THE MIGRATION OF LYMPHOCYTES ACROSS THE VASCULAR ENDOTHELIUM IN LYMPHOID TISSUE

A Reexamination

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The work of Gowans and his colleagues (1, 2) established that lymphocytes continually recirculate from blood to lymph and that most of this cell migration takes place across the wall of the so-called postcapillary venules (2) in lymphoid tissue. These vascular segments are often lined by high, nearly cylindrical endothelial cells which are encircled by a prominent connective tissue sheath. Both layers may be heavily infiltrated with lymphocytes.

Since their discovery by Thomé (3) in 1898, these special vessels have been described in lymph nodes (4–6), tonsils (7, 8), Peyer's patches (6, 9, 10), solitary lymph follicles (6, 10–12), and the thymus (13). Schumacher (4) first drew attention to the large number of leukocytes (lymphocytes) migrating across the wall of these vessels, and since then the direction and mode of penetration have been debated repeatedly. Many early workers make reference to loosely fitted endothelial cells (4, 5), or even stomata (6, 11, 14) and imply an intercellular migration of lymphocytes. An intracellular passage was apparently first suggested by Schulze (6) as an alternative pathway and later accepted by a number of other authors (8, 9, 15). It finally became largely accepted when Marchesi and Gowans (16) reported experiments which apparently demonstrated that lymphocytes migrated across the vascular wall in a different fashion from polymorphonuclear leukocytes. At the ultrastructural level, lymphocytes were described as penetrating into the endothelial cells while polymorphonuclear leukocytes migrated exclusively between the endothelial cells.

This seemingly special mode of migration has been related to the lymphocyte's unique role in immunological reactions. The possibility has been raised that the intracellular contact of lymphocytes and endothelial cells may provide a special opportunity for an interaction between lymphocytes and antigens (16). An "intracellular surveillance" by these endothelial cells has also been thought of as acting in some peculiar way to regulate the numbers of lymphocytes in the bloodstream (17). Although the interpretations of Marchesi and Gowans have been questioned (18–22), the prevalent notion on the mode of lymphocyte migration from the bloodstream seems to be that they pass through

the substance of endothelial cells. The present study was undertaken to determine whether or not this is in fact so.

Materials and Methods

Peyer's patches of normal rat and mouse intestine were used for these studies. These are flat, disc-shaped clusters of lymphoid follicles in the intestinal wall. The relatively simple architecture of this lymphoid tissue greatly assisted in locating these special venules which form a meshwork around each follicle.

The following fixatives were used: 2% osmium tetroxide in 0.1 M phosphate or cacodylate buffer at pH 7.35–7.40 and a glutaraldehyde/formaldehyde mixture (23) in the same buffers. Fixation times ranged from 2 to 4 hr for osmium tetroxide and one-half to 1 hr for the aldehydes, followed by osmium tetroxide after a wash of several hours or overnight in buffer.

Areas of gut containing Peyer's patches were briefly fixed *in situ* by dripping fixative onto the serosal surface and also injecting it into the lumen of the gut. The area of lymphoid tissue was then dissected out, cut into two or three pieces, and fixation continued for the periods mentioned above. After dehydration in acetone and infiltration with Durcupan (Fluka AG, Basel, Switzerland), the tissue segments, usually containing two or more lymphoid follicles, were embedded in flat molds. They were orientated and sections were cut at right angles to the surface of the intestine. Semithin sections of these rather large pieces were stained with an azure II/methylene blue mixture (24) and selected areas were then trimmed down for ultrathin and serial sectioning on an LKB Ultrotome (LKB Produckter, Stockholm, Sweden) using a diamond knife. Sections were picked up on parlodion and carbon-coated 100 mesh or honeycomb grids and stained with lead citrate. In most experiments, the tissue had also been stained en bloc with uranyl acetate, using either aqueous solutions before dehydration, or adding the salt to the 50 or 70% acetone. Pictures were taken on a Philips EM 200 electron microscope (Philips Electronic Instruments, Australia) operating at 60 kv and using 40-μ objective apertures. Primary magnifications ranged from 750–30,000.

Two approaches were used to evaluate the relationship of lymphocytes to endothelial cells in the vessel wall: I. The evaluation of observations in single sections and II. extensive sequences of serial sections.

I. *Observations in Single Sections.*—

(*a*) About 800 lymphocytes associated with the endothelium (omitting those beneath the endothelium or in the connective tissue sheath) were classified as follows: (1) *luminal*: if the lymphocyte bordered onto the lumen of the vessel; (2) *endothelial*: if the lymphocyte lay entirely within the endothelial layer; (3) *basal*: if any part of the lymphocyte bordered onto or projected into the periendothelial area.

In class 2 (endothelial), lymphocytes were subdivided according to their apparent position in the particular plane of section: "*internal*" = surrounded by a single endothelial cell; "*external*" = intercalated between two or more endothelial cells.

The other two groups were similarly subdivided. Lymphocytes in close contact with or projecting into or near the middle of the endothelial cell were assumed to be potentially internal; those near endothelial cell junctions potentially external. From these data, the probabilities of a sectioned lymphocyte appearing external or internal were estimated, and confidence intervals for these probabilities were derived.

(*b*) All lymphocytes of class 2 (endothelial) were further analyzed as to their group size, i.e., whether they occurred singly or in contiguous groups. In addition, for each external lymphocyte of that class, the number of junction points (points where, in section, two adjoining cells abut the lymphocyte) around its perimeter and the shape of the section profile were recorded. Finally, from these data, the probability for an intercellular or intracellular migration path for lymphocytes was estimated, on the basis of an approximate mathematical model.

II. *Observations in Serial Sections.*—The second and more tedious method involved long

sequences of serial sections. Initially, a particular endothelial cell and the lymphocytes in contact with it were selected at the light microscope level and serial sectioning was started from that point. However, with this approach, a large portion of the selected cell may already have been removed before serial sectioning is begun. So, in other series, long sequences were cut of a particular vessel and fields were then selected in which a large part or all of the endothelial cell body could be followed. Most sequences were cut at approximately right angles to the axis of the vessel, but in one series, sectioning parallel to the axis was started at the luminal surface and continued into the perivascular connective tissue sheath. On the average, a series of some 90 serial sections were required to pass through most of the cell body of a given endothelial cell.

RESULTS

The predominant cell type migrating across the endothelial lining of venules in lymphoid tissue appeared to be small lymphocytes; possibly some were also larger mononuclear leukocytes, though this question was not specifically studied. Red blood cells or granulocytes were not observed in the vascular wall. In a few instances, an eosinophil was adherent to the luminal surface, or was present in the perivascular tissue.

