

# L-Selectin and $\alpha_4\beta_7$ Integrin Homing Receptor Pathways Mediate Peripheral Lymphocyte Traffic to AKR Mouse Hyperplastic Thymus

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**Before the development of thymic lymphoma, AKR mice undergo a striking lymphoid hyperplasia of the thymic medulla. We have previously shown that there is a marked increase in traffic of B and T lymphocytes from the periphery into the preneoplastic, hyperplastic thymuses of these mice, in contrast to the scant traffic of such cells to normal thymuses. The traffic of lymphocytes to lymph nodes and Peyer's patches is controlled in part by the interaction of lymphocyte adhesion molecules called homing receptors with their tissue-selective endothelial ligands known as vascular addressins. We have investigated the roles of homing receptors and vascular addressins in the traffic of lymphocytes to the AKR hyperplastic thymus. We demonstrate that development of hyperplasia is accompanied by an increase in the number of thymic medullary blood vessels with high endothelial venule morphology and expression of the peripheral node addressin (PNAd) and the mucosal addressin (MAdCAM-1). In vitro and in vivo functional assays show that the addressin/homing receptor pairs PNAd/L-selectin and MAdCAM-1/ $\alpha_4\beta_7$  are involved in lymphocyte traffic to the hyperplastic thymus. These results indicate that molecular adhesion mechanisms involved in tissue-selective migration of lymphocytes to peripheral lymph node and to mucosal lymphoid tissues play a role in the recruitment of B and T lymphocytes to the AKR thymus and thus in the pathogenesis of thymic hyperplasia. (Am J Pathol 1995, 147:412-421)**

ing the latter part of the first year of life.<sup>1</sup> Before the onset of lymphoma, these mice develop thymic hyperplasia characterized by an enlarged medulla containing B and T lymphocytes.<sup>1,2</sup> Our previous studies have shown a marked increase in traffic of B and T lymphocytes from the periphery into the medulla of hyperplastic thymuses of old AKR mice compared with histologically normal thymuses of age-matched BALB/c and C57BL/Ka mice or young AKR mice.<sup>2</sup> However, the mechanisms by which these lymphocytes are recruited to the thymic medulla have not been determined.

Most mature B and T lymphocytes migrate continuously throughout the body, trafficking from blood through lymphoid tissues to lymph and back to blood.<sup>3</sup> A key element in this migration is the ability of blood-borne lymphocytes to recognize and bind to the luminal surface of specialized high endothelial venules (HEVs) in organized lymphoid tissues such as lymph nodes (LNs) and Peyer's patches (PPs) or to functionally analogous venules in sites of chronic inflammation.<sup>4,5</sup> Certain lymphocyte subsets are known to migrate to organized lymphoid tissues and sites of chronic inflammation in a tissue-selective manner<sup>5</sup> (reviewed in Refs. 6 and 7). Although lymphocyte migration is clearly a complex process involving sequential adhesion and activation events, one well defined key event in tissue-selective migration is the interaction of lymphocyte homing receptors (HRs) with vascular addressins.<sup>8,9</sup>

Most mature lymphocytes bear the  $\alpha_4\beta_7$  integrin mucosal HR (previously called LPAM-1 or  $\alpha_4\beta_7$ ) and/or the L-selectin peripheral LN (PLN) HR.<sup>10-13</sup> The  $\alpha_4\beta_7$  HR binds to the mucosal addressin MAdCAM-1, which is expressed at high levels by HEV in mucosal sites such as PP and mesenteric LN (MLN).<sup>14,15</sup> Although L-selectin is involved in lymphocyte traffic to PP, homing specificity to this site is dominated by  $\alpha_4\beta_7$ /MAdCAM-1 interactions.<sup>16</sup>

Virtually all AKR mice develop a retrovirus-associated T cell lymphoma that originates within the thymus dur-

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MAdCAM-1 is also expressed by venules in intestinal lamina propria, lactating mammary gland, inflamed pancreata of diabetic mice, and the central nervous system of some mice with relapsing experimental autoimmune encephalomyelitis and is thought to be involved in lymphocyte traffic to these sites.<sup>14,16-23</sup> Functional ligands for L-selectin are expressed at high levels by HEVs in PLNs and MLNs, at low levels by HEVs in PPs, and by vessels in inflamed pancreata of diabetic mice and in a variety of inflammatory sites in humans.<sup>10,20-28</sup> These ligands, known as the peripheral node addressin (PNAd), react with monoclonal antibody (MAb) MECA-79.<sup>24</sup> L-selectin ligands include appropriately glycosylated subsets of the glycoproteins GlyCAM-1, CD34, and MAdCAM-1.<sup>29-33</sup> Thus PNAd consists of structurally diverse glycoproteins that share the capacity to present specific carbohydrate ligands to L-selectin. Here we investigate the roles of these HRs and their vascular addressin ligands in the traffic of lymphocytes to the AKR mouse hyperplastic thymus.

