Immunology 1976 **31** 731

Lymphocyte emigration from high endothelial venules in rat lymph nodes

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Received 12 *February* 1976; *accepted for publication* 12 *April* 1976

Summary. Sequential events during lymphocyte emigration from high endothelial venules (HEV) were studied by scanning and transmission electron microscopy combined with regional perfusion techniques. The results indicate that blood lymphocytes selectively adhere to HEV surfaces through microvilli which attach to shallow pits on the luminal surfaces of high endothelial cells. These intercellular contact points resist hydrodynamic and osmotic shearing forces, but can be disrupted by treatments which remove endothelial glycocalyx, hydrolyse lymphocyte surface glycoproteins, or chelate divalent cations. After this initial attachment phase, lymphocytes enter apical clefts between endothelial cells where they assume a motile configuration characterized by loss of microvilli and formation of irregular surface folds. Intramural lymphocytes adhere to adjacent endothelial cells through macular and villous contacts. Fibrillar electron-dense material traverses the 15–20 nm gap at these points of adhesion. Microtubules and microfilaments are also seen around areas of cytoplasmic constriction in these motile lymphocytes.

The migrating lymphocytes show cytoplasmic polarity which is oriented in the direction of movement as they pass through extracellular spaces in the venular wall and cross successive laminations in the perivascular sheath to enter the node. Since these lymphocytes enter channels between endothelial cells which are stained by intralymphatic injections with horseradish peroxidase, it is suggested that their entry into the node depends upon migration along a chemotactic gradient.

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INTRODUCTION

Previous studies (Gowans, 1959; Gowans & Knight, 1964; Howard, Hunt & Gowans, 1972) established that recirculating T and B lymphocytes leave the blood and enter lymph nodes by migrating across the walls of high endothelial venules (HEV). However, there has been prolonged debate over the migration pathways and mechanisms which regulate this cellular traffic. Suggestions that some lymphocytes may move in the opposite direction to enter the bloodstream (Sainte-Marie, Sin & Chan, 1967) have never been proven. Lymphocytes have been reported to accumulate in HEV lumens (Sainte-Marie & Sin, 1970) where they attached to endothelial surfaces by ill-defined cellular recognition mechanisms (Woodruff & Gesner, 1968, 1969; Vincent & Gunz, 1970). Early light microscopic studies were generally interpreted as showing that lymphocytes migrated through intercellular spaces between endothelial cells as they crossed the venular wall (von Schumacher, 1899; Zimmermann, 1923;

Hummel, 1935). This tenet was discarded when electron microscopic observations by Marchesi & Gowans (1964) indicated that lymphocytes emigrated from HEV by passing directly through endothelial cell cytoplasm in a manner quite distinct from the intercellular migration of granulocytes. Subsequent ultrastructural studies (Schoefl, 1972; Wenk, Orlic, Reith & Rhodin, 1974) demonstrated that virtually all intraendothelial lymphocytes were within extracellular spaces, but Farr & De Bruyn (1975) concluded that lymphocytes reached these sites after initial passage through the luminal portions of endothelial cytoplasm. Consequently, intracellular migration is still widely regarded as a characteristic feature of lymphocyte recirculation.

This report describes the sequence of lymphocyte emigration from HEV using scanning and transmission electron microscopy in combination with regional perfusion techniques. The results indicate that lymphocytes actively migrate across HEV walls by passing through extracellular spaces which permit unidirectional flow of macromolecules from the lymph node into the venular lumen.

MATERIALS AND METHODS

Animals

Adult male Lewis and Wistar rats (Microbiological Associates, Walkersville, Maryland), weighing between 200 and 250 *g* were used.

Anaesthesia

Rats were anaesthetized for all operative procedures by intraperitoneal injections of chloral hydrate at dosages of 360 mg/kg body weight.

Scanning electron microscopy

Lymph nodes were prepared for scanning electron microscopy (SEM) using both immersion and perfusion fixation with 3% glutaraldehyde in 0·05 M cacodylate buffer, pH 7·2 with 6·8% sucrose at room temperature. After 4–6 h in fixative, the tissues were washed for 15 min in distilled water prior to post-fixation to prevent the appearance of granular deposits over their surfaces. Then they were placed in osmium tetroxide (1% in 0·05 M cacodylate buffer, pH 7·2, with 1·5% potassium ferrocyanide) for 2 h at room temperature. The tissues were cut into 2-mm cubes and dehydrated in alcohol. They were brought to Freon TF and after 1 h immersion, the tissue fragments were dried at the critical point of Freon 13 in a Bomar critical point drying apparatus (Bomar Company, Seattle, Washington). The dried tissue was mounted on carbon-coated copper discs with silver electrical paint and placed in a desiccator for 24 h. Then the tissue-bearing discs were coated with a thin layer of gold in a Jelco vacuum evaporator. The specimens were examined with a JEM-100 B high resolution transmission electron microscope modified with a scanning accessory (ASID) which was used to view sections at accelerating voltage of 20 and 40 KV with beam currents of 85 and 65 μA.

Transmission electron microscopy

Rats were killed by cervical dislocation at the termination of each experiment. Axillary and mesenteric lymph nodes were excised, minced into 1-mm cubes in cold 3 per cent glutaraldehyde in 0·1 M cacodylate buffer (pH 7·3) and transferred to fresh fixative for 2–4 h. In some studies, 1 per cent alcian blue (8 g) was added to the fixative to enhance preservation of surface coat oligosaccharides. After washing in 3 per cent sucrose 0·1 M cacodylate (pH 7·3), tissues were post-fixed in 1 per cent osmium tetroxide in Millonig's buffer (pH 7·3), dehydrated through graded alcohols and embedded in araldite. Thick sections were cut at 0·5–1·0 μ with glass knives in a Sorval MT-1 and stained with toluidine blue. Thin sections were cut at 600–900 Å with diamond knives in a Sorval MT-2 and mounted on uncoated 200 mesh copper grids. After staining with aqueous uranyl acetate and/or lead citrate, the grids were examined with an AEI 801 electron microscope.

Regional perfusion techniques for studying lymphocyte attachment to HEV

Anaesthetized rats were anticoagulated by intravenous injections with 200 u of heparin. The superior mesenteric artery was exposed through a mid-abdominal incision and cannulated with PE50 polyethylene tubing (Clay Adams, Parsippany, New Jersey) so that the distal tip of the cannula lay at the base of the mesenteric node. Extra-nodal vessels were occluded with vascular clamps after the superior mesenteric vein was cannulated with PE90 tubing. The isolated vascular bed of the mesenteric nodes was flushed with 10 per cent dextran (40,000 MW) in 0·9 per cent saline until the venous effluent contained less than 5×10^6 red cells/ml. Then perfusion was maintained with various solutions described below for an additional 12 min at 25° using 1 meter gravity pressure with flow rates

of 0·3 ml/min. The venous effluent was collected and total cellular output was determined by counting aliquots in a haemocytometer. Differential cell counts were made conventionally. At the conclusion of each study, 0·2 ml of 2% alcian blue was added to the perfusate to stain endothelial glycocalyx (Anderson & Anderson, 1975) and to identify perfused vessels within the isolated vascular bed. Then the nodes were excised, fixed in glutaraldehyde and examined by light microscopy to assess vascular integrity and determine the source of effluent cells. The perfusates used in this study were 0·9 per cent saline, dextran-saline without additives, and dextran-saline solutions containing 0·1 per cent trypsin (type III from bovine pancreas, Sigma Chemical Co., St. Louis, Missouri), 2·5 per cent trypsin, or 0·01 M trisodium ethylenediamine tetraacetic acid (EDTA).