Occasionally, lymphocytes occurred in the wall of venules lined by thin endothelium (Fig. 1) but the majority penetrated vascular segments in which the endothelium appeared cuboidal or even columnar (Fig. 2). In section, lymphocytes were in contact with the luminal surface, within the endothelium and in the layers of the connective tissue sheath. In some cases, a lymphocyte projected from the lumen, through the endothelial layer into the periendothelial space (Fig. 5); this position results in a complete separation of endothelial lateral surfaces at such a locus.

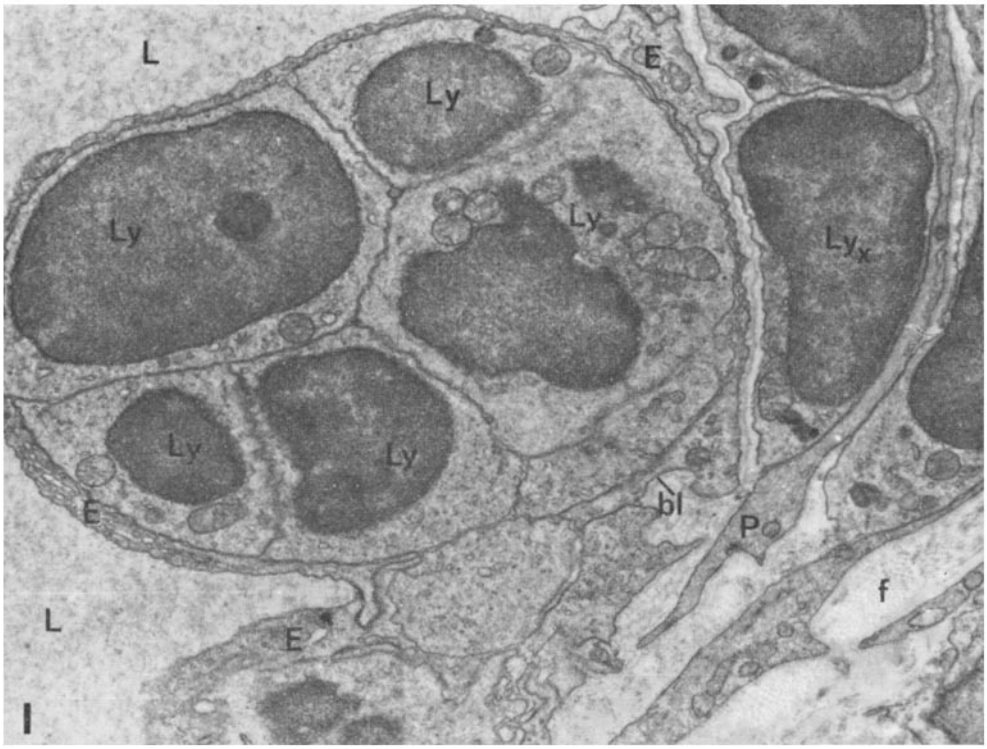
I. *Observations in Single Sections.*—

(*a*) The number of lymphocytes examined and classified according to their position within the endothelial layer (see Materials and Methods) is listed in Table I. Thus, within each group, the majority of lymphocytes was clearly located external to the endothelial cells. In class 2 (endothelial), internal lymphocytes were seen as such in section. In class 1 (luminal) and 3 (basal), some were judged potentially internal, though all of them were external.

Sections used for this analysis were not strictly random. Since these venules are often not easily identified at the light microscope level, unless the plane of sectioning passes through the lumen, there was a tendency to omit fields in which the endothelium was cut very obliquely. Also, the incidence of internal lymphocytes in any given section was rare and hence most, if not all, were

FIG. 1. From a venule lined by low endothelium. A group of lymphocytes (*Ly*), covered by a thin sheet of endothelial cytoplasm (*E*), balloons into the lumen (*L*) of the vessel. × 10,500.

FIG. 2. High endothelium lining a venule in the Peyer's patch of a rat. Three rather plump endothelial cells (E_1, E_2, E_3) are illustrated; a fourth one (E_4) molds around a cluster of lymphocytes (*Ly*). The area also includes part of the connective tissue sheath, consisting of pericytes (*P*) and collagen fibers (*f*). A lymphocyte (Ly_x) is wedged between its concentric layers. *bl* = basal lamina. × 7300.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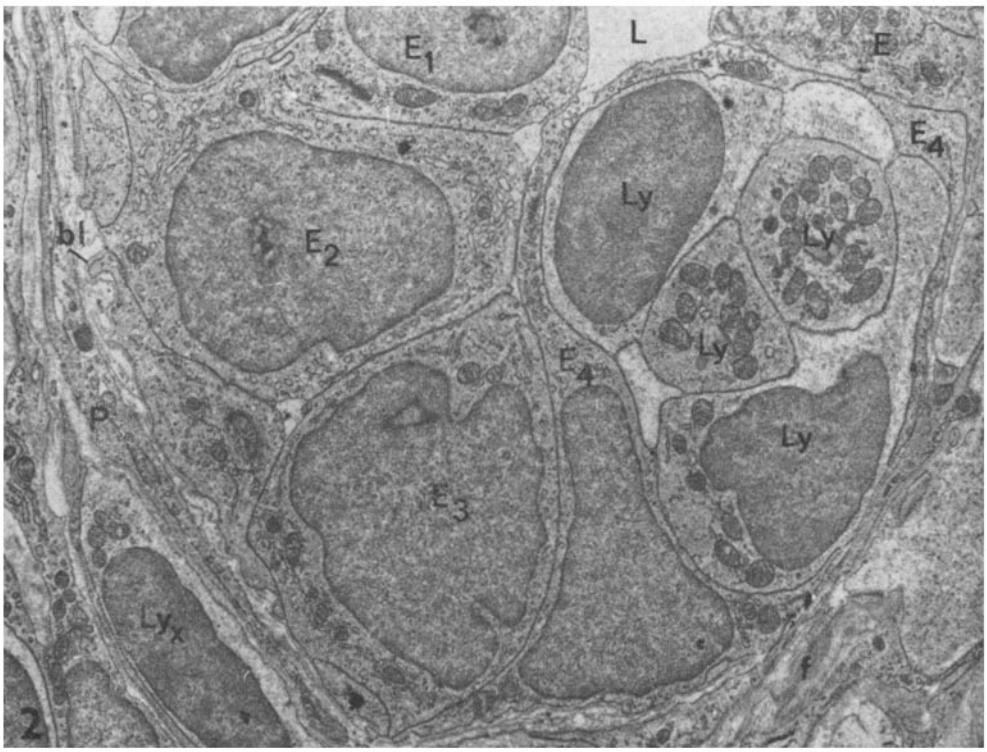
E1
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recorded photographically. Records for external lymphocytes are not as complete.