## Materials and Methods

### Mice

AKR/C and AKR/J mice, purchased from Cumberland Farms (Cumberland, TN) and Jackson Laboratories (Bar Harbor, ME), respectively, or bred in our animal facility from stock obtained from these vendors were used at ages 1 to 36 weeks. BALB/c mice, obtained from the Institute for Medical Research (San Jose, CA) or bred in our animal facility, and C57BL/Ka mice, bred in our facility from stock obtained from Dr. Irving L. Weissman, were used at ages 1 to 105 weeks. All experiments involved female mice.

Mice were sacrificed, and selected lymphoid organs including thymus were frozen in OCT compound (Miles Laboratories, Naperville, IL) for immunoperoxidase or immunofluorescence (IF) studies as described below. Adjacent sections stained with hematoxylin and eosin (H&E), as well as H&E-stained sections of formalin-fixed, paraffin-embedded thymus, were evaluated by using standard histological criteria. Any thymus showing gross or microscopic evidence of involvement by lymphoma was eliminated from the study.

### Monoclonal Antibodies

Rat MAb MEL-14 (anti-L-selectin, CD62L, IgG2a isotype), MECA-79 (anti-PNAd, IgM), and MECA-367 (anti-MAdCAM-1, IgG2a) were prepared in our laboratory.<sup>10,14,24</sup> Rat MAbs reactive with the  $\alpha_4\beta_7$  integrin

included PS/2 (anti- $\alpha_4$  chain, CD49d, IgG2b isotype; provided by P. Kincade, University of Oklahoma, Oklahoma City, OK), DATK32 (recognizes a combinatorial epitope on the  $\alpha_4\beta_7$  heterodimer, IgG2a; provided by D. Andrew, Stanford University, Stanford, CA), and Fib22 (anti- $\beta_7$  chain, IgM isotype; provided by D. Andrew).<sup>34,35</sup> MAb MK2.7 (rat IgG1), which reacts with vascular cell adhesion molecule-1 (VCAM-1), was provided by P. Kincade.<sup>36</sup> Rat MAbs used for isotype-matched negative controls included MECA-89 (reacts with an epitope on MAdCAM-1 not involved in  $\alpha_4\beta_7$ -mediated lymphocyte binding, IgG2a isotype), OZ-42 (anti-mouse cerebellar granule cell, IgM), 1G (reacts with retroviral gp90, IgG2b), GK1.5 (anti-mouse CD4, IgG2b), 53-2.1 (anti-mouse Thy-1.2, IgG2a), and 9B5 (anti-human CD44, IgG2a).

### Tissue Section Immunoperoxidase Staining

Frozen sections of lymphoid organs were stained by a three-stage immunoperoxidase technique involving sequential incubations with unconjugated rat MAb, biotin-conjugated rabbit anti-rat Ig in 5% normal mouse serum, peroxidase-avidin, and 3,3'-diaminobenzidine/hydrogen peroxide solution.<sup>37</sup> As negative controls, primary MAbs were replaced with species and isotype-matched irrelevant MAbs or phosphate-buffered saline.

### Tissue Section IF Staining and Evaluation

Frozen sections of AKR, BALB/c, and C57BL/Ka thymus and BALB/c MLN were stained with biotin-MECA-367 followed by fluorescein isothiocyanate (FITC)-MECA-79 mixed with Texas Red-avidin (Cappel Laboratories, Malvern, PA). Adjacent sections of thymus stained with FITC-peanut agglutinin (Vector Laboratories, Burlingame, CA) were examined to differentiate cortex from medulla. Sections stained with the anti-addressin MAb were examined by fluorescence microscopy; the number, location (thymic cortex *versus* medulla), and morphological appearance (high *versus* low endothelial) of vessels expressing one or both addressins were determined. These slides were then restained with H&E and the areas of thymic medulla, thymic cortex, and MLN paracortex were determined for each slide by using an image analysis system as previously described.<sup>37</sup> The number of addressin-expressing HEVs per unit area in each microenvironment was then calculated.

### *In Vitro Lymphocyte-Endothelial Cell Binding Assay*

The Stamper-Woodruff *in vitro* assay of lymphocyte-endothelial cell binding was performed as described.<sup>20,38,39</sup> Briefly, cell suspensions were prepared from BALB/c MLN or from one of two lymphoma cell lines that exhibit tissue-selective binding to HEVs in organized lymphoid tissues: 38C13, a C3H B cell lymphoma that is L-selectin<sup>hi</sup>,  $\alpha_4\beta_7^{-10,24,25}$ ; and TK1, an AKR T cell lymphoma that is  $\alpha_4\beta_7^+$ ,  $\alpha_4\beta_1^-$ , L-selectin<sup>-</sup>.<sup>5,13,25</sup> AKR and BALB/c mice were injected intravenously with 0.2 ml of the intravascular dye luconyl blue (40% in saline) and sacrificed 5 minutes later, and lymphoid organs (AKR thymus; BALB/c PLN, MLN, PP) were removed and frozen in OCT for preparation of frozen sections.

To determine the ability of MAbs against HRs or addressins to interfere with binding of lymphoid cells to endothelium in hyperplastic thymuses, tissue sections or lymphoid cells were incubated with specific MAb, isotype- and species-matched negative control MAb, or media before the assay.<sup>20</sup> Tissue sections were then incubated with  $1 \times 10^6$  to  $3 \times 10^6$  lymphocytes or lymphoma cells for 30 minutes at 7°C with gentle rotation. Nonadherent cells were decanted and adherent cells fixed to tissue sections by immersion in cold phosphate-buffered saline containing 1.5% glutaraldehyde. Cell binding to vessels was evaluated microscopically under phase or dark-field illumination; the mean number of cells bound per HEV was determined for each treatment of each individual tissue. Binding results after MAb treatment are presented as percent of binding in the media control.