Methods for delineating extracellular spaces in HEV

Several techniques were utilized to determine whether lymphocytes moved intra- or extracellularly as they crossed HEV walls. In some studies hypertonic solutions were used to enlarge extracellular spaces during fixation. Lymph node fragments were fixed in 4 per cent glutaraldehyde in 0·1 M cacodylate with osmolarity adjusted to 450–900 milliosmoles by addition of sucrose. After 90 min fixation at 4°, these specimens were washed in hypertonic sucrose and immediately post-fixed in cold osmium tetroxide to prevent reversal of the hypertonic changes (Bennett, Spira & Pappas, 1972). Then they were processed for electron microscopy as described previously.

Intravascular injections with electron-dense tracers were used to localize all lymphocytes moving across the HEV wall through spaces contiguous with the vascular lumen. This was achieved by retrograde infusion of 0·2 ml of tracer solution into the brachial artery using injection pressures which slightly exceed systolic blood pressure (Anderson & Anderson, 1975). The regional axillary lymph nodes were processed for electron microscopy in the usual manner following rapid excision and fixation in the cold to minimize endocytosis. Tracers employed in these studies were: stabilized, colloidal thorium dioxide (Thorotrast, Fellows Testagar Incorporated, Detroit, Michigan); 0·5% horseradish peroxidase (type II, Sigma Chemical Company, St Louis, Missouri); colloidal lanthanum in 1·5% glutaraldehyde, and dilute India ink (Pelikan, Gunther Wagner, Germany). In studies employing horseradish peroxidase, the electron-dense reaction product was developed with diaminobenzidine tetrahydrochloride (Sigma Chemical Company, St Louis, Missouri) and hydrogen peroxide using methods described by Graham & Karnovsky (1966).

Similar tracer techniques were employed to determine whether the walls of HEV served as functional lymph node-venous communications. After ligating the thoracic duct at the level of the diaphragm, 0·05 ml of physiologic saline containing 0·05% horseradish peroxidase and 0·05% trypan blue was injected into the subserosal lymphatic arcades of the small bowel using a 30-gauge needle. Flow of these tracers into mesenteric lymphatics was monitored by following the distribution of trypan blue. The draining mesenteric nodes were excised when their cortical sinuses turned blue. Then these nodes were fixed in 3 per cent glutaraldehyde, sectioned and the peroxidase reaction product was developed as described previously. Tissue sections were prepared for light and electron microscopy to define the intranodal distribution of the reaction product and its relation to lymphocytes migrating across HEV walls. The same experimental design was utilized to localize carbon particles in mesenteric nodes following intralymphatic injections with dilute India ink.

RESULTS

Lymphocyte accumulation within HEV lumens

HEV were readily identified in toluidine blue-stained thick sections by their polygonal endothelial cell lining and prominent perivascular sheaths. These multibranched venules appeared randomly dispersed in the lymph node cortex and extended from beneath the marginal sinus to the corticomedullary junction. Numerous lymphocytes were seen within the lumen, wall and sheath of each HEV segment. While some venules appeared to be filled with pure populations of lymphocytes, the ratio of lymphocytes to erythrocytes in HEV lumens was usually 2:1. Since granulocytes and monocytes were rarely seen in these vessels, attempts were made to characterize the mechanisms responsible for the selective sequestration of lymphocytes within HEV.

Examination of HEV in serial thick sections through normal nodes showed that the ratio of luminal lymphocytes to endothelial cells ranged from 0·26–0·34, and 76 per cent of the lymphocytes were in direct contact with endothelial surfaces. Similar

Table 1. Lymphocyte output from perfused mesenteric nodes

Perfusate	Lymphocyte output ($\times 10^3$) Mean	Range	No. of rats
10 per cent Dextran in 0·9 per cent NaCl (DS)	85	30–140	6
0·1 per cent Trypsin in DS	254	190–340	9
2·5 per cent Trypsin in DS	589	400–920	6
0·01 M EDTA in DS	137	70–190	6
0·9 per cent NaCl alone	184	140–240	6

Table 2. Effects of perfusion on lymphocyte adherence to HEV endothelium in mesenteric lymph nodes (summarized histological data)

Perfusate	AL	LL	ESC	EI
10 per cent Dextran in 0·9 per cent NaC l(DS)	+	0	+	intact
0·1 per cent Trypsin in DS	0	0	+	intact
2·5 per cent Trypsin in DS	0	+	0	damaged
0·1 M EDTA in DS	0	0	+	intact
0·9 per cent NaCl alone	0	0	0	intact

AL = Adherent lymphocytes, LL = luminal lymphocytes, ESC = endothelial surface coat, EI = endothelial cell integrity.

studies on nodes flushed with dextran–saline solution revealed that this ratio persisted at 0·18–0·30 and virtually all of the lymphocytes remaining in perfused vessels were in contact with high endothelial cells. Regional perfusion of isolated mesenteric nodes with normal saline and dextran–saline solutions containing trypsin or EDTA significantly increased cellular output in the effluent fluid (Table 1). Histologic examination of these nodes showed that perfusates containing 0·1 per cent trypsin or EDTA completely removed all luminal lymphocytes without altering surface coat staining or producing detectable damage in HEV (Table 2). While perfusion with normal saline displaced most lymphocytes from HEV surfaces, this was accompanied by diffuse loss of alcian blue staining for endothelial glycocalyx. Concentrated trypsin solutions produced extensive vascular damage and the resulting leakage of parenchymal cells into nodal vessels probably contributed to the increased effluent cellular output seen during perfusion with this solution. Together, these findings suggested that the attachment of lymphocytes to endothelial surfaces through trypsin–EDTA-sensitive binding sites was a major component in the selective accumulation of these cells within HEV lumens.

Sites of lymphocyte migration in HEV

Longitudinal sections through HEV demonstrated that these vessels progressively increased in size as they coursed from the outer cortex towards the medulla. The number of high endothelial cells seen in cross-section of these venules ranged from four in the smaller proximal segments to more than sixteen in distal portions near the corticomedullary junction. While lymphocytes were seen within the wall in all HEV segments, the number of these cells and their pattern of migration varied in different regions. Only occasional lymphocytes were seen emigrating from proximal HEV. Numerous intramural lymphocytes were present in mid-portions of HEV where cells appeared to migrate singly. This contrasted with distal segments where clusters of two to six lymphocytes were frequently found migrating through deep intercellular clefts between adjacent endothelial cells. These apparent differences in cellular traffic were confirmed when the ratio of

migrating lymphocytes to endothelial cells was determined in different HEV segments (Table 3). Since the ratio of 0·25 seen in proximal HEV consistently differed from the 0·81–0·69 observed in mid- and distal regions, variations in lymphocyte migration could not be attributed to quantitative differences in the number of endothelial cells lining different vascular segments. Despite this finding, no unique structural characteristics were identified in the endothelium of proximal HEV by light and electron microscopy.

Table 3. Lymphocyte migration index (LMI) in high endothelial venules

Region of HEV	LMI		No. of HEV counted
	Mean	Range	
Proximal segments	0·25	0·21–0·59	600
Middle segments	0·81	0·67–1·07	250
Distal segments	0·69	0·40–0·94	1000