If E_3 and I_3 represent the percentages of external and internal lymphocytes migrating across the vascular wall in three-dimensional reality, then $E_3 + I_3 = 100$; and if E_2 and I_2 are the corresponding percentages for two-dimensional sections, then $I_2 + E_2 = 100$. Moreover, $I_2 = I_3 + \theta E_3$, where, θ is the probability that a lymphocyte externally located in three dimensions

TABLE I

Over-All Count of Lymphocytes Associated with the Endothelium

Class of lymphocyte	Number	"External"	"Internal"	"External"	"Internal"
				%	%
Luminal	130	102	28	78.5	21.5
Endothelial	377	344	33	91.2	8.8
Basal	289	200	89	69.2	31.8
Total	796	646	150		

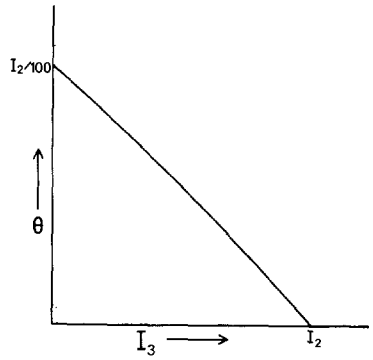


FIG. 3

is sectioned so as to appear internal. (Note here that we are using the fact that an internally located lymphocyte in three dimensions is always sectioned so as to appear internal.) Thus,

$$\theta = \frac{I_2 - I_3}{E_3} = \frac{I_2 - I_3}{100 - I_3}.$$

For any given value of I_2, the possible values of θ and I_3 lie on the curve shown in Fig. 3 and must lie between 0 and $I_2/100$, and between 0 and I_2, respectively.

Now, according to Table I, the total number of observed lymphocytes in class 2 (endothelial) was 377, of which 344 were external and 33 internal. There

will be little error in regarding the 377 lymphocytes as statistically independent, in which case 344 is a realized value of a binomial (377, $E_2/100$) random variable, and 33 is a realized value of a binomial (377, $I_2/100$) random variable. Thus, E_2 and I_2 are estimated by

$$\hat{E}_2 = 100\frac{344}{377} = 91.2, \qquad \hat{I}_2 = 100\frac{33}{377} = 8.8.$$

Using the binomial sampling theory in Wilks (25), we obtain the following

TABLE II

Classification by Group Size and Number of Junction Points for Each of the 377 Lymphocytes Observed in the Endothelial Layer

External								
No. of junction points	Group size							Total
	1	2*	3	4	5	6	7	
1	4	0	0	0	0	0	0	4
2	37	10	4	0	0	1	0	52
3	30	13	16	6	6	3	1	75
4	30	23	14	11	8	3	1	90
5	18	12	11	6	9	3	6	65
6	6	12	8	5	5	6	3	45
7	4	0	2	3	0	2	1	12
8	0	0	0	0	0	0	0	0
9	0	0	1	0	0	0	0	1
	129	70	56	31	28	18	12	344
Mean No. of junction points	3.42	4.00	4.25	4.61	4.47	4.89	5.16	4.02

Internal								
	Group size							Total
	1	2	3	4	5	6	7	
No. of lymphocytes	20	6	3	4	0	0	0	33

* For groups of two and above, counts of junction points include those made with either adjoining endothelial cells or other lymphocytes. One or more lymphocytes of any group may have been classified other than "endothelial."

90% confidence intervals for E_2 and I_2 : $88.6 < E_2 < 93.3$ and $6.7 < I_2 < 11.4$. Thus, with at least 95% confidence, we have $0 < I_3 < 11.4$ and $0 < \theta < 0.114$.

In words, simply from the data given in Table I, we may conclude with at least 95% confidence, that at least 88.6% of all lymphocytes take a path external to the endothelial cells and, correspondingly, at most 11.4% of them may pass internally.

(*b*) The further classification of lymphocytes in class 2 (endothelial) is shown in Table II.

It is evident from Table II that over 60% of all external lymphocytes oc-

curred in clusters of two or more. This confirmed the subjective impression gained during their study in the electron microscope that lymphocytes were more often seen in groups than singly, which is also the case for those in the surrounding connective tissue sheath. A similar tendency can be observed for internal lymphocytes.

In the Appendix, a value of 0.07 is derived for θ, for a specific mathematical model in which the lymphocytes migrate singly rather than in groups. Taking into account that over 60% of the lymphocytes observed traveled in contiguous groups, it is there argued that the true value for θ is of the order of 0.05 which gives a 90% confidence interval of 93–99 for E_3 and a corresponding point estimate of $\hat{E}_3 = 96$.

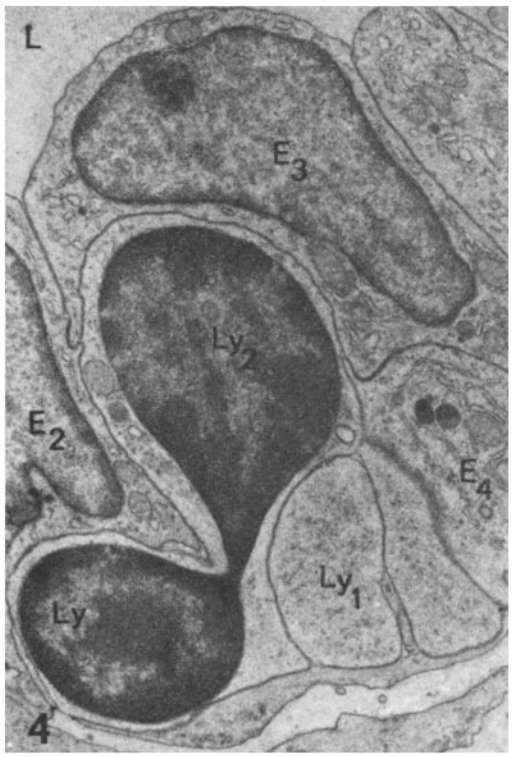
As to the general shape of sectioned lymphocytes, the majority appeared round or oval, though extreme deformations (Fig. 4) were seen occasionally. In contrast, profiles of endothelial cells suggested a much greater plasticity and pliability. Endothelial cells were highly pleomorphic and the shape of the nucleus frequently suggested compression and displacement (Figs. 2, 4, 6). Many examples were seen in which a lymphocyte protruded into the endothelial cell body (Fig. 6). Cut at planes indicated on Fig. 6, this lymphocyte would presumably have been judged internal over many sections. Images such as these prompted the use of serial sections as another approach to study the relationship of migrating lymphocytes to the endothelial cells.

