Lymphocyte Migration into Three-dimensional Collagen Matrices: A Quantitative Study

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ABSTRACT Lymphocytes have been plated onto the surface of three-dimensional gels of native collagen fibers, and their distribution throughout the three-dimensional collagen matrix has been determined in a quantitative fashion at various times thereafter. Information regarding the total number of applied cells may be obtained by this means. Lymphocyte penetration into the collagen gel does not appear to involve the expression of collagenolytic activity, nor does it require the presence of serum. Analysis of the kinetics of lymphocyte penetration into the gel matrix indicates that lymphocytes are migrating in a "random-walk" fashion. Our objective has been to establish a model system for studying the cell-matrix and cell-cell interactions which influence the pattern of lymphocyte recirculation in vivo and the results presented here are discussed in this context.

Lymphocytes are highly mobile cells in vivo capable of moving back and forth between the peripheral blood and various tissue compartments. This active traffic of cells, referred to as lymphocyte recirculation, was first demonstrated to occur by Gowans (7). The recirculation of lymphocytes is a complex process involving the following sequence of events (2, 5, 6): (*a*) the adherence of peripheral blood lymphocytes to endothelial cells in specific regions of the microvasculature, (*b*) the extravasation of adherent lymphocytes into the perivascular tissue space, (*c*) the migration and sorting out of lymphocyte subsets into defined regions of the tissue, and (*d*) the passage of lymphocytes through the lymphatic circulation back into the peripheral blood.

The overall pattern of lymphocyte recirculation must be examined in vivo. Related studies in vitro have tended to focus attention on the effects of various soluble factors on lymphocyte migration into filter disks in order to identify putative chemotactic agents (14, 15, 27, 28). Stamper and Woodruff (24) have introduced an in vitro method for examining the specific adhesive interactions between lymphocytes and the high endothelium found in lymph nodes.

Our own interest in lymphocyte recirculation is specifically concerned with identifying the various cell-matrix and cell-cell interactions which contribute to the control of lymphocyte migration and sorting out once they are within the stroma of relevant tissues. Collagen fibers are a constituent of the tissue stroma through which lymphocytes migrate in vivo (12), and three-dimensional gels of native collagen fibers have previously been used to study the migratory behavior of a number of cell types in vitro (4, 17–22). Data are presented in this communication regarding the migration of normal human peripheral blood lymphocytes into three-dimensional collagen gels. These data indicate that lymphocyte migration through this matrix proceeds in a "random-walk" fashion in a manner which does not involve the lytic degradation of collagen fibers. These results are discussed in the context of our interest in lymphocyte recirculation.

MATERIALS AND METHODS

Isolation of Lymphocytes: Human peripheral blood lymphocytes were obtained from normal volunteer donors. Between 40 and 60 ml of blood were removed by venipuncture and defibrinated by shaking gently for 20 min at room temperature with glass beads in 20-ml glass beads in 20-ml glass bottles. The fibrin clot was removed and the resultant defibrinated blood mixed in 1:1 ratio with Dulbecco's A phosphate-buffered saline (PBS). 20-ml aliquots of this diluted preparation were then layered over 20-ml of Lymphocyte Separation Medium (cat. no. 16-920-54; Flow Laboratories, Irvine, Scotland) and centrifuged at 400 *g* for 30 min at 20°C. Leukocytes were recovered from the interface, washed 3× in PBS, and resuspended in Eagle's Minimal Essential Medium (MEM) supplemented with 15% donor calf serum and 100 U/ml penicillin and streptomycin (complete growth medium). Adherent cells were removed by incubating the leukocyte suspension in tissue culture dishes for 60 min at 37°C. The nonadherent cells were recovered in the growth medium, washed 3× in complete growth medium, and finally suspended in complete growth medium at a concentration of 10^6 cells/ml.

