM-1 can confer the organ specificity of binding, since fibronectin is nonspecifically absorbed to the surface of all cells of vascular lining, and VCAM-1 is not present to any significant extent in either normal or inflamed mucosa (31, 59). In mouse, the importance of  $\alpha 4/\beta 7$  interaction with MAdCAM-1 has been directly proven (16) and our current studies suggest that similar molecular mechanisms are used in humans although the human homologue of MAdCAM-1 remains to be found. On the other hand, involvement of VLA-4 in the binding of mucosal immunoblasts to synovial HEV strongly suggests that VCAM-1 will be its principal counterpart in synovial endothelium (18). VCAM-1 was, in fact, moderately expressed in the vessels of the synovial membranes used in the present HEV-binding assays, and also in other studies it has been shown to be present in the inflamed synovium (60, 61). Furthermore, our results indirectly indicate that in synovial venules VCAM-1 is not a physiologically significant ligand for  $\alpha 4/\beta 7$  of the mucosal T cell line.

Earlier animal studies have suggested that mucosal immunoblasts specifically home back to mucosa-associated lymphatic tissues. In their classical work, Gowans and Knight (62) showed that small lymphocytes isolated from the rat thoracic duct rapidly homed to all lymphoid tissues when injected into syngeneic animals. In contrast, large lymphocytes, which presumably consisted mainly of activated mucosal blasts, preferentially localized in the lymphoid tissues of the gut, and practically none were found at other locations. Selective migration of immunoblasts from mesenteric nodes and lymph back into mucosa-associated lymphatic tissues has been con-

firmed in later reports (63–66). However, our results for the first time elucidate the interactions of lamina propria immunoblasts with endothelium, and provide the molecular explanation for these interactions in humans. Moreover, a previously unknown interaction between mucosal immunoblasts and synovial HEV was found. Based on our HEV-binding data, we propose a putative model of mucosal immunoblast interaction with endothelial cells in gut, synovium, and peripheral lymph node (Fig. 9).

Several observations indicate that synovial HEV binding of mucosal immunoblasts is not merely due to the inflammatory status of the target tissue. Mucosal immunoblasts from the T cell line did not bind to human peripheral lymph nodes that are severely inflamed any better than to noninflamed lymph nodes, although endothelial ligands of  $\alpha 4/\beta 1$ , CD44, and LFA-1 are expressed both in the inflamed synovium and lymph node. Moreover, IL-2-activated T cell lines originated from peripheral lymph nodes and most of those generated from PBL show only marginal binding to inflamed synovial HEV (33). Finally, IL-2-propagated tumor infiltrating lymphocytes that adhere efficiently to the tumor vasculature, show negligible binding to HEV in the inflamed synovium (Salmi, M., and S. Jalkanen, manuscript submitted for publication). Together, these data suggest that mucosal immunoblasts have some unique properties (e.g., response to activation factors in the second step of adhesion cascade; 7, 10) that render them capable of dual binding. It is of interest to note, however, that chronic inflammation of the gut alters the HEV-binding specificity of mucosal immunoblasts. Thus, immunoblasts from ulcerative colitis and Crohn's disease bowels bind to normal peripheral nodes, and this binding is mediated by interaction of peripheral lymph node addressin with a currently unknown, non-L-selectin ligand (31).

The present findings suggest that the normal lamina propria contains immunoblasts that are able to home either to the



**Figure 9.** Schematic model of mucosal immunoblast interactions with different endothelial recognition specificities in humans. Mucosal immunoblasts express  $\alpha 4/\beta 1$ ,  $\alpha 4/\beta 7$ , LFA-1, and CD44, but not L-selectin. The putative interactions with endothelial ligands in mucosal lymphatic tissues, synovium and peripheral lymph nodes are illustrated. The thickness of the line connecting the lymphocyte adhesion receptor and its endothelial cell ligand is proportional to the importance of each receptor-ligand pair in recognition of distinct endothelial cell specificities as revealed in our current in vitro model.

mucosa or to the synovium. This may explain the development of extraintestinal manifestations not uncommonly encountered in the infectious and inflammatory bowel diseases (29, 30). In the case of arthritis, the following hypothesis may be put forth: after recognizing their cognate antigen at mucosal sites, activated immunoblasts will return to the blood circulation. Thereafter, blast cells can home either back to mucosal sites or alternately to synovium. Accumulation of antigen-specific blasts in joints would result in aggravation of synovitis, especially if the triggering antigen is trapped at synovial sites (67).

In conclusion, we have shown here that mucosal immu-

noblasts have a dual endothelial binding capacity—they bind both to mucosal and synovial HEV, but not to peripheral lymph nodes. Binding of mucosal blasts to gut-associated lymphatic tissues is mainly mediated by  $\alpha 4/\beta 7$ . In contrast, the same cells utilize different, mostly yet-to-be-described adhesion molecules in adherence to synovial sites. This kind of elucidation of the molecular mechanisms of lymphocyte-endothelial interactions between mucosal immunoblasts and inflamed synovium may in the future be helpful in designing new methods to alleviate joint complications connected to inflammatory and infectious bowel diseases.

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