II. *Observations in Serial Sections.*—21 endothelial cells were followed through 40–133 sections. Assuming a section thickness of 750–1000 A (26, 27), these series ranged in depth from approximately 3–4 to 10–13 μ. In 13 of these endothelial cells, a lymphocyte appeared internal at certain levels of sectioning. The depth of internal location varied from 2 to 23 sections, or a depth of approximately 0.15–0.2 to 1.7–2.3 μ. In no instance was a lymphocyte truly within the body of the endothelial cell. Often, it became apparent that an internal lymphocyte was not only located outside the endothelial cell but actually belonged to a group of lymphocytes wedged between two or more endothelial cells. As mentioned in the preceding section, this clustering of migrating lymphocytes was also readily apparent in single sections (Figs. 1 and 2).

FIG. 4. Example of extensive deformation of a lymphocyte (*Ly*) as it insinuates itself between endothelial cells (E_2, E_3, E_4). This micrograph is part of the sequence of serial sections shown in Fig. 7 and represents sectioning level 40. × 10,800.

FIG. 5. A lymphocyte (*Ly*) which extends from the lumen (*L*) into the connective tissue sheath thus completely separating two endothelial cells (E_1, E_2). Arrows mark the margins of the endothelial basal lamina. The over-all shape of the lymphocyte suggests that the nucleus leads and that the cytoplasm is being trailed behind, a pattern of locomotion which has been described for lymphocytes in vitro. *c* = centriole. × 13,500.

FIG. 6. Detail from the endothelial lining of a venule. A lymphocyte (*Ly*) is almost completely surrounded by an endothelial cell (*E*). The lines indicate planes of sectioning in which this lymphocyte would presumably have appeared "internal" in many consecutive sections. × 13,000.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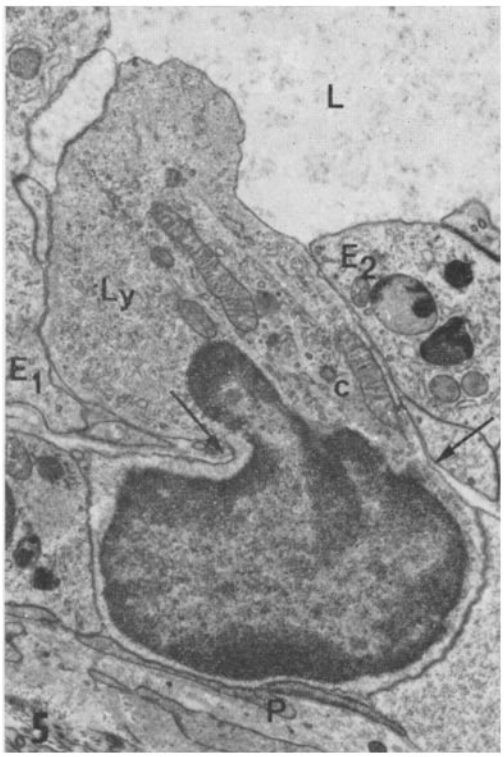
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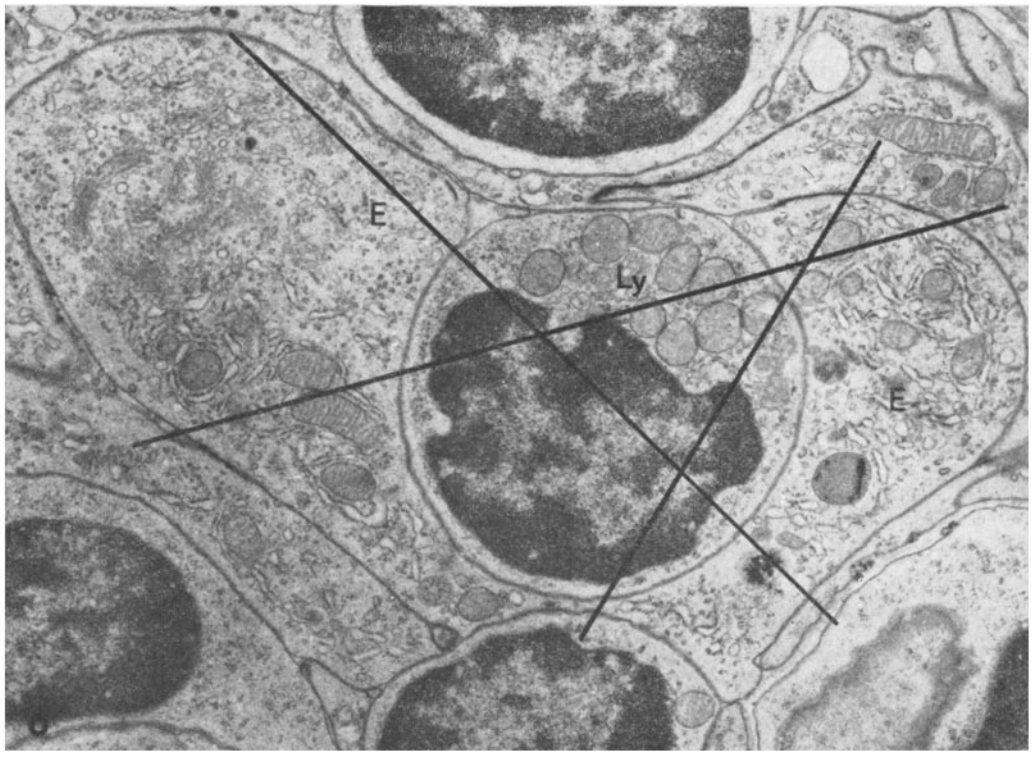
E
Ly
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6

Figs. 7 (A–D) and 8 (A–C) are selected photographs from series in which a lymphocyte appeared internal over 16 (Fig. 7) and 21 (Fig. 8) levels. In each case, a large portion of the lymphocyte was associated with two or more endothelial cells and was in direct contact with another lymphocyte.