Cell numbers were routinely determined with a Neubauer hemocytometer. In a small number of experiments, cell number was measured with a Becton, Dickinson fluorescent activated cell sorter (Model 4; Becton, Dickinson & Co.). Contaminating monocytes in the final cell suspension were identified by staining

for nonspecific esterase as described by Yam et al. (29) and estimated to account for <3% of the population. The polymorphonuclear leukocytes were identified morphologically in Giemsa-stained preparations and estimated to account for <1.8% of the population.

Determination of Lymphocyte "Adhesion" and Migration: Type I collagen was extracted from rat tail tendons and used to prepare 2-ml gels in 35-mm plastic tissue culture dishes (Gibco-Brocult, Uxbridge, England; cat. no. 53066) as previously described (17).

Two-dimensional films consisting of native collagen fibers or denatured collagen (i.e., gelatin) were prepared as previously described (19). The adhesion of lymphocytes to these films was ascertained as described below for the three-dimensional gels. Collagen labeled to a high specific activity in vitro with ^{3}H-acetic anhydride was prepared as described previously (18) and used to make radio-labeled gels. The release of radio-labeled collagen degradation products into the growth medium overlying such gels was monitored with a Beckman liquid scintillation counter (model LS7500; Beckman Instruments, Palo Alto, CA).

Unless otherwise indicated, 1-ml aliquots of the lymphocytes suspended in complete growth medium were pipetted onto collagen gels previously overlaid with 1 ml of complete growth medium. Immediately after plating, 1-ml samples were removed from two replicate gels and mixed with an equal volume of 1% glacial acetic acid (to lyse erythrocytes), and an aliquot of this mixture was taken for cell counting. Cell numbers so determined were used to calculate the number of lymphocytes initially plated onto the gels (i.e., zero-time count). The remaining gels were incubated at 37°C in a humidified incubator gassed with 5% CO_2 in air. At various times thereafter, the number of lymphocytes remaining in the growth medium was determined in each of two replicate cultures as described above and, by substraction of this number from the zero-time count, it was then possible to calculate the percentage of lymphocyte remaining "adherent" to the gel. It must be emphasized, however, that the term "adherent" is used here simply to distinguish those cells remaining associated with the collagen gel from those recovered in the growth medium and is not meant to suggest any particular mode of lymphocyte-collagen interaction.

The migration of lymphocytes into the collagen matrix was measured in the same replicate gels used to determine cell "adhesion." The remaining 1 ml of growth medium (with floating cells) was removed from these cultures, and the gels were overlaid with 2 ml of 10% formalin. The migration of cells into the gel matrix was determined by the "microscopic" method previously described by Schor (17). Information regarding the number of lymphocytes present at different depths within the collagen matrix may be obtained by optical sectioning of the gel into four equal levels (using the micrometer calibration scale), moving from the gel surface down to the plastic dish (Fig. 1). Data obtained in this fashion are expressed as the percentage of "adherent" (i.e., gel-associated) lymphocytes which are present at the gel surface (*S*) and within levels *1*, *2*, *3*, and *4* of the gel matrix (Fig. 1). The distribution of lymphocytes within the gel matrix may also be expressed as a percentage of the total number of cells initially applied.

All microscopic observations were done at × 320 with a Leitz Diavert microscope fitted with an SY_2 photographic graticule defining a rectangular field of 0.9 × 0.65 cm^2.

In certain experiments lymphocytes were incorporated directly into the three-dimensional collagen matrix when the gel was initially cast (as described by Schor, reference 17). The distribution of cells within the gel matrix was subsequently monitored as described above. Cells which migrated up into the growth medium were recovered at periodic intervals and counted.

