

LYMPHOCYTE HOMING INTO LYMPH NODES:  
IN VITRO DEMONSTRATION OF THE SELECTIVE  
AFFINITY OF RECIRCULATING  
LYMPHOCYTES FOR HIGH-ENDOTHELIAL VENULES\*

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Recirculating lymphocytes emigrate from the blood into lymph nodes via a system of specialized venules which are distinguished by endothelium composed of tall cells containing large pale vesicular nuclei and abundant cytoplasm. The site of entry is specific for high-endothelial venules (HEV) and no migration of lymphocytes into the tissue occurs through other vascular endothelia (1-5). The basis of this selectivity is unknown although it has been suggested that lymphocytes have surface receptors which recognize endothelium of the specialized venules (6, 7).

This report describes an in vitro model developed to study the mechanism of lymphocyte homing into lymph nodes. When murine lymphocyte suspensions are layered over fixed sections of syngeneic lymph nodes, the cells adhere specifically to HEV but not to other vascular structures. Lymphocytes obtained from thoracic duct lymph, lymph nodes, and spleen adhere to the vast majority of HEV in each section, whereas thymus and bone marrow are deficient in cells capable of binding to HEV in vitro.

### Materials and Methods

*Animals.* Wistar-Furth rats were originally purchased from Microbiological Associates, Bethesda, Md. and then bred and maintained in the animal facilities at Downstate Medical Center. Males weighing 170-200 g were used. Female BALB/cJ mice obtained from The Jackson Laboratory, Bar Harbor, Maine were used at 6-12 wk of age.

*Cell Suspension.* Thoracic duct lymphocytes (TDL) were obtained from rats during the first 28 h after thoracic duct cannulation (1). Lymph was collected at room temperature in 20 ml of Dulbecco's phosphate-buffered saline (PBS; pH 7.4) containing heparin (5 U/ml), penicillin (500 U/ml), and streptomycin (100  $\mu$ g/ml). Cells used in experiments were from lymph obtained during the last 4-6 h of collection. Lymph was centrifuged at 400 g for 10 min at 4°C and the cells washed two to three times in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (pH 7.4). TDL were resuspended at a concentration of  $3 \times 10^7$ /ml of RPMI, placed in an ice bath, and used in experiments 30-60 min later. In most experiments the RPMI medium was prepared without  $\text{NaHCO}_3$  in order to maintain the pH at 7.4 during the in vitro binding reaction. NaCl was added to make this medium isotonic. Identical results were obtained when the assays were performed using RPMI containing  $\text{NaHCO}_3$ , although the pH of the lymphocyte suspensions at the end of the experiment was frequently at or above 8.0.

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Single cell suspensions of thymus, spleen, and peripheral lymph nodes were obtained by gently teasing tissues into cold RPMI medium ( $\text{NaHCO}_3$  deficient). Bone marrow cells were collected by flushing the tibia and fibula with medium and the cells were dispersed by pipetting. After allowing debris to settle by gravity, each cell population was washed three times in RPMI medium and resuspended at a concentration of  $3 \times 10^7$  mononuclear cells/ml. All suspensions were kept on ice before use.

*Preparation of Lymph Node Sections.* Cervical lymph nodes were obtained from ether-anesthetized rats and axillary or inguinal nodes from mice killed by cervical dislocation. The tissues were rapidly frozen to  $-15^\circ\text{C}$  in a Cryo-Cut Cryostat Microtome (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). Cryostat sections ( $8 \mu\text{m}$ ) were mounted on microslides near one end, air dried for about 5 min, and fixed for 10 min at  $4^\circ\text{C}$  in freshly prepared 3% glutaraldehyde (Fisher Scientific Co., Fair Lawn, N. J.) in PBS. After fixing, the sections were washed in running cold deionized water for 30 min, the water decanted, and the slides kept in Coplin jars on ice. The sections were used in experiments 30–90 min later.