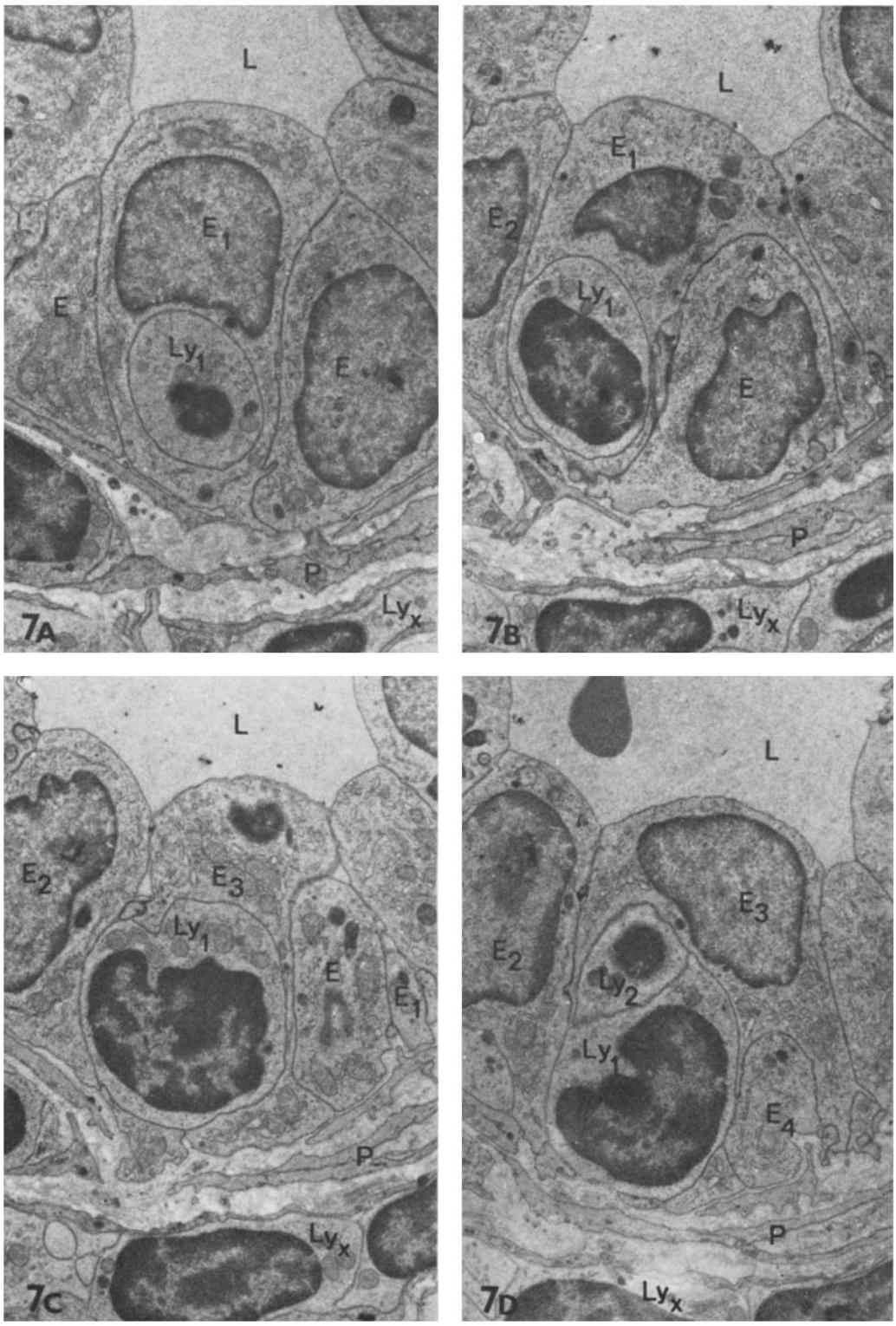
DISCUSSION

For about 80 yr (4), the selective presence of lymphocytes in the walls of venules in lymphoid tissue has been recognized as a special relationship between the endothelium and these migrating cells. In most early descriptions, and also in some more recent ones, it was reasoned that lymphocytes migrate along a concentration gradient, i.e., from the dense lymphatic tissue into the bloodstream (4–6, 9, 15). Though Gulland (28) in 1894 suggested that "adenoid tissue is merely an extremely vascular variety of connective tissue . . . adapted to retain for a while the leucocytes which wander out of the vessels, and to further their reproduction," subsequent workers accepted von Schumacher's (4) argument that a higher count of lymphocytes in veins of lymphoid tissue, as compared with arteries, signified their entry into the blood from the parenchyma (29). Using labeled lymphocytes, Gowans and Knight (2) demonstrated, however, that the predominant traffic of cells is from blood into the lymphoid tissue; furthermore, Hall and Morris (30) showed that approximately 95% of the lymphocytes in the efferent lymph from the popliteal node of the sheep are normally derived from the bloodstream.

As to the mechanism by which lymphocytes pass through the vascular wall, older publications refer to lymphocytes between endothelial cells (4, 5, 31), though illustrations may show the endothelium as a syncytium (31). Since endothelial cell boundaries in these venules could not be demonstrated by silver nitrate, the cells were thought to be only loosely cemented together (5) thus permitting the passage of lymphocytes.

The first detailed morphological analysis of these vessels was made by Schulze (6) who concluded, on the basis of supra vitam perfusions, that the vascular wall contained oblique channels (stomata) which permitted the passage of cells and particles in both directions. Due to the peculiar relationship of some lymphocytes to endothelial cells in section, he suggested that an intracellular pathway may also exist. Many authors accepted the stomata theory, or at

FIG. 7. Selected levels of a sequence of serial sections cut at approximately right angles to the axis of the venule. At level 107 (Fig. 7 A) a lymphocyte (Ly_1) is completely surrounded by one endothelial cell (E_1). This lymphocyte appeared "internal" over 16 levels of sectioning. In Fig. 7 B (level 96), the same lymphocyte makes contact with a second endothelial cell (E_2). Only a small portion of the first endothelial cell (E_1) remains at level 77 (Fig. 7 C) and lymphocyte Ly_1 is now also in contact with a third endothelial cell (E_3). Finally, in Figure 7 D (level 59), it becomes apparent that a second lymphocyte (Ly_2) is occupying the same intercellular space and lymphocyte Ly_1 makes contact with a fourth endothelial cell (E_4). Fig. 4 shows level 40 of this sequence. × 6400.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7C
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7D

least an intercellular migration (5, 7, 10, 11, 12, 14) though an intracellular passage eventually became more widely accepted (8, 9, 15) and finally appeared to be confirmed at the ultrastructural level (16). Reports that, in vitro, normal and leukemic lymphocytes can penetrate into the cytoplasm of certain tumor cells and macrophages (32) and are apparently present in some epithelial cells (33) helped establish this concept.

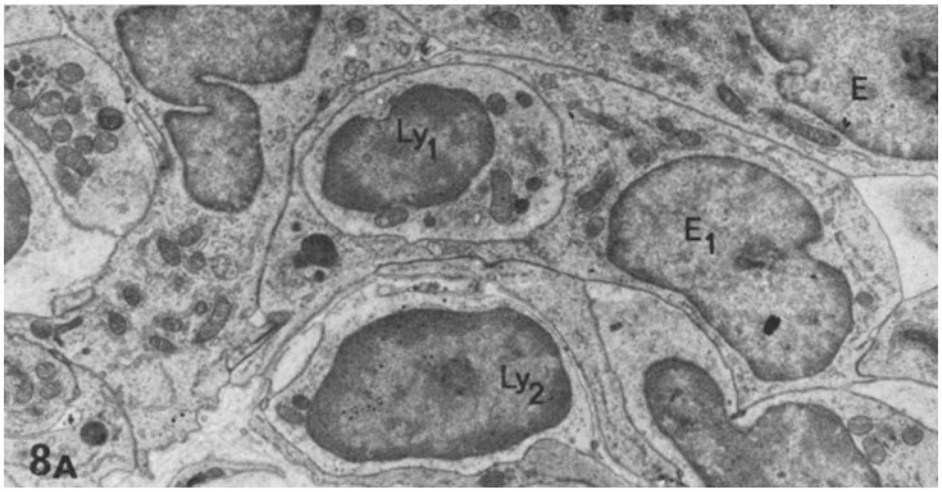
In agreement with several previous reports (18–21), the present data demonstrate, however, that lymphocytes follow an intercellular route. From the material analyzed, it could be shown that at least 93–99% of the lymphocytes are located external to the endothelial cells. The occurrence of internal lymphocytes in certain sections is rare and appears to depend on a sectioning artifact. The frequent incidence of clustering of lymphocytes further suggests that particular "weak spots" in the cohesion of the endothelial cell sheet favor the successive passage of lymphocytes at those loci.