Electron Microscopy: Lymphocytes were fixed directly in suspension or in situ within the collagen matrix. The method described by Saunders et al. (16) was used for fixation of cells in suspension. For fixation of lymphocytes in situ, the gels were flooded with 3% glutaraldehyde (as above), given three rinses with buffer, left for 1 h, and postfixed for 1 h with 1% OsO_4. The fixed gels were washed 3× buffer, and a 15-mm cork borer was then used to remove "plugs" from the gel. These "plugs" were subsequently dehydrated by passage through a graded series of ethanol and critical-point-dried from CO_2, using Freon 113 as the transitional fluid. For viewing the surface morphology of lymphocytes which had migrated deep within the gel matrix, the gel "plugs" were attached to stubs and a portion of the gel surface was dissected out with a pair of watchmaker's forceps under a binocular microscope. The texture of the fixed, dried gel is such that removal of superficial portions of the gel in this manner produces clean fractures, leaving depressions in which it is possible to view the surface morphology of lymphocytes which had migrated into the gel matrix. The gels were then sputter-coated with ~10 nm of gold and viewed in a Cambridge S-410 scanning electron microscope (SEM) at an accelerating voltage of 20 kV.

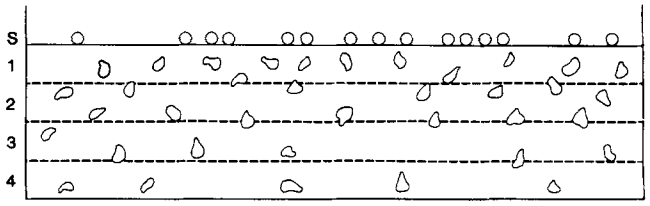


FIGURE 1 A schematic representation of the optical "sectioning" method used to obtain data regarding the distribution of lymphocytes within the collagen gel matrix. Cells are plated on the gel surface and then the number of cells present at the gel surface (S) and within four approximately equal zones within the gel (levels 1, 2, 3, and 4) are determined as described in Materials and Methods. The gel is 4 mm thick and each level is therefore ~1 mm in height.

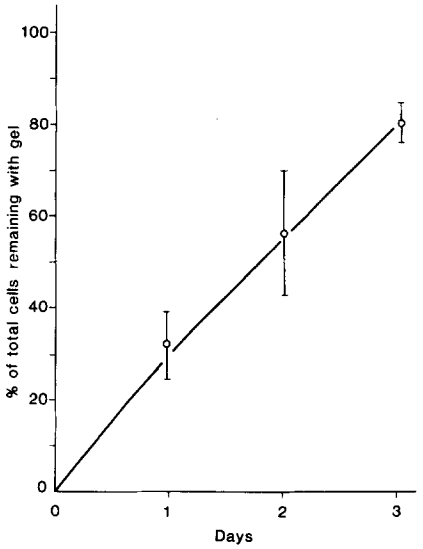


FIGURE 2 The "adhesion" of lymphocytes to three-dimensional collagen gels. Lymphocytes were plated onto the gel surface at an initial density of 10^6 cells/culture and the number of cells remaining floating in the growth medium at various times thereafter was determined as described in Materials and Methods. The percent "adhesion" was calculated simply by subtracting these values from 100%. The data presented are the means ±SD of 20 experiments. Lymphocytes were actually migrating into the gel matrix (see Fig. 3) and therefore the data presented here simply reflect the association of lymphocytes with the gel and do not imply that these cells are establishing adhesive interactions with the collagen fibers.

RESULTS

Lymphocyte Adhesion and Migration into the Gel Matrix

Human peripheral blood lymphocytes were collected and suspended in complete growth medium at a concentration of 10^6 cells/ml as described in Materials and Methods. The cells were plated onto collagen gel cultures, and the percentage of "adherent" cells was determined after 24, 48, and 72 h of incubation. Mean values from 20 individual experiments (conducted over a period of 2 yr) were used to calculate the means and SDs shown in Fig. 2. There is an approximately linear increase in the number of "adherent" cells during the incubation period. The viability of the floating cells recovered in the growth medium was assessed by the ability of the cells to exclude the dye trypan blue and was found to be >90% at all times.