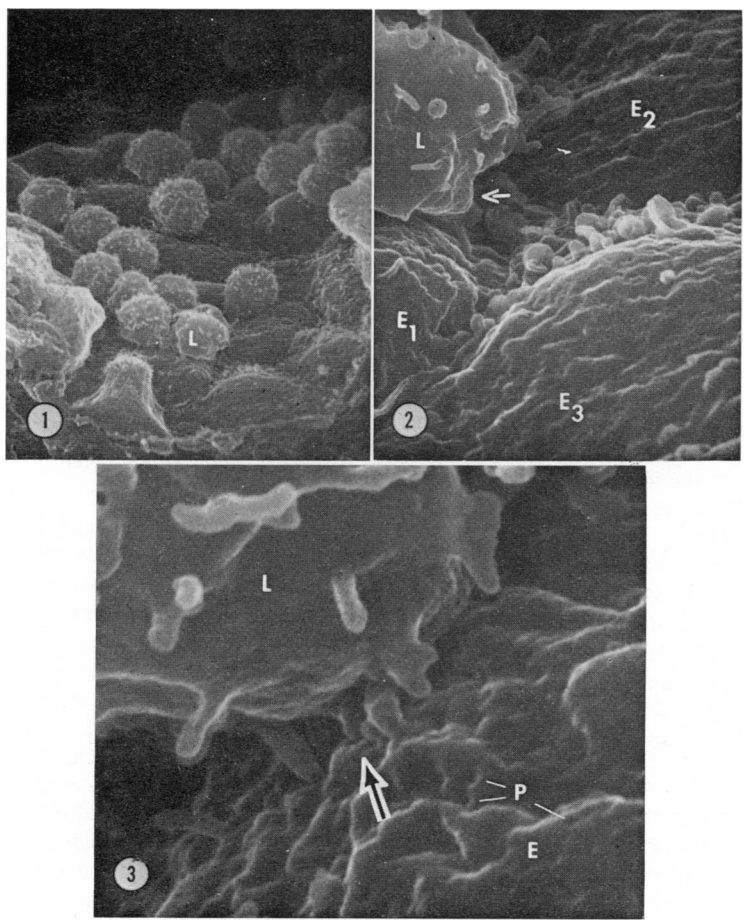


Figure 1. SEM of luminal surface of a high endothelial venule showing adherent lymphocytes (L) with microvillous surfaces, and bulging endothelium characteristic of HEV. (Magnification × 875.)

Figure 2. Detail from Fig. 1 showing interendothelial cleft with central ridges composed of vesiculatory processes. The lymphocyte (L) shown has begun migrating into the space between two endothelial cells (E) and reduction in the number of microvilli on its surface can be seen (arrow). (Magnification × 7000.)

Figure 3. Microvilli (arrow) on the surface of this recirculating lymphocyte attach to discrete pits (P) on the endothelial cell surface. (Magnification × 21,000.)

Lymphocyte attachment to HEV

Light microscopy of nodes prepared by both immersion and perfusion fixation revealed an irregular contour of HEV surfaces caused by bulging of endothelial cells into their lumens. HEV usually displayed cobblestone luminal surfaces where high endothelial cells formed dome-like protrusions surrounded by intercellular clefts (Fig. 1). Lateral folds and vesiculatory processes from adjacent endothelial cells formed ridges in the centre of these clefts (Fig. 2). Lymphocytes within the vascular lumens possessed numerous microvillous projections and these cells attached to HEV at sites where the microvilli interdigitated with randomly spaced pits studding the endothelial surface (Figs 1 and 3). Occasional lymphocytes established surface contacts with mid-portions of high endothelial cells, but they did not penetrate into the endothelial cytoplasm (Table 4). Over 80 per cent of the adherent lymphocytes were found within clefts between endothelial cells (Table 4). Several lymphocytes were seen entering the venular wall by insinuating themselves between endothelial cell junctions, and their surface characteristics appeared to change at various stages of emigration (Figs 1 and 2). If only a small portion of the lymphocyte mass lay within clefts, numerous folds and long, branched microvilli projected from their luminal surface. When the bulk of the lymphocyte was contained in the venular wall, the luminal side of the cell surface was usually smooth or exhibited only a few folds.

When sixty HEV were examined by transmission electron microscopy (TEM), 165 of 218 luminal lymphocytes were attached to endothelial surfaces through villous and plate-like contact points (Figs 4–7). Although migrating cells within the endothelial layer exhibited few villous projections, discrete intercellular contacts were frequently observed between these lymphocytes and lateral walls of endothelial cells (Figs 6 and 7). Membrane specialization was present at these sites where outer leaflets of juxtaposed membranes showed increased electron density (Figs 8–11). When surface coat oligosaccharides were preserved by adding alcian blue to the fixative, fibrillar, electron-dense material was seen traversing the 12–26 nm gap at contact points between lymphocyte and endothelial cell membranes (Fig. 11). Occasionally, lymphocyte processes invaginated endothelial cell membranes at sites where smooth or granular endoplasmic reticulum approximated cytoplasmic borders (Figs 10 and 11).

Table 4. Location of lymphocytes on HEV luminal surface

Location	No. of lymphocytes	Percent
Within endothelial clefts	129	83·3
Over endothelial cell centers	5	3·2
Indeterminant*	21	13·5
Total	155	100·0

* The precise location of these lymphocytes was obscured by other cells attached to the luminal surface.

These contact points appeared to be maintained by significant adhesive forces. Examination of HEV within perfused nodes by light microscopy, SEM and TEM demonstrated that many lymphocytes remained attached to endothelial surfaces despite the use of pressure and flow rates sufficient to flush away other blood elements and dilate venular lumens (Figs 1–4). When lymph nodes were fixed in hyperosmolar solutions, TEM showed that contact points persisted as osmotic forces distorted cellular contours and ruptured adjacent membrane segments (Figs 6 and 9).

Location of intra-endothelial lymphocytes

TEM revealed that HEV were lined by endothelial cells measuring 10–12 μm in height which were linked together by macular-tight junctions located near their luminal and basilar surfaces. Occasionally, a third junctional complex formed by loosely interlocking cell processes joined the mid-portions of adjacent cells, but typical zonulae occludentes were not seen. A lattice of interdigitating foot processes extended beneath basilar portions of adjacent endothelial cells. The endothelium rested upon a thin basement membrane and was surrounded by a complex sheath composed of reticular cell processes and collagen bundles which were contiguous with the reticular meshwork of the node.

With TEM, occasional lymphocytes were seen separating lateral surfaces of adjacent endothelial cells with one end touching the basal lamina while the other protruded into the venular lumen (Fig. 12). However, such demonstrations of trans-endothelial intercellular migration were unusual. In most instances, lymphocytes were surrounded by endothelial cell cytoplasm and the membranes were closely