The seemingly unusual relationship of lymphocytes and endothelial cells, as seen in section, may find a ready explanation if one considers some general properties of these migrating cells and the endothelium. In vitro, lymphocytes have a distinct polarity and, frequently, the nucleus leads while most of the cytoplasm is trailed behind (32, 34). Examples were seen which suggest that a similar pattern may be followed during their migration across the vascular wall (Fig. 5). If this is so, a blunt rounded process probing between and against endothelial cells would, in certain planes of sectioning, result in the occasional apparent internal lymphocyte even if one assumed that endothelial cells are relatively unyielding.

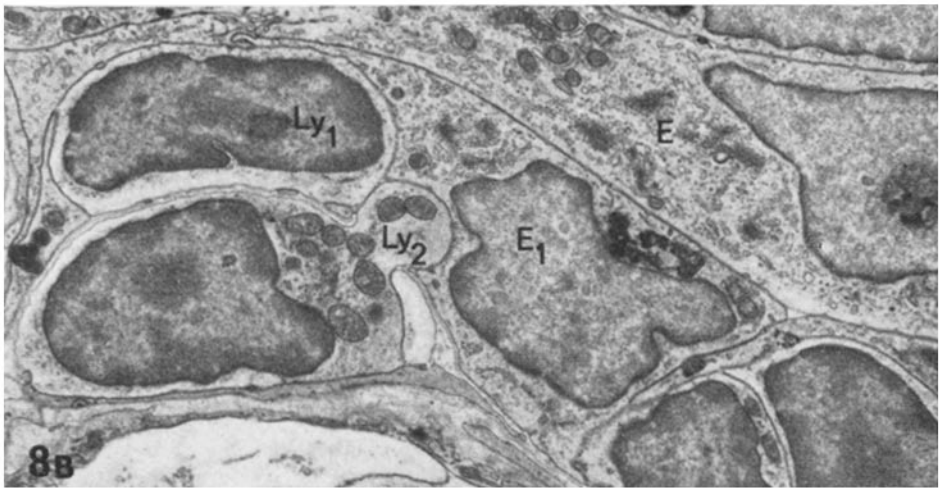
A considerable degree of rigidity of the endothelial cells is in fact implied in the intracellular theory of lymphocyte migration. This follows the widespread notion that the endothelium is composed, as it were, of immutable plates comparable to tiles in a mosaic. The increased height of endothelial cells in these special venules then necessitates a special mechanism to penetrate this barrier. The present observations give ample evidence that endothelial cells are far from being rigid (Figs. 2, 4, 6). They appear to be soft and extremely pliable cells capable of being molded into almost any shape. This is particularly evident in reconstructions of their cell bodies from serial sections (unpublished observations).

Furthermore, there is evidence from in vivo observations that they have some motility. In growing vessels, for example, they are apparently capable of migrating though still retaining their context within a sheet of cells (35, see also reference 36). The same has also been observed in organ cultures (37). It has been shown that endothelial cells, as other lining epithelial cells, tend to migrate

FIG. 8. From a sequence of serial sections cut approximately along the axis of the vessel. In Fig. 8 A (level 31) a lymphocyte (Ly_1) appears "internal." In Fig. 8 B (level 63) it is in direct contact with another lymphocyte (Ly_2), and in Fig. 8 C (level 89) it is primarily associated with a second endothelial cell (E_2). × 8000.



8A



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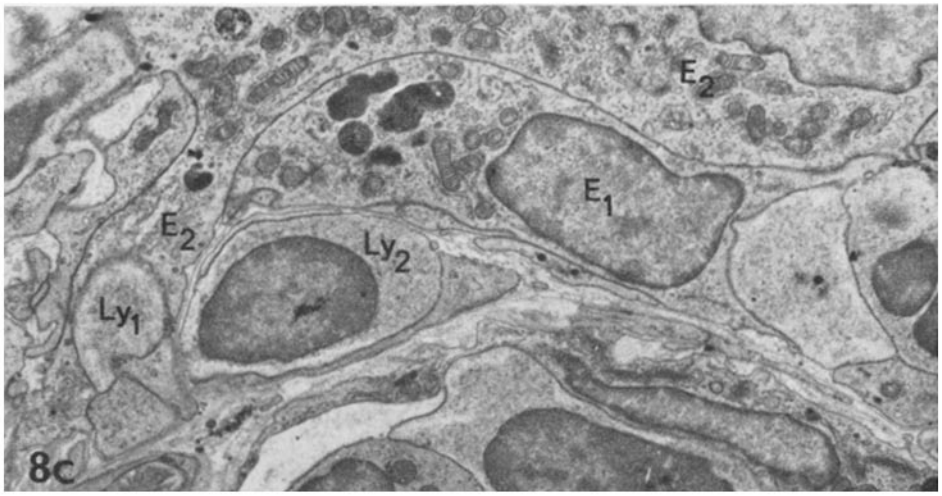

8C

over and eventually cover denuded areas (38, 39), or sites with abnormal materials, such as fibrin, adhering to the luminal surface. Now, if one concedes that endothelial cells are capable of movement and have a tendency to form closed sheets, their increased height in these venules of lymphoid tissue may be regarded as a special adaptation rather than as an increased barrier, a suggestion which was already advanced by Dabelow (10) in 1938.