As previously mentioned, the term "adherent" is used here solely to distinguish between those cells associated with the gel matrix and those recovered floating in the growth medium. To obtain further information regarding the adhesive behavior of

the lymphocytes, we compared the ability of these cells to adhere to three-dimensional collagen gels, two-dimensional collagen films and two-dimensional films of denatured collagen (i.e., gelatin). Data presented in Table I indicate that lymphocytes suspended in complete growth medium do not adhere in significant numbers to the two-dimensional films of either native or denatured collagen. These data suggest that the "adherent" lymphocytes on the gels represent cells which have insinuated themselves amongst the collagen fibers of the three-dimensional matrix. This conclusion is consistent with the appearance of the cells in the SEM (to be discussed in a subsequent section).

The depth of lymphocyte migration into the three-dimensional collagen matrix was measured in the same 20 individual experiments used to determine the "adhesion" data shown in Fig. 2. Data in Fig. 3 are expressed as the percentage of the total applied cells present at the gel surface and within each of the four layers. These data indicate that there is a progressive movement of lymphocytes down into the three-dimensional collagen matrix during the 3-d incubation period of the experiment. It should also be noted that these normal human lymphocytes migrate in a highly reproducible fashion into the collagen gel matrix (as evidenced by the relatively small standard deviations obtained), thus making it possible to compare the migration of different types of cells (e.g., specific subsets, malignant lymphocytes, etc.) in a meaningful fashion. Preliminary data indicate that peripheral blood lymphocytes from patients with chronic lymphocytic leukemia (CLL) migrate significantly more slowly into the gel matrix than do normal cells (J. Wagstaff and S. L. Schor, unpublished observations).

Lymphocyte migration into the gel is inhibited by incubation at 4°C and by the presence of 10 mM sodium azide, thus indicating that it is an active process requiring the expenditure of cellular energy (data not shown). Formalin-fixed lymphocytes plated onto the gel surface do not infiltrate into the gel (even after 6 d of incubation), again indicating that cell movement into the collagen matrix is not a passive occurrence simply involving the sedimentation of cells through fiber matrix.

The data presented in Fig. 3 were replotted according to the diffusion equation as first suggested by Zigmond and Hirsch (32). The linear nature of the resultant plot (Fig. 4) suggests that lymphocyte migration into the three-dimensional collagen matrix proceeds in a random-walk fashion. The slope (i.e., $1/4DT$) may be used to calculate the "diffusion" coefficient (D) of lymphocyte movement into the gel at a given time (T), with values of $D = 1.79$ mm^2/d obtained on day 2 and $D = 1.85$ mm^2/d obtained on day 3.

TABLE I

Adhesion of Lymphocytes to Three-dimensional Collagen Gels and Two-dimensional Collagen Films

Time	Native collagen gel	Native collagen film	Denatured collagen film
d			
1	51.4	21.8	17.9
2	71.0	16.1	21.4
3	82.2	19.6	21.4

Lymphocytes were plated onto the surface of three-dimensional collagen gels and two-dimensional films of either native or denatured collagen (17). The number of cells recovered floating in the growth medium was determined at various times thereafter and used to calculate the adhesion of cells to these substrata. The data presented are the mean values of triplicate cultures at each time point.