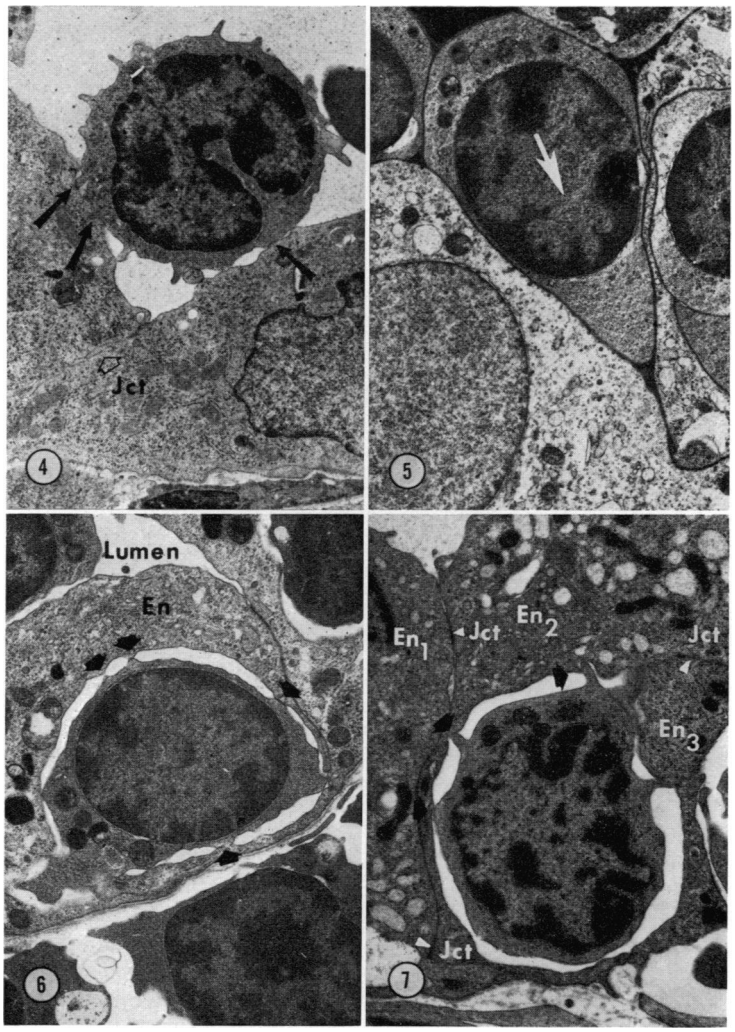
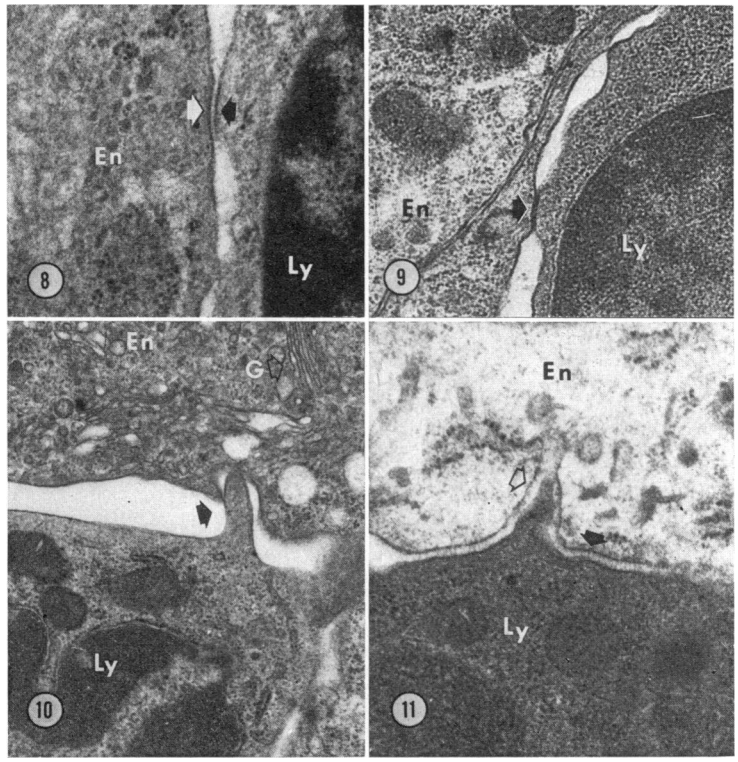


Figure 4. Lymphocytes adhere to luminal surfaces of high endothelial venules by villous and membranous contact points (arrows). (Magnification × 5950.)

Figure 5. Emigrating lymphocytes show cytoplasmic polarity as they insinuate between endothelial cells. (Magnification × 5950.)

Figure 6. Lymphocyte-endothelial cell contact points persist after hypertonic fixation has produced cell shrinkage which distorts adjacent membrane segments. (Magnification × 6125.)

Figure 7. The intercellular spaces surrounding emigrating lymphocytes are enlarged after fixatives were injected directly into the node to raise interstitial pressure. (Magnification × 8120.)



Figures 8–11. Lymphocyte-endothelial cell-contact points. **Figure 8.** Example of the plate-like contacts seen in standard EM preparations. (Magnification × 28,700.) **Figure 9.** Hypertonic fixation distort membranes adjacent to contact sites. (Magnification × 25,200.) **Figure 10.** Villous contact site (arrow) where a lymphocyte process is closely associated with smooth-surfaced organelles in the endothelial cell cytoplasm. (Magnification ×21910.) **Figure 11.** Fibrillar, electron-dense material is seen traversing the 26 nm gap between membranes at this contact point after fixation with glutaraldehyde containing Alcian Blue. (Magnification × 36,400.)

apposed making it difficult to determine whether the lymphocytes were migrating intra- or extracellularly (Fig. 13). Attempts were made to characterize lymphocyte-endothelial cell relationships in electron micrographs of HEV using methods proposed by Schoefl (1972). Lymphocytes situated between the venular lumen and basement membrane were considered to be in an 'endothelial' location. Lymphocytes were designated as 'intracellular' when they were surrounded by the cytoplasm of a single endothelial cell. If lymphocytes were enclosed by more than one endothelial cell or lay within spaces communicating with the vascular lumen or basement membrane, they were classified as 'extracellular' in location. By these criteria, 373 of 467 migrating lymphocytes were situated in extracellular spaces, while the remaining ninety-five appeared to be incarcerated within endothelial cell cytoplasm (Table 5). Similar TEM studies were made in nodes where hypertonic fixatives had enlarged the extracellular compartment. In these sections, lymphocytes surrounded by clear spaces were classified as extracellular while

Table 5. Location of recirculating lymphocytes during migration across high endothelial venular endothelium

Method	Extracellular percentage (between cells)	No. counted
Standard EM*	79·7	467
Hypertonic fixatives	97·7	301
Intra-arterial Thoratrast	99·2	128
Intra-arterial peroxidase	100·0	187
Intra-lymphatic peroxidase	100·0	269

* Criteria from Schoefl (1973).

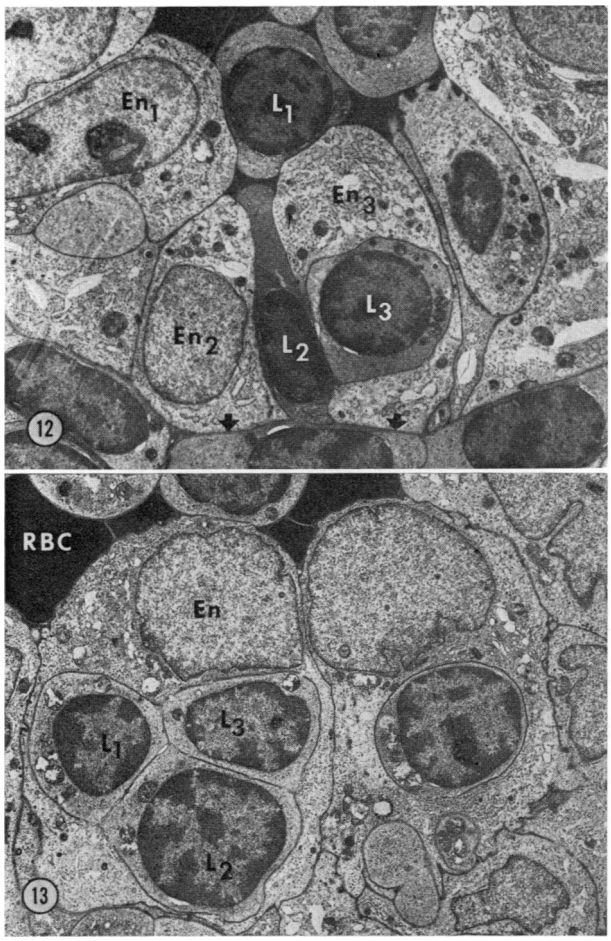
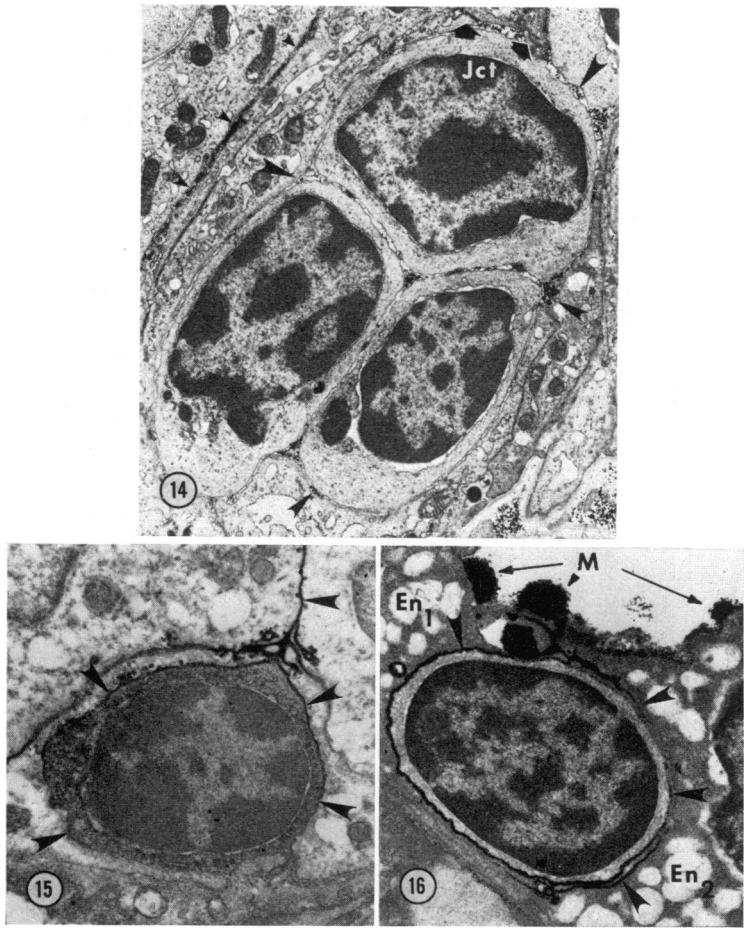