In one set of binding assays, FITC-labeled 38C13 cells were mixed with unlabeled TK1 cells in a ratio shown by preliminary assay to give an overall 1:1 binding ratio on BALB/c MLN HEV.<sup>39</sup> The cell suspension was then incubated on frozen sections of AKR thymus and BALB/c MLN as described above. Slides were fixed in glutaraldehyde and examined by fluorescence microscopy, and the number of FITC-38C13 and TK1 cells binding to each vessel was determined. Results are expressed as the overall ratio of 38C13 to TK1 binding on AKR thymic vessels with the ratio of such binding on MLN designated as one.

### *In Vivo Lymphocyte Transfer Studies*

MAbs against HRs or addressins were tested for their ability to block lymphocyte traffic to hyperplastic thymus using one of the following protocols. 1) Host mice (16-week AKR/J or 28-week AKR/C) were injected intravenously with 0.5 mg of anti-addressin MAb

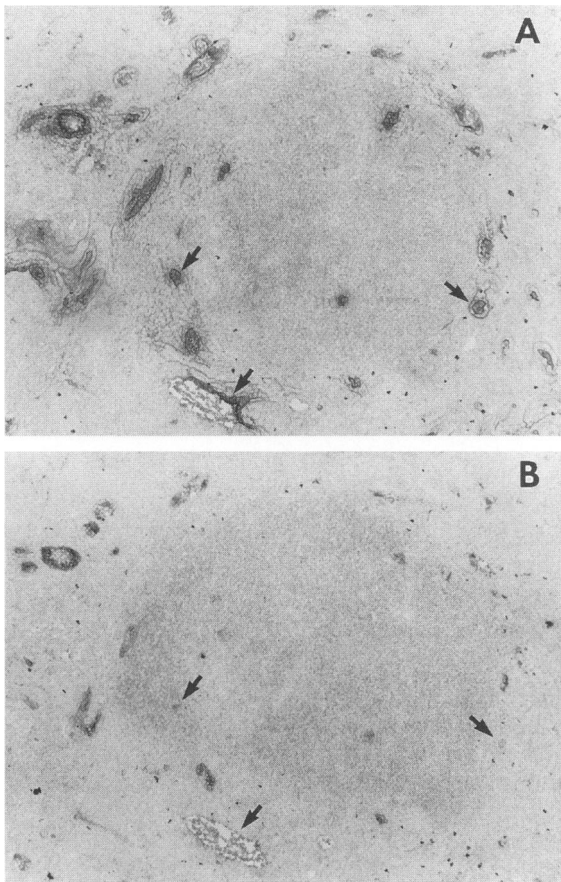
(MECA-79 or MECA-367) or negative control MAb (OZ-42, MECA-89, or 9B5) in 0.2 ml of saline or with saline alone, and 30 minutes later each host received intravenously another 0.5 mg of the original MAb or saline mixed with  $5 \times 10^7$  rhodamine-labeled MLN lymphocytes from 6- to 8-week-old syngeneic donors.<sup>40</sup> 2) Host mice received intravenously  $5 \times 10^7$  rhodamine-labeled donor lymphocytes that had been incubated for 15 minutes with anti-L-selectin MAb (100  $\mu\text{g/ml}$  MEL-14), negative control MAb, or media alone and washed twice before injection. One hour after receiving donor cells, all host mice were sacrificed, blood collected in heparinized saline, and lymphocytes isolated by Lympholyte-M density centrifugation (Cedarlane Laboratories, Hornby, Ontario, Canada). One-half of each of the following lymphoid organs was taken for suspension and the remaining half frozen: thymus, spleen, MLN, PLN, PP. The percentage of rhodamine-labeled cells in each suspension was determined by fluorescence microscopy as previously reported.<sup>41</sup> Frozen tissue was used for immunoperoxidase or IF staining.

## **Results**

### *Vascular Morphology and Adhesion Molecule Expression*

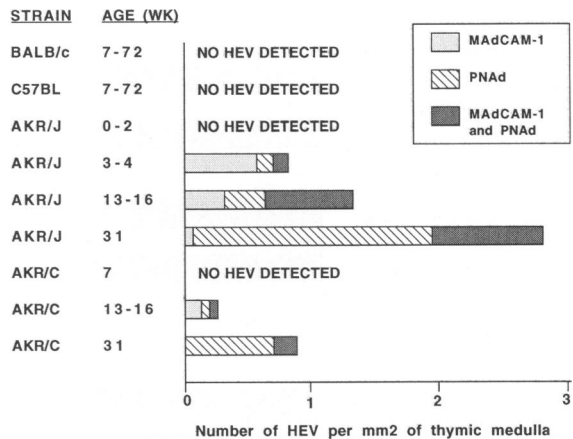
To investigate the role of vascular adhesion molecules in lymphocyte traffic to the AKR thymus, we stained frozen sections of thymuses from 1- to 36-week-old AKR/C and AKR/J and 1- to 105-week-old BALB/c and C57BL/Ka mice with MAb against PNA<sup>d</sup>, MAdCAM-1, and VCAM-1 by an immunoperoxidase technique. Adjacent sections stained with H&E or with MAb reactive with B cells or L-selectin were examined to evaluate the presence of medullary hyperplasia within these thymuses.<sup>2</sup> As previously reported, thymic medullary hyperplasia was first seen in AKR/J mice at 3 to 4 weeks of age and in AKR/C mice at approximately 16 weeks of age; the hyperplasia became more prominent with increasing age.<sup>2</sup> There was no evidence of hyperplasia in the thymuses of the BALB/c and C57BL/Ka mice.