*Assay for In Vitro Binding of Lymphocytes to HEV in Lymph Node Sections.* All steps were carried out in a cold room ( $7^\circ\text{C}$ ). Aliquots containing  $6 \times 10^6$  lymphocytes in 0.2 ml of RPMI were placed over each lymph node section and the slides immediately placed on a rotating table (Clinical Rotator; Eberbach Corp., Ann Arbor, Mich.) operating at 80 rpm. The slides were placed so that the sections were directed toward one edge of the table with the opposite edge raised approximately 1 cm. This ensured that the cell suspension remained on the section and did not flow freely over the entire slide. After incubation for 30 min the sections were washed by dipping the slides repeatedly in cold PBS and then fixed for 10 min in 3% glutaraldehyde on ice. After washing with deionized water the sections were stained with methyl-green-thionin at  $37^\circ\text{C}$  for 15 min and examined under the light microscope. Each experiment was performed using sections obtained from a single lymph node and the assays were carried out in triplicate or quadruplicate.

## Results

HEV in fixed sections of lymph node were identified by the characteristic appearance of the endothelial cells (1). The vessels were found throughout the paracortex but were not present in the subcapsular area or the medulla. The number of HEV in each section varied depending on the size of the lymph node; rat nodes generally had 100–200 HEV per section and mouse lymph nodes 20–40 per section (Tables I and III). Usually 20 sections were obtained from a rat lymph node and 8 to 12 from a mouse node. Fixed sections were used since preliminary experiments showed that loss of endothelial cells from HEV occurred when the assay was carried out with unfixed tissue.

When rat TDL were incubated with rat lymph node sections the overlaid lymphocytes adhered selectively to endothelial cells of HEV. The adherent TDL were easily identified as small, intensely staining round cells which were not in the same plane as tissue lymphocytes. Fig. 1 illustrates the typical appearance of HEV with adherent TDL. Lymphocytes bound both to dilated HEV and to those with no apparent lumen and were usually found directly over the endothelium. Generally, 70–85% of the TDL which adhered to each section were localized over the HEV with the remainder scattered randomly over the node. TDL were not found in the subcapsular marginal sinuses and did not bind to the flat endothelium of cortical capillaries, medullary venules, or lymphatic sinuses.

Table I presents the results of three representative experiments. Approximately 75–90% of the HEV in each rat lymph node section contained two or more adherent TDL. The number of TDL which attached to each vessel varied but was generally proportional to the area of the endothelium exposed. Approximately 40–60% of positive HEV (venules binding two or more lymphocytes) had more than five TDL with some containing as many as 60–80 adherent cells.

TABLE I  
*Pattern of Adherence of Rat TDL to HEV in Rat Lymph Node Sections*

Exp.	% of HEV per section with indicated number of adherent TDL						No. of HEV per section‡	% Positive HEV per section§
	0-1*	2-5	6-10	11-20	21-30	>30		
1	13 ± 3‡	39 ± 3	26 ± 4	15 ± 1	4 ± 1	2 ± 1	244 ± 17	87 ± 3
2	24 ± 8	46 ± 4	17 ± 4	10 ± 4	3 ± 2	1 ± 1	117 ± 11	76 ± 8
3	16 ± 4	36 ± 1	25 ± 1	17 ± 2	4 ± 2	2 ± 1	172 ± 16	84 ± 4

\* Number of TDL adhering to each HEV.

‡ Mean ± SE of three to four sections.

§ Mean ± SE of HEV per lymph node section with two or more adherent lymphocytes.