Figure 12. The surfaces of HEV endothelial cells (En 1–3) mould about emigrating lymphocytes: (L_1)—lymphocyte adhering to luminal surface of an endothelial cell; (L_2)—lymphocyte extending from the HEV lumen to the basement membrane (arrows), and (L_3)—lymphocyte indenting endothelial cell cytoplasm. (Magnification × 3580.)

Figure 13. In this plane of sectioning, lymphocytes (L) appear encarcerated within the cytoplasm of endothelial cells (En). (Magnification × 3580.)

lymphocytes maintaining contact with endothelial cell membranes over more than a 30° sector were designated as 'indeterminate' in location. By these techniques 294 of 301 lymphocytes classified as 'internal' by Schoefl's criteria lay within extracellular spaces.

This conclusion was corroborated by using electron-dense tracers to identify extracellular spaces. Immediately after intra-arterial infusion, thorotrast particles were found in HEV lumens and between endothelial cells in narrow (15–30 nm) extracellular spaces which extended down to the basal lamina (Fig. 14). This tracer surrounded all lymphocytes within the endothelium regardless of whether they were classified as extra- or intracellular by morphological criteria. Intra-arterial injections with horseradish peroxidase yielded similar results, with the added advantage that lymphocytes could be localized by both light and electron microscopy. This tracer accumulated about migrating lymphocytes at all levels in the venular wall (Fig. 15), and each of the 187 lymphocytes which appeared incarcerated within endothelial cells were surrounded by spaces labelled with reac-



Figures 14–16. The location of tracers in HEV walls following intra-arterial perfusion. Thorotrast (Fig. 14), horesradish peroxidase (Fig. 15), and lanthanum (Fig. 16) penetrate intercellular spaces contiguous with the vascular lumen and surround lymphocytes at all levels of migration. These tracers are seen on both sides of junctional complexes (Jct) between endothelial cells. Lanthanum appears to bind on cell surfaces and form micelles (M) with serum proteins. (Magnification × 7000, 9310 and 10,500 respectively.)

tion product. There was a continuous gradient in reaction product density ranging from intense staining of HEV lumens to faint staining near the basal lamina which was unaltered by the presence of lymphocytes (Fig. 15). This suggested that tracer diffused into spaces surrounding lymphocytes rather than being carried there on the surface of migrating cells.

The other tracers employed in these studies did not consistently label extracellular spaces between high endothelial cells. Intra-arterial infusions with aqueous suspensions of lanthanum hydroxide or lanthanum in glutaraldehyde caused convulsions and rapid death of the injected rats. While this tracer did surround some migrating lymphocytes in HEV, it also bound to cell-surface coat and formed aggregates in the circulation which made it unsuitable for use (Fig. 16). Intra-arterial injections with dilute India ink heavily stained vascular lumens, but the large carbon particles (35–45 nm) rarely penetrated extracellular spaces in the endothelium.

Despite morphological evidence for massive lymphocyte traffic across HEV walls, extravasation was seen infrequently in normal nodes. Erythrocytes

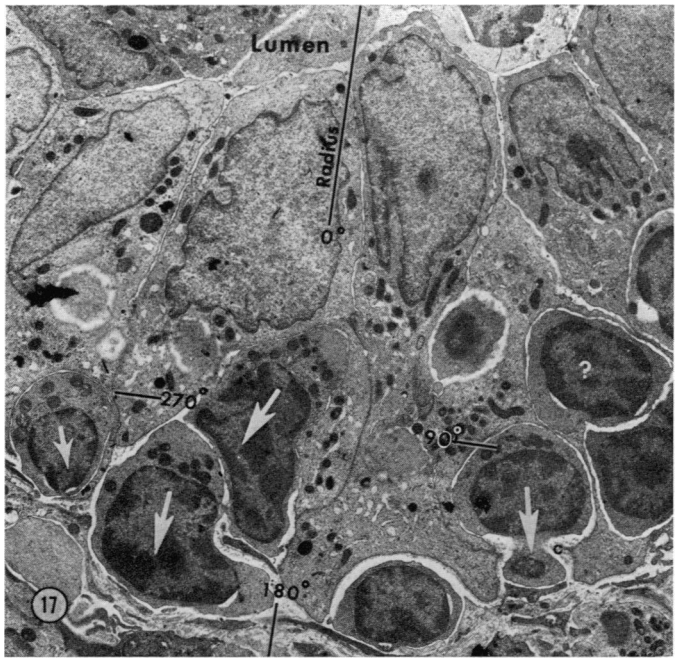


Figure 17. The distribution of cytoplasmic organelles was used to assess the direction of lymphocyte movement through HEV walls. Lymphocytes (L) were oriented with the nucleus leading and the organelle-rich tail following during migration. The angle formed by a line drawn through the polar axis of each lymphocyte (arrows) was measured using a 360° navigational compass which was aligned with the radius of the vessel. (C) Cytoplasmic constriction. (Magnification × 3850.)

and platelets were never observed within extracellular spaces between high endothelial cells. While tracer studies indicated that colloidal carbon particles were usually retained within the venular lumen, thorotrast and horseradish peroxidase permeated all spaces between endothelial cells and diffused down to the level of the basal lamina. Thorotrast particles were occasionally found in the interstitium adjacent to HEV, but this appeared to be restricted to sites where lymphocytes crossed the basal lamina and separated plates of the surrounding reticular sheath.

Direction of lymphocyte migration

Lymphocytes are motile cells which exhibit characteristic cytoplasmic polarity while in motion with the ruffled border and nucleus leading, and the organelle-rich tail following (Lewis, 1931). In the TEM study, 146 of 216 lymphocytes migrating across HEV walls were in a motile configuration, while cytoplasmic polarity could not be determined in the plane of sectioning through the remaining cells. Since motile lymphocytes appeared to be oriented between adjacent endothelial cells, cytoplasmic polarity was used to determine their direction of migration. A line was drawn through the polar axis of these lymphocytes and the angle formed where this line intercepted the HEV radius was measured with a 360° navigational compass (Fig. 17). By this technique polar angles approximating 180° indicated movement from HEV lumens into the node, while angles near 0°/360° were consistent with migration in the opposite direction. Chi-squared analysis indicated that the difference between these values were highly significant (P = 0·00025). If the population of indeterminate cells where polar angles could not be determined in the plane of sectioning were excluded from these calculations, 92 per cent of the migrating lymphocytes appeared to be moving towards the nodal parenchyma at the time of fixation.