The immunoperoxidase stains showed prominent expression of the vascular addressins (MAdCAM-1 and PNA<sup>d</sup>) by HEVs in the medulla of hyperplastic thymuses from AKR mice (Figure 1). We used two-color IF staining followed by quantitative morphometry<sup>37</sup> to further evaluate the addressin expression by HEVs in thymuses of selected mice (Figure 2). In the AKR thymuses, these HEVs not only increased in number with increasing age and hyperplasia but also



**Figure 1.** Vascular addressin expression in AKR hyperplastic thymus. Serial frozen sections of thymus from 31-week-old AKR/J mouse demonstrate expression of PNA (A) and MAdCAM-1 (B) by vascular endothelium in the thymic medulla. Several of the vessels express both addressins; other vessels, three of which are highlighted by arrows, express PNA but not MAdCAM-1. Immunoperoxidase stain with MAb MECA-79 (A) and MECA-367 (B); methylene blue counterstain;  $\times 100$ .

showed a change in their patterns of addressin expression. In AKR mice (3- to 4-week AKR/J and 13- to 16-week AKR/C) with early thymic hyperplasia, the majority of the HEVs expressed MAdCAM-1 but not PNA (Figure 2). As the mice increased in age, there was a dramatic increase in the number of thymic PNA-expressing HEVs but little to no increase in the number of such vessels expressing MAdCAM-1. In 31-week-old AKR mice with well developed thymic hyperplasia, PNA expression on HEV clearly predominated over MAdCAM-1 expression. Some of the PNA-expressing vessels in the hyperplastic thymuses co-expressed MAdCAM-1, with the majority of individual endothelial cells in each such HEV expressing both addressins (Figure 1). In all of the AKR thymuses examined, the addressin-expressing HEVs were confined exclusively to the extrafollicular (non-B cell) areas of the hyperplastic medulla.



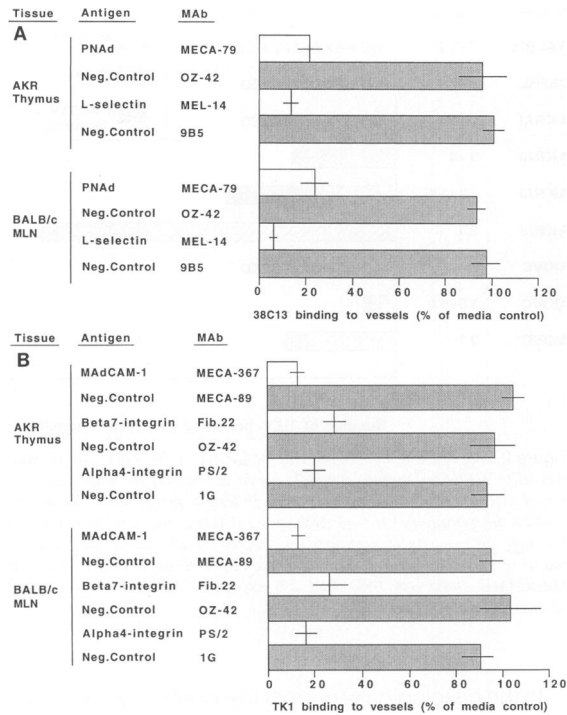
**Figure 2.** Expression of PNA and MAdCAM-1 by HEVs in thymic medulla. HEVs in thymic medulla were evaluated for expression of PNA and MAdCAM-1 by a two-color IF technique followed by quantitative morphometry ( $n = 3$  AKR or 10 BALB/c and C57BL thymuses per age group). As a comparison, BALB/c MLN paracortex had a mean of 10.1 HEVs/mm<sup>2</sup>; 84% of these HEVs co-expressed PNA and MAdCAM-1, whereas 14% and 2% expressed MAdCAM-1 or PNA alone, respectively.

In the histologically normal thymuses of young AKR mice, the patterns of addressin expression were identical with those seen in BALB/c or C57BL/Ka thymuses (SA Michie and EC Butcher, unpublished observation); MAdCAM-1 was expressed by a few vessels with low endothelial morphology in the medulla and at the cortico-medullary junction, whereas PNA expression was limited to a very small number of cortical vessels with low to medium height endothelium.

There were no detectable differences in endothelial VCAM-1 expression between AKR, BALB/c, or C57BL/Ka thymuses. Specifically, VCAM-1 was expressed by a few vessels in the medulla of all thymuses examined (data not shown).