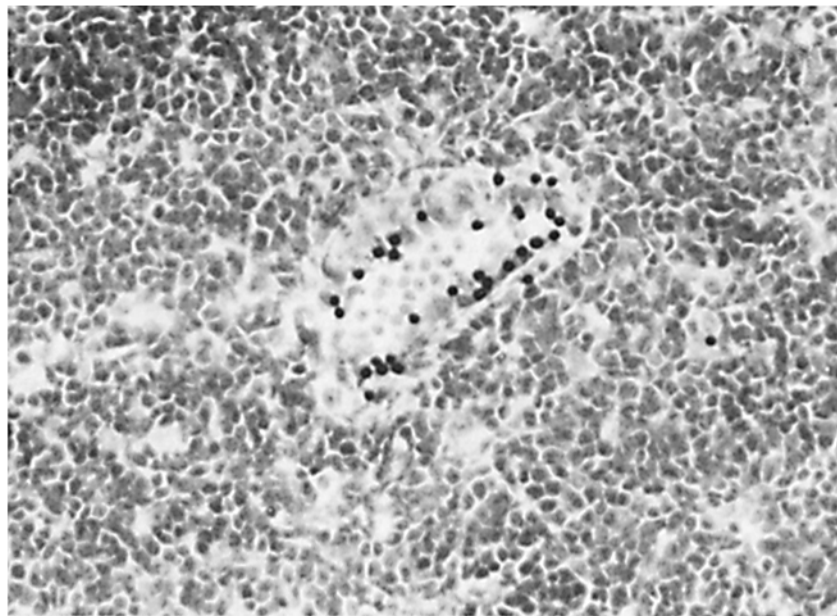


FIG. 1. Section of a rat lymph node showing overlaid TDL adhering to a HEV. TDL are darkly stained and not in the same plane as the tissue section. (Methyl-green-thionin, × 200.)

Thoracic duct lymph, lymph nodes, and spleen contain substantial numbers of recirculating lymphocytes capable of homing into lymph nodes when infused intravenously, whereas very few recirculating cells are found within the thymus and bone marrow (8). If lymphocyte adherence to HEV in lymph node sections correlates with the capacity of the cells to recirculate in vivo, then it might be expected that lymph node or spleen cells would also attach to HEV in vitro, while binding would be minimal with thymus or bone marrow cells. The results of two representative experiments in which the prediction was tested using rat lymphoid cells are shown in Table II. On the average 86-88% of the HEV in each rat lymph node section showed two or more adherent lymphocytes when the tissue was incubated with TDL, lymph node, or spleen lymphocytes. In contrast, only 6-10% of the HEV showed adherent lymphocytes when the sections were overlaid with thymus or bone marrow cells. Differences in the binding activity

TABLE II  
Tissue Distribution of Rat Lymphocytes Capable of Adhering to HEV in Lymph Node Sections

Exp.	Cell source	% Positive HEV per section*
1	Thoracic duct lymph	87 ± 3
	Lymph nodes	88 ± 7
	Spleen	86 ± 2
	Thymus	10 ± 5‡
2	Lymph nodes	86 ± 4
	Spleen	81 ± 3
	Thymus	6 ± 3‡
	Bone marrow	8 ± 3‡

One rat lymph node was used for each experiment and alternate sections were treated in sequence with the indicated cell suspension; three to four sections per group.

\* Mean ± SE of HEV per section with two or more adherent lymphocytes.

‡ Significantly lower ( $P < 0.005$ ) than values obtained with TDL, spleen, or lymph node cells.

TABLE III  
Pattern of Adherence of Mouse Lymphocytes to HEV in Lymph Node Sections

Exp.	Cell source	% of HEV per section with indicated number of adherent lymphocytes					No. of HEV per section‡	% Positive HEV per section§
		0-1*	2-5	6-10	11-20	>20		
1	Spleen	47 ± 11‡	30 ± 3	15 ± 7	9 ± 4	3 ± 2	41 ± 6	53 ± 11
2	Spleen	28 ± 7	43 ± 3	19 ± 2	8 ± 3	2 ± 1	41 ± 2	72 ± 7
3	Spleen	34 ± 8	41 ± 6	17 ± 3	6 ± 3	2 ± 1	23 ± 3	67 ± 8
	Lymph nodes	38 ± 13	41 ± 3	19 ± 9	3 ± 2	0	28 ± 5	62 ± 13
	Thymus	97 ± 1	3 ± 3	0	0	0	20 ± 2	3 ± 3

In each experiment sections were prepared from a single mouse lymph node. In exp. 3 alternate lymph node sections were treated in sequence with the indicated cell suspension.

\* Number of lymphocytes adhering to each HEV.

‡ Mean ± SE of three to four sections.

§ Mean ± SE of HEV per section with two or more adherent lymphocytes.

|| Significantly lower ( $P < 0.001$ ) than values obtained with spleen or lymph node cells.

of thymocytes and bone marrow cells on the one hand and TDL, lymph node, and spleen cells on the other were statistically significant ( $P < 0.005$ ).

The results presented in Table III show that mouse lymphocytes obtained from spleen or lymph nodes also possessed the capacity to adhere to HEV in vitro. In three separate experiments 53-72% of the HEV per section contained adherent lymphocytes when the sections were treated with spleen cells and comparable results were obtained using lymph node cells. Approximately 80% of the adherent cells were attached to HEV. With both cell suspensions the overlaid lymphocytes were not found associated with other vascular structures. When sections were incubated with thymocytes, only 3% of the HEV in each section were positive for adherent cells and the extent of binding was relatively low with only two to five lymphocytes adhering to each HEV. In contrast, when lymph node sections were incubated with lymph node or spleen cells approximately 20-30% of the HEV contained six or more adherent lymphocytes.