Unidirectional permeability of the HEV wall

Morphologic observations in the accompanying

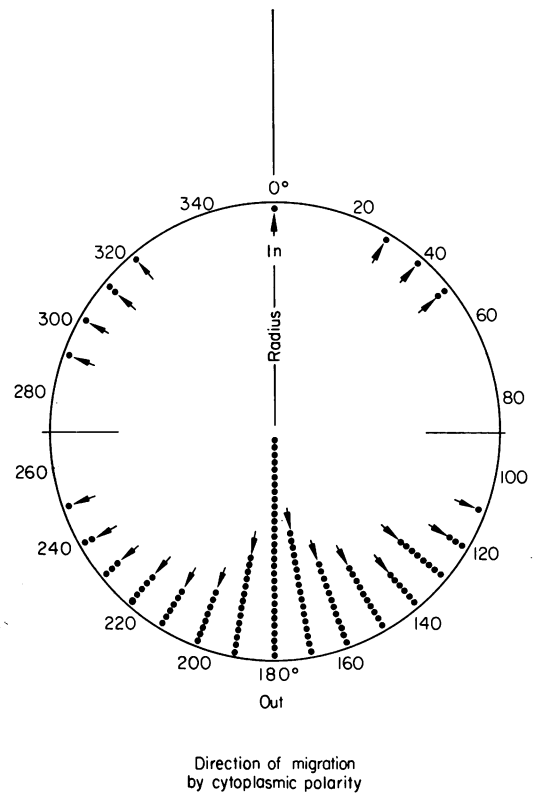
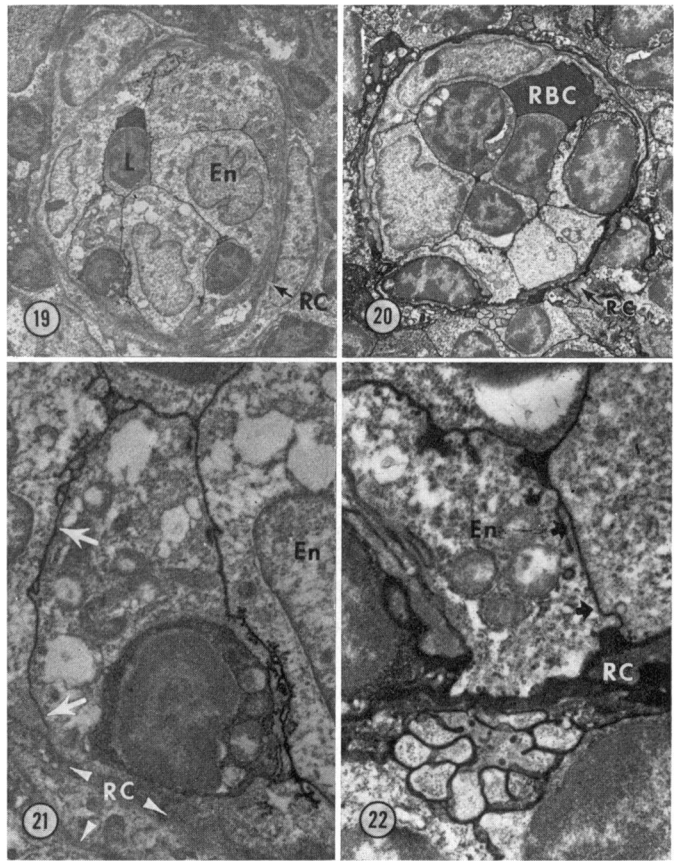


Figure 18. Direction of lymphocyte migration. Each dot in this scattergram represents the polar angle observed in one lymphocyte migrating across HEV walls. Most of these cells showed polar angles approximately 180° indicating directional migration away from the venular lumen.



Figures 19–22. Unidirectional permeability of HEV to horseradish peroxidase (HRP). Following intra-arterial injection (Fig. 19 and Fig. 21), HRP penetrates spaces between endothelial cells, but does not cross the basement membrane. (Magnification × 1820 and 6790.) When this tracer was infused into afferent lymphatics (Figs 20 and 22), HRP crosses the reticular cell sheath (RC), penetrates spaces between endothelial cells (arrows) where it surrounds emigrating lymphocytes, and then enters the venular lumen. (Magnification × 1666 and 11,100.)

report (Anderson, Anderson & Wyllie, 1976) demonstrated that the walls of HEV were constructed in a manner which could provide valve-like regulation of macromolecular permeability. This thesis was tested directly in the present study by injecting solutions containing horseradish peroxidase and trypan blue into the subserosal lymphatic arcade of the small intestine. After carefully injecting a small bolus to minimize pressure changes, the tracers flowed slowly through mesenteric lymphatics and stained the draining nodes. When these nodes were examined by light and electron microscopy, peroxidase reaction product was dispersed along the reticular meshwork of the nodal parenchyma and concentrated with in perivascular sheaths surrounding HEV. The tracer consistently permeated extracellular spaces in the HEV wall and entered the venular lumens. Each of the 269 intramural lymphocytes seen in these vessels lay within spaces stained by reaction product. Since the lumens of capillaries and arterioles were devoid of tracer, the diffusion of peroxidase from the node into the vascular system appeared to be restricted to the HEV wall. Similar intralymphatic injections with dilute India ink caused intense staining of subcapsular and medullary sinuses in the regional nodes where carbon particles were phagocytosed by littoral cells and macrophages. In addition, a few macrophages containing endocytosed carbon were seen adjacent to HEV, and occasional carbon particles were found in extracellular spaces between high endothelial cells and within venular lumens. Since neither tracer leaked from HEV following intra-arterial infusions, these findings provided evidence for unidirectional flow of macromolecules from the node into HEV lumens which were consistent with structural adaptations of the venular wall (Figs 19–22).

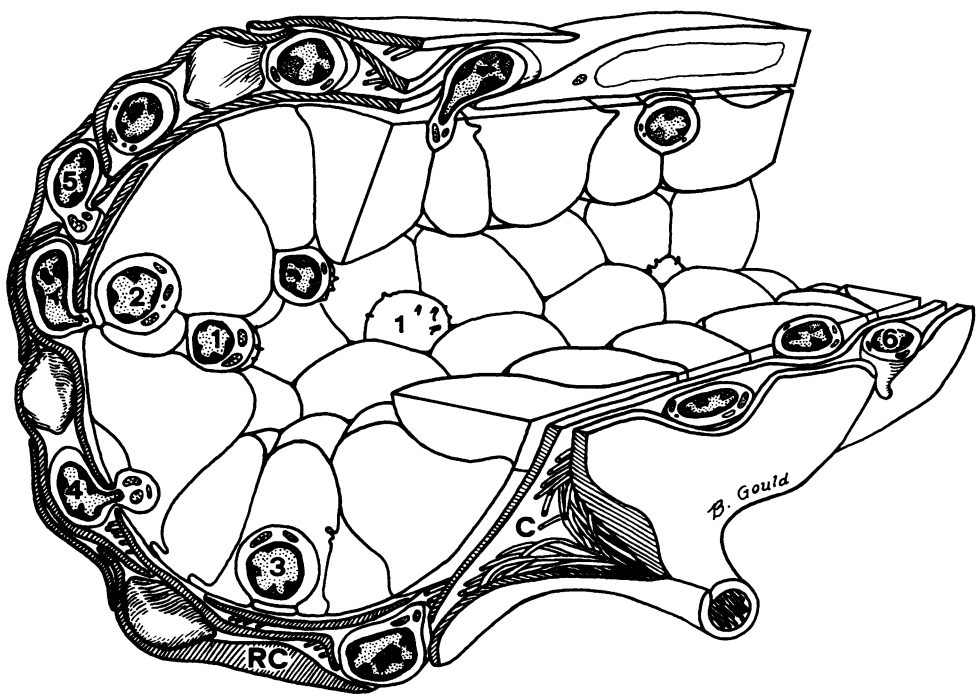


Figure 23. The sequence of lymphocyte emigration across the walls of HEV. This reconstruction drawing shows the characteristic structure of HEV with their cuboidal endothelium, delicate basement membrane, and multilaminated reticular cell sheath (RC) which is anchored to the lymph node reticulum by collagen bundles (C). Sequential stages of lymphocyte emigration are designated: (1) attachment to endothelial surfaces; (2) position after migrating around intact macular junctional complexes between endothelial cells; (3) location of lymphocytes moving beneath endothelial cells and above basal foot processes extending from adjacent endothelial cells; (4) migration into the reticular cell sheath (RC) after crossing the basement membrane; (5) radial movement across successive laminations of the sheath; and (6) emigration from the sheath into the nodal parenchyma.