### In Vitro Lymphocyte-Endothelial Cell Binding Assays

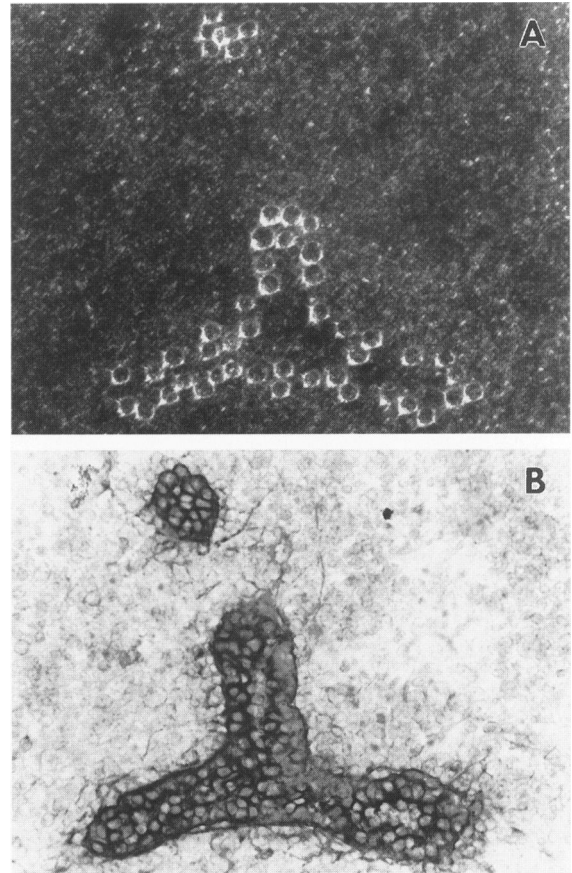
The Woodruff-Stamper *in vitro* assay was used to study the specific molecular interactions involved in the adhesion of lymphoid cells to vascular endothelium in hyperplastic thymuses of 30- to 36-week-old AKR/J and AKR/C mice.<sup>38,39</sup> To investigate the roles of lymphocyte HRs and vascular addressins in this adhesion, we used two mouse lymphoma cell lines that show tissue-selective binding to HEVs in organized lymphoid organs.<sup>5,10,13,24,25</sup> Results from a representative experiment are illustrated in Figures 3 and 4. 38C13 lymphoma cells, which selectively bind to PLN but not mucosal HEVs with an L-selectin/PNA recognition system, bound to many vessels in the



**Figure 3.** The ability of MAb against HRs or addressins to interfere with binding of lymphoid cells to endothelium in AKR hyperplastic thymuses and for comparison with MLN HEVs was determined by using an *in vitro* assay of lymphoid cell-endothelial binding. Binding results after MAb treatment are presented as percentage of binding in the media control. **A:** 38C13 lymphoma cells, which bind to HEVs via an L-selectin/PNAd interaction, bound to vessels in medulla of hyperplastic thymuses from 31-week-old AKR/J mice ( $n = 3$ ). There was similar binding of 38C13 cells to BALB/c MLN ( $n = 2$ ) where most HEVs co-express PNAd and MAdCAM-1. Binding to vessels in thymus and MLN was inhibited by MAb against L-selectin (MEL-14) or PNAd (MECA-79) but not by isotype-matched control MAb (shaded bars; mean  $\pm$  SD). **B:** TK1 lymphoma cells, which bind to endothelium via an  $\alpha_4\beta_7$ /MAdCAM-1 interaction, bound to vessels in 31-week-old AKR/J hyperplastic thymus and BALB/c MLN. Binding was inhibited by MAb against MAdCAM-1 (MECA-367) or against either chain of the  $\alpha_4\beta_7$  integrin (anti- $\beta_7$  MAb Fib 22; anti- $\alpha_4$  MAb PS/2). There was no significant inhibition of binding by negative control MAb (shaded bars; mean  $\pm$  SD).

AKR thymic medulla (Figures 3A and 4). This binding was almost completely inhibited by preincubation of the lymphoma cells with anti-L-selectin MAb MEL-14 or by preincubation of the thymus tissue sections with anti-PNAd MAb MECA-79 (Figure 3A). TK1 lymphoma cells, which bind selectively to mucosal but not PLN HEVs with an  $\alpha_4\beta_7$ /MAdCAM-1 recognition system, also bound to vessels in the thymic medulla; much of this binding was blocked by anti-MAdCAM-1 antibody (MAb MECA-367, Figure 3B) or by MAbs against the  $\alpha_4\beta_7$  molecule (anti- $\alpha_4$  MAb PS/2 and anti- $\beta_7$  MAb Fib22, Figure 3B; anti- $\alpha_4\beta_7$  heterodimer MAb DATK32, data not shown).