Fig. 9 shows diagrammatically the pathway of migration of lymphocytes across the vascular endothelium. It takes into account observations made in this investigation and some properties of lymphocytes and endothelial cells just discussed. At the luminal surface, lymphocytes presumably probe for a path of least resistance. As adjacent endothelial cells are separated from each other, they tend to close over the "defect" in the vascular lining and to reseal the luminal surface as the lymphocyte continues to insinuate itself between cells towards the connective tissue sheath. The obvious plasticity of these rather large endothelial cells and the basic pattern of lymphocyte locomotion

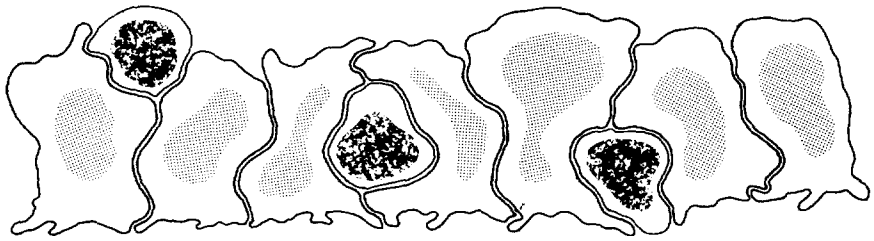

FIG. 9. Diagrammatic representation of the stages of a lymphocyte migrating through the endothelial layer. See text.

would account for the intricate relationship of these two cell types in sectioned material.

The net effect of such a process would be that of a valve which allows the passage of great numbers of cells without excessive fluid loss. Sustained migration of lymphocytes across the vascular wall apparently results in some leakage of plasma which can be demonstrated with the use of intravenously injected marker particles (21, 40).

The suggested scheme has made no reference to the difficult question of the cellular recognition that makes lymphocytes "home" into these vessels. Endothelial cells have been viewed as regulating in some way the cell traffic into areas where antigens are normally concentrated. The work of Gesner and his colleagues (41–43) has shown that a variety of procedures which alter the surface coat of the lymphocyte inhibit the homing of lymphocytes into these vessels. Based on his extensive studies of the vascular supply in lymphatic organs, Dabelow (10) has suggested an additional regulatory mechanism depending on blood being shunted into these vascular beds or bypassing them. An interesting finding was recently reported by Sordat, Hess, and Cottier (44).

Endothelial cell borders of these venules in human tonsils gave a positive reaction for IgG. The origin or function of this antibody are not known at present, but, as these authors point out, it may have some significance in regard to lymphocyte recirculation.

The emigration of lymphocytes from the bloodstream is not restricted to venules in lymphoid tissue or to vessels lined by high endothelium. Some lymphocytes are present in the lymph of all organs (45) and the possibility has been raised that the vascular endothelium is modified in response to a sustained traffic of lymphocytes across the vascular wall (8, 46). In rats, neonatally thymectomized, with greatly reduced numbers of circulating lymphocytes, the endothelial lining of these venules is low but is reported to increase in height after the intravenous injection of additional lymphocytes (46). Similar modifications of blood vessels have been reported in experimental granulomas in sheep (47). During the postnatal development of Peyer's patches in the rat, the appearance of vessels lined by high endothelium coincides with the presence of large numbers of migrating lymphocytes (9). In general, the formation of "reaction centers" or secondary nodules is apparently accompanied by an increase in the number of "high-endothelium" venules (8, 48).

Though little is known about the precise mechanisms of any of these processes, it now appears certain that the migration of lymphocytes across the vascular wall does not involve the continual perforation of endothelial cells. Like other white cells, lymphocytes cross the endothelial layer by insinuating between cells. Their pattern of locomotion, however, and the abundant cytoplasm of endothelial cells in these vessels result in an intricate spatial relationship which is often difficult to interpret in section.

SUMMARY

An electron microscope study was made of the mode of lymphocyte migration across the endothelial layer of venules in the Peyer's patches of mice and rats. Single and serial sections were examined.

Of a total of about 800 lymphocytes observed in single sections, 91% were located between endothelial cells and 9% were surrounded by endothelial cytoplasm in the particular plane of section. 62% of the lymphocytes occurred in groups of two or more. In long sequences of serial sections through 21 endothelial cells, all lymphocytes were located external to the endothelial cells though some appeared "internal" at certain levels of sectioning.

The probability that a lymphocyte which appears to be surrounded by endothelial cell cytoplasm actually lies within the cell was analyzed with a mathematical model derived from data obtained from single sections. The results of this analysis suggested that at least 93–99% of lymphocytes (within 90% limits of confidence) take an intercellular path in their migration from blood to lymph.

It is concluded that lymphocytes migrate across the vascular endothelium by insinuating themselves between endothelial cells and not by passing through

them. Rather than constituting an increased barrier to cell migration, the unusual height of the endothelial cells in these vessels is interpreted to be a special adaptation which allows sustained cell traffic without excessive fluid loss taking place concomitantly.

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APPENDIX

*Probability Analysis**

By R. E. Miles

The following argument involves the calculation of θ, the probability that an "external" lymphocyte appears to be "internal" in the plane of section, for an approximate mathematical model suggested by an examination of the section profiles of the 129 singleton external lymphocytes (Table II).

The surface of a singleton lymphocyte contains a net of junction curves, the curves common to the surfaces of the lymphocyte and a pair of endothelial cells (Fig. 1).

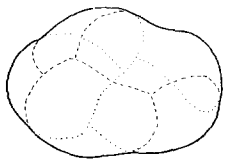

Fig. 1

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Thus a planar section of the surface of a singleton lymphocyte consists of a closed curve punctuated by junction points at which the sectioning plane intersects junction curves (Fig. 2).

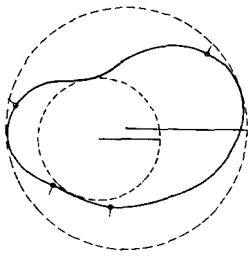

FIG. 2

Close examination of the 129 external lymphocytes indicated that the shapes of these sections are generally convex (a curve is convex if it has no "dents"), with the average value of the ratio of the radius of the largest contained circle to that of the smallest contained circle about $\frac{1}{2}$ (see Fig. 2). The boundary curves between junction points generally bulged outwards, indicating that singleton lymphocytes are fairly spherical and are deformed relatively little by endothelial resistance.

Although the planes of section were generally approximately perpendicular to the axis of the blood vessel, the positions of the junction points on the section perimeters were isotropic (in a two-dimensional sense), from which it was concluded that the positions of the junction curves on the lymphocyte surfaces were also isotropic. This enables us to assume below that the random sectioning plane is isotropic with respect to the lymphocytes being sectioned.

Thus, we take as our model of a lymphocyte a sphere of radius r, and first consider the case where the lymphocyte is in contact with just two endothelial cells, the corresponding junction curve being a circle of angular radius ϕ (see Fig. 3).