Route of lymphocyte emigration from HEV

The sequence of lymphocyte emigration across HEV walls was reconstructed from the study of more than 3000 lymphocytes studied by SEM and TEM (Fig. 23). Emigration began when circulating lymphocytes attached to HEV surfaces through microvillous processes which interdigitated with pits on the surface of high endothelial cells. Then these motile cells actively migrated into potential spaces between endothelial cells by insinuating themselves between macular junctions. The walls of endothelial cells were markedly indented as they 'moulded' over surfaces of the emigrating lymphocytes. The leading front of lymphocytes established new contact points along the lateral walls of adjacent endothelial cells. In the outer endothelium, lymphocytes maintained directional migration away from the vascular lumen, but this altered as they passed around abluminal junctional complexes and moved between interdigitating basal foot processes. Lymphocytes penetrated the basal lamina and moved into the perivascular sheath where they migrated longitudinally for undetermined distances, or followed an erratic course as they traversed successive laminations in the sheath to enter the node.

DISCUSSION

The presence of numerous lymphocytes within the walls and lumens of HEV has been widely accepted as a characteristic feature of these vessels. Early histologic studies (Zimmermann, 1923; Schulze, 1925; Hummel, 1935) attributed this finding to the movement of lymphocytes produced in the node into the blood stream. However, tracer studies with radiolabelled cells (Gowans, 1959; Gowans & Knight, 1964) clearly demonstrated that circulating lymphocytes left the blood and entered nodes by emigrating at this site. This conclusion was supported by subsequent experiments where prolonged thoracic duct drainage depleted lymphocytes within HEV (McGregor & Gowans, 1963) and regional cannulation studies revealed that over 90 per cent of the lymphocytes in efferent lymph from unstimulated nodes were derived from the blood stream (Hall & Morris, 1965). Since these results did not completely exclude the possibility that some cells might be migrating in the opposite direction, other investigators (Sainte-Marie *et al.*, 1967; Sainte-Marie & Sin, 1970) described an apparent increase in lymphocyte concentration as blood passed from arteries into veins within the node and they interpreted this finding as evidence for direct entry of nodal lymphocytes into the microcirculation. In the present study TEM was used to assess the direction of lymphocyte movement across HEV. This approach was based upon previous *in vitro* and *in vivo* demonstrations that lymphocytes display a polarized distribution of cytoplasmic organelles while in motion (Lewis, 1931; Bessis & de Boisfleury, 1971; Schoefl, 1972; Norberg, Rydgren & Söderström, 1973). The results showed that two-thirds of the lymphocytes seen in random sections exhibited a motile configuration and areas of cytoplasmic constriction consistent with active movement at the time of fixation. This supported the prevailing concept that lymphocytes actively migrate across HEV walls. Since cellular polarity could not be determined in the plane of sectioning through the remaining cells, this study could not establish whether migration was characterized by continuous motility of all lymphocytes or intermittent motion with periods of rest interposed between phases of active movement. The observation that 92 per cent of the lymphocytes exhibiting a motile configuration were oriented between endothelial cells with an apparent directional movement away from the venular lumen supported Gowans' concept (1959) of cellular traffic from the blood into the node. Since 8 per cent of the intramural lymphocytes were oriented in the opposite direction, this study did not preclude cellular emigration from the node into HEV. However, these findings could simply reflect lymphocyte movement through tortuous intercellular pathways leading into the node.

Other mechanisms have been proposed to explain the accumulation of lymphocytes within HEV lumens. Schulze (1925) postulated that a sudden slowing of blood flow produced as capillaries emptied into large HEV lumens would concentrate circulating leucocytes within venules. However, this explanation failed to consider that blood flow might increase at this site since the total volume of the capillary bed was greater than that in draining veins. Pollicard's (1963) conclusions that blood lymphocytes sequestered in HEV where endothelial cells protruded into the lumen were probably based upon artifacts caused by vascular collapse during immersion fixation. Although neither of these concepts of HEV blood flow have been substantiated, there seems to be little reason for

doubting that venous stasis might facilitate lymphocyte-endothelial cell interactions. In the present study at least three-quarters of the luminal lymphocytes were attached to the endothelial surface through contact points which resisted hydrodynamic shear forces sufficient to flush away other blood elements. *In vivo* observations of other vascular beds by Atherton & Born (1972), have established that leucocytic sticking on endothelium depends upon the net effects produced by intercellular adhesion and haemodynamic shearing. Since similar forces probably influence lymphocyte attachment to endothelial surfaces *in vivo*, accelerated blood flow through HEV could dislodge adherent lymphocytes while sluggish flow might facilitate their interactions with venular surfaces. Such variations in velocity of blood flow may occur in HEV as these vessels are situated between arteriovenous communications and venous sphincters which provide regional hemodynamic control of the nodal microcirculation (Anderson & Anderson, 1975). This mechanism would readily explain the wide variation in numbers of luminal lymphocytes within different HEV in the same node, and the differences between arterial and venous lymphocyte concentrations observed by Sainte-Marie & Sin (1970).

Gowans and his colleagues (Gowans, 1959; Gowans & Knight, 1964; Howard *et al.*, 1972) established that long-lived T and B lymphocytes continually recirculate through lymphatic tissues by emigrating from HEV. While related studies provided evidence for modest traffic of these cells into other body sites (Morris, 1968; Smith, McIntosh & Morris, 1970), this 'homing instinct' remains as one of the keystones determining normal patterns of lymphocyte recirculation. Since other blood leucocytes rarely emigrated from HEV in normal nodes, several investigators (Goldschneider & McGregor, 1968; Vincent & Gunz, 1970; Sordat, Hess & Cottier, 1971; Schoefl, 1972) suggested that the selective movement of lymphocytes across these venules was dependent upon cell-surface recognition mechanisms. Although HEV possess characteristic structural and metabolic features (Anderson *et al.*, 1976), none of these have been shown to regulate cellular traffic. In this study, SEM demonstrated that the luminal surface of HEV was studded with shallow pits which were not seen in other blood vessels. Since lymphocytes attached to HEV through villous projections which adhered to these surface foci and membrane specialization was observed at each contact point, the endothelial determinants (receptors) of selective lymphocyte traffic in HEV may be contained within these focal depressions. When surface coat oligosaccharides were preserved by special fixation techniques, TEM showed electron-dense material crossing intermembranous gaps at these contact points. This appearance was similar to reported descriptions of leucocytic margination (Jones, 1970), where acid mucosubstances of the cell coat bound granulocytes to venular endothelial cells. The demonstration that regional perfusion with normal saline prevented alcian blue staining of endothelial glycocalyx and freed luminal lymphocytes from HEV provided further evidence for the attachment of lymphocytes to saline extractable surface coat constituents in high endothelial cells. The presence of a complementary surface receptor on lymphocytes was first suggested by Gesner & Ginsburg (1964) when they found that removal of membrane glycoproteins by trypsin digestion prevented lymphocytes from 'homing' in HEV. Since the present study showed that vascular perfusion with 0·1 per cent trypsin completely dislodged adherent lymphocytes without altering HEV staining, it seemed likely that both cellular recognition and attachment were dependent upon interactions between membrane glycoproteins on lymphocytes and endothelial surface coat. The chemical nature of this binding was not established. However, the demonstration that similar perfusion with 0·1 M EDTA mobilized lymphocytes from HEV without perturbing surface-coat staining suggested that divalent cations contributed to cellular adhesion as they may form cationic bridges between negatively charged carboxyl groups on apposing membranes (Bangham, 1964).