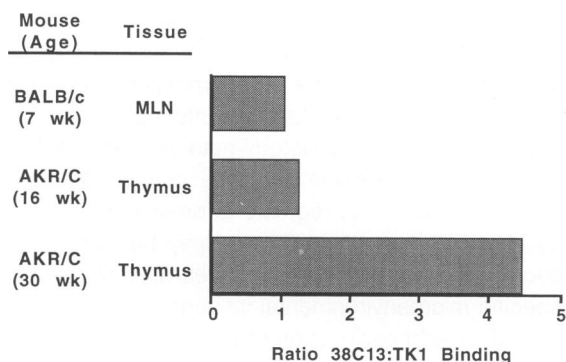
To further delineate the relative functional contributions of L-selectin interactions to  $\alpha_4\beta_7$  interactions in the binding of lymphoid cells to vessels in AKR hyperplastic



**Figure 4.** Serial frozen sections from hyperplastic thymus of 31-week-old AKR/J mouse show specific binding of 38C13 lymphoma cells (**A**) to PNAd-expressing HEV (**B**) in thymic medulla (**A**: dark-field microscopy of unstained slide from *in vitro* lymphocyte-endothelial binding assay  $\times 300$ ; **B**: immunoperoxidase stain with MAb MECA-79 (methylene blue counterstain,  $\times 300$ ).

thymus, the *in vitro* assay was performed with a mix of FITC-labeled 38C13 and unlabeled TK1 cells. The ratio of 38C13 cells to TK1 cells binding to HEVs in hyperplastic thymuses was compared with the ratio of binding to MLN HEVs, which express both PNAd and MAdCAM-1.<sup>14</sup> As illustrated in Figure 5, both adhesion systems were represented in hyperplastic thymuses of 16-week-old AKR/C mice. In contrast, in hyperplastic thymuses of 30-week-old AKR/C mice, there was clear predominance of L-selectin/PNAd-mediated lymphocyte/endothelial binding (Figure 5).

Most MLN lymphocytes express both the L-selectin and  $\alpha_4\beta_7$  HR and would thus be expected to bind to vessels expressing PNAd and/or MAdCAM-1.<sup>10,15,26</sup> We did not detect specific binding of MLN lymphocytes to vessels in histologically normal thymuses from BALB/c or 6- to 8-week-old AKR/C mice (data not shown). In contrast, MLN lymphocytes bound well to HEVs in hyperplastic thymuses from 31-week-old AKR/J mice. A large portion of this binding could be

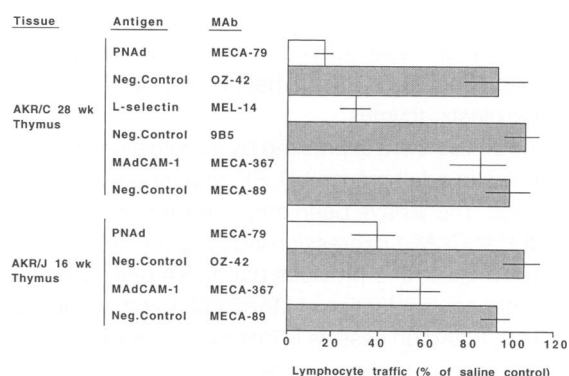


**Figure 5.** Relative contributions of L-selectin and  $\alpha_4\beta_7$  integrin HRs in lymphoid cell binding to endothelium in AKR hyperplastic thymus. The ratio of the vascular binding of 38C13 cells (L-selectin<sup>+</sup>,  $\alpha_4\beta_7$ <sup>-</sup>) to binding of TK1 cells (L-selectin<sup>-</sup>,  $\alpha_4\beta_7$ <sup>+</sup>) was determined by using an in vitro lymphocyte-endothelial binding assay. The contributions of L-selectin-mediated interactions relative to  $\alpha_4\beta_7$ -mediated interactions in the binding of lymphoid cells to vessels in 16-week-old AKR/C thymus were similar to that seen in BALB/c MLN. In contrast, L-selectin interactions dominated in thymuses of 30-week-old AKR/C mice with marked thymic hyperplasia (*n* = 3 mice per group).

blocked with MAb against L-selectin or PNAd (percent inhibition (mean  $\pm$  SD for three mice) was 68  $\pm$  8 and 60  $\pm$  13, respectively) and a smaller portion blocked with MAb against  $\alpha_4$  or MAdCAM-1 (percent inhibition was 25  $\pm$  3 and 31  $\pm$  10, respectively). MAb MECA-79 (anti-PNAd) mixed with MECA-367 (anti-MAdCAM-1) produced almost complete inhibition of lymphocyte binding to vessels in the hyperplastic thymus (84% inhibition to vessels in thymus as compared with 87% inhibition to vessels in MLN). These data indicate that PNAd and MAdCAM-1 are major ligands for lymphocyte binding to HEV in the hyperplastic thymuses of AKR mice.