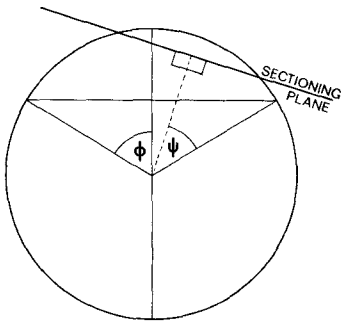

FIG. 3

Given that the sectioning plane makes an angle $\psi(0 \leq \psi \leq \pi/2)$ with the axis of symmetry, the conditional probability that the plane hits the smaller cap but not the circle of angular radius ϕ is

$$p(\phi \mid \psi) = \tfrac{1}{2}\{1 - \cos(\phi - \psi)\}. \tag{1}$$

Since the sectioning plane is isotropic, the frequency function of ψ is

$$f(\psi) = \sin\psi \qquad \left(0 \leq \psi \leq \frac{\pi}{2}\right), \tag{2}$$

and so the absolute probability that the sectioning plane hits the smaller cap but not the circle of angular radius ϕ is

$$\begin{aligned} p(\phi) &= \int_0^{\pi/2} p(\phi \mid \psi) f(\psi)\, d\psi , \\ &= \tfrac{1}{4}(2 - 2\cos\phi - \phi\sin\phi) . \end{aligned} \tag{3}$$

Next, we suppose that, approximately, the junction curves partition the surface of the spherical lymphocyte into equal circles of angular radius ϕ. Since the surface area of the smaller cap above is $2(1 - \cos\phi)\pi r^2$, there will be approximately $2/(1 - \cos\phi)$ of these circles, and so

$$\begin{aligned} \theta &= \{2/(1 - \cos\phi)\}p(\phi) \\ &= 1 - \frac{\phi}{2}\cot\frac{\phi}{2} . \end{aligned} \tag{4}$$

Since each circle perimeter is $2\pi r \sin\phi$, the total edge length of these circles in the lymphocyte surface is approximately

$$\begin{aligned} L &= \frac{1}{2} \cdot \frac{2}{1 - \cos\phi} \cdot 2\pi r \sin\phi \\ &= 2\pi r \cot\frac{\phi}{2} , \end{aligned} \tag{5}$$

the factor $\frac{1}{2}$ arising since it is supposed that the circles are contiguous, with each perimeter point lying in the perimeters of two circles.

Let N be the number of junction points in the plane section. Since the plane is random, so also is N. The mean value of N can be shown to be

$$E(N) = L/4r, \tag{6}$$

whatever the location of the total length L on the spherical surface; the proof is a matter of standard geometric probability, but is rather too long to include here.

Now, standard probability theory gives

$$E(N) = E(N \mid N = 0)P(N = 0) + E(N \mid N \geq 1)P(N \geq 1), \tag{7}$$

where $E(.)$ denotes "expectation," $E(.\mid .)$ denotes "conditional expectation," and $P(.)$ denotes "probability."

Clearly $E(N \mid N = 0) = 0$ and $P(N \geq 1) = 1 - \theta$, so

$$E(N) = (1 - \theta)\, E(N \mid N \geq 1). \tag{8}$$

Substituting in equation 8 for $E(N)$ from equations 5 and 6, and for θ from equation 4 we get

$$\phi\, E(N \mid N \geq 1) = \pi, \tag{9}$$

and so, by equation 4,

$$\theta = 1 - \frac{\pi}{2E(N \mid N \geq 1)} \cot \frac{\pi}{2E(N \mid N \geq 1)}. \tag{10}$$

It was shown in the main body of the text that the percentages of E_2 and E_3 are related by

$$E_2 = (1 - \theta)E_3. \tag{11}$$

Eliminating θ between equations 10 and 11, we arrive at the final fundamental equation

$$E_3 = \frac{2E(N \mid N \geq 1)}{\pi} \left\{\tan \frac{\pi}{2E(N \mid N \geq 1)}\right\} E_2 \tag{12}$$

expressing E_3 in terms of the two observable quantities E_2 and $E(N \mid N \geq 1)$. The ratio E_3/E_2 as a function of $E(N \mid N \geq 1)$ is shown in Fig. 4.

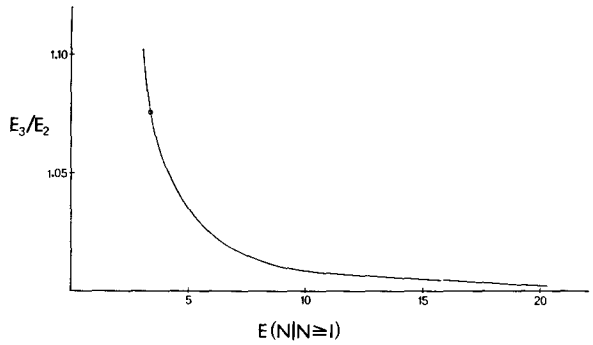

FIG. 4. E_3/E_2 as a function of E (N $N \geq 1$). The experimental value for the singleton lymphocytes (see Table II) is marked.

As expected, this ratio tends to unity as $E(N \mid N \geq 1)$ increases, since then the junction curve net intensifies and so θ rapidly tends to zero.

For the singleton lymphocytes in the present experimental study, the conditional expectation of N given that $N \geq 1$ is

$$E(N \mid N \geq 1) = 442/129 = 3.42. \tag{13}$$

Substituting this value in equations 9, 10, and 12, we obtain

$$\phi = 52°, \theta = 0.07, E_3/E_2 = 1.08. \tag{14}$$

For the same lymphocytes,

$$\hat{E}_2 = 129/149 = 86.5, \hat{I}_2 = 20/149 = 13.5, \tag{15}$$

and so by equation 14

$$\hat{E}_3 = 93.5, \hat{I}_3 = 6.5. \tag{16}$$

These estimates fall well within the 95% confidence intervals

$$0 < \theta < 0.141, 85.9 < E_3 < 100, 0 < I_3 < 14.1, \tag{17}$$

corresponding to the confidence intervals derived in the main text for all lymphocytes in class 2 (endothelial).

These results must be regarded as hypothetical, however, since in this model we have ignored lymphocytes belonging to groups of contiguous lymphocytes. Approximately 60% of the sectioned lymphocytes belong to such groups and a significant proportion of the sections which appear to be those of singleton lymphocytes may, in fact, be sections of lymphocytes in groups. The above theory does suggest, however, a likely value, 0.07, for θ in a specific model.

From Table II we may conclude that the junction curves on the surfaces of the aggregate of all lymphocytes in class 2 (endothelial) are roughly similar in distribution to those of the singleton lymphocytes, with a slight intensification. For this reason, and because of their very presence in groups, the value of θ for the group lymphocytes is probably somewhat smaller than that for singleton lymphocytes, 0.04 say. This would mean that the true value of θ is of the order of 0.05. Combining this value with equations 11 and 15, we obtain the approximate estimates

$$\hat{E}_3 = 96 \text{ and } \hat{I}_3 = 4. \tag{18}$$

To illustrate the precision of these estimates, if (as does not seem too unreasonable) a 90% confidence interval for θ is (0.03, 0.07), then the corresponding 90% confidence interval for E_3 is approximately (93, 99).