There has been prolonged controversy over the route followed by lymphocytes emigrating from HEV. Early light microscopic studies (von Schumacher, 1899; Zimmermann, 1923; Hummel, 1935) were interpreted as showing intercellular migration, although Schulze (1925) suggested that some lymphocytes might move intracellularly. Ultrastructural studies by Marchesi & Gowans (1964) indicated that lymphocytes were engulfed by HEV endothelial cells and then released on the abluminal side of the vessel. This intracellular migration pathway has been supported (Messier & Sainte-Marie, 1972) and disputed (Sugimura, 1964; Clark, 1962; Mikata & Niki, 1966; Claesson, Jørgensen & Røpke, 1971) in other reports of TEM

on random sections through HEV. Attempts to resolve this controversy by ultrastructural studies of serial sections from Peyer's patches (Schoefl, 1972) and lymph nodes (Wenk *et al.*, 1974) indicated that virtually all lymphocytes were found within extracellular spaces in the endothelium. The same conclusions were reached from evaluation of both random and serial sections in the present study. However, none of these findings could be considered as conclusive proof for intercellular migration across the endothelium since similar observations by Farr & De Bruyn (1975) indicated that lymphocytes arrived at these extracellular sites after an initial phase of intracellular migration through the apical cytoplasm of high endothelial cells. This concept of a combined intracellular–intercellular migration pathway was invalidated by a series of experiments using different methodology. Our SEM confirmed a report by Van Ewijk, Brons & Rozing (1975) that lymphocytes usually attached to the luminal surface of HEV near junctions between endothelial cells and then entered the endothelial layer by moving into intercellular clefts. Direct penetration into the apical surface of high endothelial cells was never seen by us. When hypertonic fixatives or intravascular tracers were used to identify extracellular spaces for TEM, 99–100 per cent of the lymphocytes in the endothelial layer were shown to be within extracellular spaces which were contiguous with the venular lumen in serial sections. Since peroxidase reaction product surrounded lymphocytes at all levels of penetration into the venular wall and there were virtually no signs of pinocytosis of the tracer by high endothelial cells, these findings excluded intracellular transport as a potential pathway for lymphocyte emigration.

Previous investigators (Marchesi & Gowans, 1944; Goldschneider & McGregor, 1968; Vincent & Gunz, 1970) ascribed movement of lymphocytes across the HEV wall to unique attributes of high endothelial cells which either guided or transported lymphocytes through their cytoplasm. However, the overwhelming evidence for intercellular migration pathways has made these explanations untenable. In the present study, SEM showed that microvillous projections from the lymphocyte surface descreased in number and size as these cells entered the venular wall. Similar findings were described by Van Ewijk *et al.* (1975), and they suggested that the presence of microvilli indicated that lymphocytes were in an 'activated' state while conversion to a smooth surface represented a 'quiescent' stage associated with residence in the nodal cortex. Although microvilli may be involved in cellular recognition, these surface changes could not be equated with active motility since our TEM studies showed that most lymphocytes within the HEV wall and sheath were in a motile configuration. These cells appeared to re-establish contact points with lateral surfaces of adjacent endothelial cells, but it was difficult to attribute their continued directional movement along circuitous intercellular pathways to motility guided solely by membrane interactions. However, intralymphatic injections with horseradish peroxidase resulted in flow of this tracer across the node into HEV lumens. Since all lymphocytes within the walls and sheaths of these venules were surrounded by reaction product, this suggested that lymphocytes were migrating toward increasing concentrations of macromolecules which were flowing into HEV lumens from the lymphatic tissue. Although there are no known agents which are selectively chemotactic for recirculating lymphocytes *in vivo*, Ward, Offen & Montgomery (1971) identified an *in vitro* prototype for lymphocyte chemotactic factor in antigen-containing supernatants from activated lymph node cultures. If similar agents can be demonstrated *in vivo*, the entire sequence of intercellular lymphocyte emigration could be explained by directional migration of these motile cells along a chemotactic gradient.

Schulze (1925) originally suggested that 'stomata' in HEV-permitted bidirectional exchange between blood and lymph within the node. Subsequent studies showed that proteins (Dunn, Burtz & Ward, 1972a) soluble and particulate dyes (Shanbrom & Zheutlin, 1959) bacteria (Pressman, Simon, Hand & Miller, 1962), erythrocytes (Dunn, Burtz & Ward, 1972b) and tumour cells (Dunn, Strahan & Ward, 1973) passed from lymph into the nodal microcirculation under a variety of different experimental conditions. Since this exchange of macromolecules and cells required high injection pressures, Dunn & Strahan (1972) concluded that anatomical connections between saccular lymph sinuses and small blood vessels were guarded by specialized valves which opened when sinusoidal pressure increased and permitted lymph to flow directly into the blood. However, Fukuda (1968) observed that proteins and dyes injected into afferent lymphatics under nearly physiological conditions could be recovered in venous blood from normal lymph nodes. After demonstrating colloidal iron particles

within walls and lumens of HEV in these nodes, he suggested that macromolecules were exchanged between blood and lymph at this site under conditions normally occurring *in vivo*. Subsequent TEM studies (Anderson *et al.*, 1976) demonstrated that the structure of the venular wall and its surrounding sheath appeared specially designed to permit unidirectional flow from the node into HEV lumens. This interpretation was supported by tracer experiments reported here. Following intra-arterial infusion, colloidal carbon was usually excluded from intercellular spaces in the endothelial lining of HEV, while thorotrast and horseradish peroxidase penetrated deeply into these spaces. Since extravasation of the latter tracers was limited to sites where lymphocytes were entering the reticular sheath, it seemed likely that interdigitating-basilar foot process and the basal lamina served as barriers to particle leakage. However, the injection of peroxidase into afferent lymphatics resulted in passage of the tracer from lymph sinuses through the perivascular sheath, between endothelial cells and into the lumens of HEV in the draining mesenteric node. The absence of reaction product in the wall and lumens of other blood vessels, and the failure to prevent this staining pattern by thoracic duct ligation clearly indicated that peroxidase flowed across HEV walls to enter the blood stream. Since this tracer flowed spontaneously into the draining node, this pattern of intranodal distribution could not be attributed to high injection pressures. The failure to produce similar vascular staining by intralymphatic injections with colloidal carbon suggested that absorption of materials across HEV may be restricted to small particles. Further studies will be required to determine whether these functional lymph node-venous communications regulate fluid, macromolecular and cellular exchange during local immune responses. Since both antibody and lymphocyte activation products have been identified in afferent and efferent lymph during immune stimulation (Kelly, Wolstencroft, Dumonde & Balfour, 1972) it is possible that these agents could flow through HEV walls. They would then produce altered cellular traffic (Burwell, 1962; Kelly *et al.*, 1972; Kelly & Wolstencroft, 1974); structural and metabolic changes within high endothelial cells (Anderson & Anderson, 1975) and modulation of immune reactions (Fukuda, 1968; Kelly & Wolstencroft, 1974; Kelly, Harvey, Sadler & Dumonde, 1975).

ACKNOWLEDGMENTS

The authors are grateful to Dr John D. White for the scanning electron micrographs; Philip Rutledge and Raymond Lund for photographic assistance; and Phebe W. Summers for editorial assistance.

This study was supported in part by National Institute of Health Grants, HL-17596 and GM-00415, and an Army contract DAMD-17-74-C-4095.

In conducting the research described in this report, the investigators adhered to the 'Guide for the Care and Use of Laboratory Animals', as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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