### In Vivo Lymphocyte Traffic Studies

We used lymphocyte transfer experiments to determine the *in vivo* significance of the L-selectin/PNAd and  $\alpha_4\beta_7$ /MAdCAM-1 pathways in the traffic of lymphocytes to hyperplastic AKR thymus. In these experiments we evaluated the ability of MAb against L-selectin, PNAd, and MAdCAM-1 to block the homing of the transferred LN lymphocytes to the host thymus. Two groups of host mice were used in these experiments: 28-week-old AKR/C mice in which thymic PNAd-expressing HEVs predominate in numbers over MAdCAM-1-expressing HEVs; and 16-week-old AKR/J mice in which there are roughly equal numbers of PNAd-expressing and MAdCAM-1-expressing HEVs in the thymus (Figure 2). Within each of these two groups of hosts, previous studies have shown little variability among individual mice in the amount of lymphocyte traffic to the thymus.<sup>2</sup> Results of the current experiments are illustrated in Figure 6. Anti-



**Figure 6.** The ability of MAbs against HRs or addressins to interfere with trafficking of LN lymphocytes to AKR hyperplastic thymuses was determined by using short-term adoptive transfer experiments. Results are presented as a percentage of donor lymphocytes migrating into thymuses of host mice in which the specific or negative control MAb was replaced with saline. MAb against PNAd (MECA-79) blocked much of the traffic of adoptively transferred MLN lymphocytes to hyperplastic thymuses of 28-week-old AKR/C and 16-week-old AKR/J mice. Anti-MAdCAM-1 (MAb MECA-367) blocked some of the lymphocyte traffic to the AKR/J thymuses but had only a slight effect on traffic to the AKR/C thymuses (mean  $\pm$  SD; *n* = 3 mice per treatment).

L-selectin (MAb MEL-14) and anti-PNAd (MAb MECA-79) significantly blocked lymphocyte traffic to hyperplastic thymuses of the 28-week-old AKR/C mice. Although there was little blocking of traffic by anti-MAdCAM-1 (MAb MECA-367), immunohistochemistry revealed very few MAdCAM-1-expressing vessels in these thymuses (data not shown). In contrast, in the 16-week-old AKR/J host mice, both anti-PNAd and anti-MAdCAM-1 MAbs produced significant blocking of traffic to the thymuses.

### Discussion

The migration of lymphocytes from blood into organized lymphoid tissues such as LN and PP involves the interaction of lymphocyte HRs with vascular addressins on specialized HEVs in these tissues.<sup>6-16,24-26,29-33</sup> In an earlier study of lymphocyte traffic into the hyperplastic thymus of AKR mice, we showed that there is increased migration of B and T lymphocytes, most expressing high levels of the L-selectin HR, to the hyperplastic as compared with the normal thymic medulla.<sup>2</sup> After publication of that study, MAbs reactive with the  $\alpha_4\beta_7$  integrin HR and peripheral and mucosal addressins became available; these MAbs have enabled us to directly examine the molecular mechanisms that regulate traffic of mature B and T lymphocytes from the periphery to the hyperplastic thymus. We demonstrate that morphologically classic HEVs develop in the medulla of the hyperplastic thymus and that many of these HEVs express PNAd whereas a smaller number express

MAdCAM-1. Furthermore, we have directly demonstrated that these addressin molecules function *in vivo* to direct the traffic of mature lymphocytes to the hyperplastic thymus.

Addressins are expressed by endothelium of many venules in adult mouse LN, PP, and intestinal lamina propria. There have been only a few reports of PNA<sub>d</sub> and MAdCAM-1 expression induction in other tissues in the mouse. In a previous paper, we described the spontaneous induction of MAdCAM-1 and, to a lesser extent, PNA<sub>d</sub> on vessels in inflamed pancreatic islets of nonobese diabetic mice and used an *in vitro* lymphocyte-endothelial cell binding assay to demonstrate that these addressins function to bind lymphocytes.<sup>20</sup> The expression of addressins in nonobese diabetic pancreas has been confirmed by Faveeu and colleagues.<sup>23</sup> Sarvetnick and colleagues demonstrated similar induction of MAdCAM-1 and PNA<sub>d</sub> expression on vessels in inflamed islets of diabetic interferon- $\gamma$  transgenic and interleukin-10 transgenic mice.<sup>21,22</sup> Two groups have shown that MAbs to MAdCAM-1 react with a small number of vessels in the central nervous system of mice with relapsing experimental autoimmune encephalomyelitis. The functional significance of MAdCAM-1 expression by these vessels is unclear, as lymphocyte binding studies were not conducted.<sup>18,19</sup> Addressin expression in the central nervous system of mice with acute experimental autoimmune encephalomyelitis or *Corynebacterium parvum*-induced inflammation has not been reported.<sup>18,42,43</sup> Addressin expression was not observed at a variety of other sites of chronic inflammation in the mouse. These sites include inflamed skin induced by a variety of stimuli including complete Freund's adjuvant, lipopolysaccharide, concanavalin A, tumor necrosis factor- $\alpha$ , interleukin-1, interferon- $\gamma$ , skin allografts, subcutaneous sponge allografts, and DTH responses, and sheep red blood cell-induced inflammation of lung (B Englehardt, MT Martin, SA Michie, and EC Butcher, unpublished observations).