### Discussion

This report describes an in vitro approach to study the affinity of recirculating lymphocytes for endothelium of HEV. Fixed lymph node sections from rats were overlaid with suspensions of syngeneic TDL, incubated at 7°C for 30 min on a

rotating table, and then washed. TDL adhered to the section in a highly regular and reproducible fashion. They were found localized over the endothelium of HEV but did not bind to other vascular structures in the nodes. The vast majority of HEV in each section showed adherent lymphocytes. Usually 2-20 TDL attached to each vessel although some HEV showed as many as 60 or more adherent lymphocytes. Approximately 15-30% of the TDL which adhered to the section were not found attached to HEV. This "nonspecific" binding occurred in a random and irregular fashion and was present in germinal centers and medulla, as well as the cortex. When sections were counted for adherent TDL using a calibrated ocular grid, the mean lymphocyte density over HEV was approximately 100-fold greater per unit area than that found in non-HEV sites.

The selective adherence of TDL to HEV in lymph node sections correlates precisely with observations in animals showing that lymphocytes emigrate from the blood into nodes only via the specialized venules (1-4, 8). Further evidence of the correlation between lymphocyte adherence to HEV *in vitro* and homing into the nodes *in vivo* was provided by experiments determining the tissue distribution of lymphocytes with the capacity to bind to HEV *in vitro*. Very low levels of binding were observed when HEV were exposed to rat thymus or bone marrow cells. However, adherence of lymph node and spleen cells to HEV was comparable to that observed with TDL. Similarly, in the mouse, lymphocytes capable of binding to HEV *in vitro* were present in spleen and lymph nodes but very few could be detected in the thymus.

These results provide direct evidence that recirculating lymphocytes possess surface receptors for recognition of HEV. Observations with bone marrow and thymus cells suggest that nonrecirculating lymphocytes lack the appropriate receptors although it cannot be ruled out that their poor binding activity reflects metabolic rather than surface membrane properties.

The assay described in this communication should prove useful in elucidating the molecular basis of homing and improve understanding of factors which affect lymphocyte traffic into lymph nodes. Clearly, more direct analysis of lymphocyte-HEV interaction can be accomplished by this technique than by *in vivo* transfer studies. The technique may also permit investigation of homing mechanisms in humans and detection of possible defects in lymphocyte recirculation in patients with various immunologic disorders.

### Summary

An *in vitro* model is described for studying the interaction between lymphocytes and high-endothelial venules (HEV) of lymph nodes. Rat or mouse lymphocytes which were layered over fixed sections of syngeneic lymph nodes adhered selectively to the endothelium of HEV but did not bind to other vascular endothelia. Evidence is presented that adherence to HEV *in vitro* is a property of recirculating lymphocytes and not a characteristic of cells which are unable to home into lymph nodes *in vivo*.

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## References

1. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. Roy. Soc. Ser. B.* 159:257.
2. Howard, J. C. 1972. The life span and recirculation of marrow-derived small lymphocytes from rat thoracic duct. *J. Exp. Med.* 135:185.
3. Sprent, J. 1973. Migration of T and B lymphocytes in the mouse. I. Migratory properties. *Cell. Immunol.* 7:10.
4. Gutman, G. A., and I. L. Weissman. 1973. Homing properties of thymus-independent follicular lymphocytes. *Transplantation (Baltimore)*. 16:621.
5. Herman, P. G., I. Yamamoto, and H. Z. Mellins. 1972. Blood microcirculation in the lymph node during the primary immune response. *J. Exp. Med.* 136:697.
6. Gesner, B. M., and V. Ginsburg. 1964. Effect of glycosidases on the fate of transfused lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 52:750.
7. Woodruff, J., and B. M. Gesner. 1968. Lymphocytes: circulation altered by trypsin. *Science (Wash. D. C.)*. 161:176.
8. Ford, W. L. 1975. Lymphocyte migration and immune responses. *Prog. Allergy*. 19:1.