In contrast to the situation in the mouse, PNA<sub>d</sub> is expressed by a wide variety of chronically inflamed human tissues including skin, synovium, and thyroid.<sup>27,28</sup> Although the existence of a human equivalent of MAdCAM-1 has been inferred through functional studies of lymphocyte-endothelial cell binding, its immunohistological distribution in sites of inflammation has not been studied because of a lack of MAbs against human MAdCAM-1.<sup>44</sup>

Several factors may affect expression of addressins by endothelial cells in organized lymphoid tissues and extranodal sites of chronic inflammation. Immune response cytokines may play some role, as

suggested by the recent finding that expression of MAdCAM-1 but not PNA<sub>d</sub> can be induced on a cultured endothelial cell line by inflammatory mediators such as tumor necrosis factor- $\alpha$ , interleukin-1, and lipopolysaccharide.<sup>45</sup> Indeterminate lymph-borne factors, which may include cytokines, cells, or antigens have been shown to regulate addressin expression by LN HEVs<sup>46,47</sup>; these factors may be involved in such regulation at extranodal sites as well. Tissue-specific microenvironmental influences are likely involved in addressin expression, as suggested by transplantation studies<sup>48</sup> and by the tissue-specific differences in addressin expression between PLN and PP of adult mice.<sup>14,24</sup>

A large body of work suggests that lymphocyte binding to LN HEVs involves the interaction of the lectin domain of lymphocyte L-selectin with PNA<sub>d</sub> carbohydrate determinants. These carbohydrate determinants are specifically recognized by the MECA-79 MAb. Although MECA-79 reacts with several glycoproteins found in mouse LN, only three of these have been characterized at the molecular level: GlyCAM-1 and CD34 are sulfated, mucin-like molecules with a high content of O-linked carbohydrates, whereas MAdCAM-1 includes a mucin domain that may be capable of presenting carbohydrate determinants.<sup>29-33</sup> The functional moiety on murine GlyCAM-1 is a sulfated variant of the sialylated Lewis<sup>x</sup> (sLe<sup>x</sup>) blood group structure.<sup>49</sup> The functionally important L-selectin binding carbohydrates on CD34 and MAdCAM-1 have not been identified.

The induction of PNA<sub>d</sub> expression by vascular endothelium in AKR hyperplastic thymus can be explained by several different mechanisms: 1) there may be induction of glycosylation mechanisms that then decorate pre-existing proteins with carbohydrate side chains that react with MECA-79 and support L-selectin-dependent lymphocyte binding; 2) synthesis of protein backbones may be induced, which are then decorated with MECA-79-reactive carbohydrates by pre-existing glycosylation mechanisms; or 3) some combination of the above mechanisms.

Most HEVs in mouse MLN react with MECA-79 and MECA-367.<sup>14,24</sup> Berg and colleagues have recently shown that a subset of MLN MAdCAM-1 expresses MECA-79-reactive carbohydrates that support L-selectin-dependent lymphocyte rolling.<sup>33</sup> We have shown that some HEVs in AKR hyperplastic thymus react with both MECA-79 and MECA-367, raising the possibility that these vessels may express MAdCAM-1 that bears MECA-79 carbohydrate epitopes. A similar situation may exist in pancreata of diabetic mice, in which a subset of HEVs in inflamed

islets react with both MECA-79 and MECA-367.<sup>20,23</sup> Additional study is needed to identify the molecules that express MECA-79 epitopes in HEVs of hyperplastic thymus and inflamed pancreas and to determine the physiological roles of the various L-selectin ligands in leukocyte traffic to organized lymphoid tissues and sites of inflammation.

In the normal adult mouse, a small number of mature B and T lymphocytes migrate to the thymic medulla.<sup>41,50,51</sup> The significance of this low level of traffic to the thymus is unclear, although it has been suggested that these lymphocytes are involved in immunological memory or tolerance induction.<sup>51-53</sup> The roles of addressins and HRs in lymphocyte traffic to normal thymus have not been delineated. Most of the B and T lymphocytes having recently entered the normal thymus are L-selectin<sup>lo/neg</sup>,<sup>2,41</sup> suggesting that the L-selectin HR does not play a major role in mature lymphocyte migration to normal thymus. It is possible, however, that these cells are L-selectin<sup>hi</sup> when they enter the thymus and that receptor expression is down-regulated during or soon after entry.<sup>54</sup> To clarify the roles of addressins and HRs in the migration of lymphocytes to normal thymus, *in vivo* studies need to be carried out to determine whether MAb against HRs or addressins can block this migration.

The amount of lymphocyte traffic to histologically normal thymuses of young AKR mice is similar to that of adult BALB/c and C57BL mice.<sup>2,41</sup> However, with increasing age and increasing thymic medullary hyperplasia, there is a marked increase in traffic of peripheral B and T cells to the AKR thymus.<sup>2</sup> Although the physiological significance of this increase in lymphocyte traffic is unclear, it correlates temporally with the increase in expression of retroviral antigens within the thymus, suggesting that the lymphocytes might be involved in an immune response against the viral antigens.<sup>55</sup>

In this study, we demonstrate that morphologically classic HEVs develop in the medulla of the hyperplastic thymus of AKR mice and that these HEVs express PNA<sup>d</sup> and MAdCAM-1. Furthermore, we demonstrate that these addressins function *in vivo* to control the migration of lymphocytes to the hyperplastic thymus. These results, together with those of our previous study showing increased migration primarily of L-selectin<sup>hi</sup> lymphocytes to the hyperplastic thymus as compared with the normal thymus, show that mechanisms involved in traffic of lymphocytes to hyperplastic thymus are similar to those involved in lymphocyte traffic to organized lymphoid tissues such as LN and PP.

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