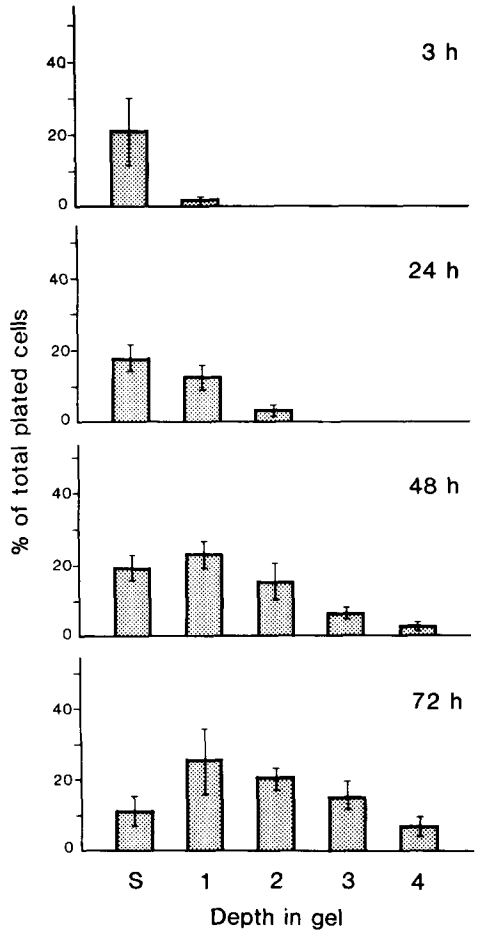


FIGURE 3 Lymphocyte migration into the collagen gel. Cells were plated onto the surface of collagen gel cultures and their distribution on the gel surface (*S*) and within the collagen matrix (levels *1*, *2*, *3*, and *4*) was determined as described in Materials and Methods at various times thereafter. Data expressed as a percentage of the total applied cells (mean ±SD of 20 experiments).

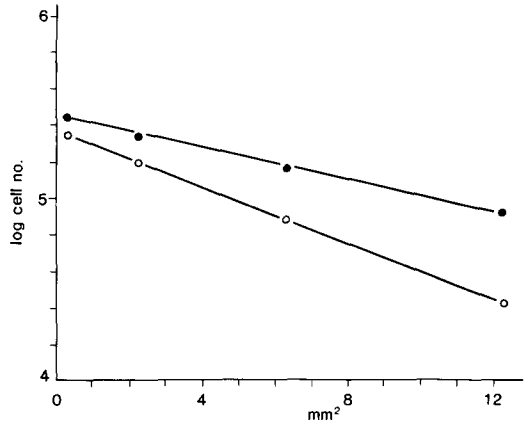


FIGURE 4 The data presented in Fig. 3 for day 2 (○) and day 3 (●) are plotted as the log of cell number in each level of the gel against the square of the mean distance of that level from the gel surface (NB-each level is 1 mm high). The resultant straight line plot indicates that the movement of cells into the gel conforms to the diffusion equation and allows a "diffusion coefficient" (D) to be calculated (see reference 32).

The random nature of lymphocyte movement within the collagen gel matrix was further indicated in an experiment in which lymphocytes were incorporated within the three-dimen-

sional collagen matrix when the gel was initially cast (17). As expected, lymphocytes were observed to be homogeneously distributed throughout the gel matrix at the beginning of the experiment, with no cells present in the overlying growth medium (Table II). The medium was withdrawn from replicate cultures on day 1, fresh medium pipetted onto the cultures, and the distribution of lymphocytes then determined within the gel matrix. This operation was repeated on the same cultures on the following 2 d. The data in Table II reveal that the lymphocytes remained homogeneously distributed throughout the gel matrix during this time. The maintenance of such a homogeneous distribution after 3 d of active cell movement (confirmed by direct observation of living cells under phase-contrast optics and data in Fig. 3) indicates that lymphocytes display no preferential directionality in their migration within this simple collagenous matrix.

The data in Table II also reveal that a small number of lymphocytes (i.e., 2% of the total) were recovered floating in the growth medium after each of the 24-h incubation periods during the 3-d duration of the experiment. The same number of floating cells were recovered in the medium of parallel cultures left undisturbed for 3 d. These data suggest that lymphocytes migrate out of the gel matrix into the overlying growth medium and establish a dynamic equilibrium (at a time less than or equal to 24 h) in which ~2% of the total cells are in the medium. When these floating cells were collected and plated back onto the surface of a collagen gel, the cells moved into the gel matrix at the same rate as the Fig. 3 (data not shown).

The effects of cell density on lymphocyte migration into the collagen gel are shown in Table III. Cells were plated onto the gel surface at concentrations ranging between 10^5 and 2×10^6/gel. Migration into the gel matrix was assessed after 3 d of incubation. The data presented in Table III indicate that lymphocyte migration into the collagen matrix is independent of plating density within the experimental range examined. These results are in contrast with those previously reported with a variety of both normal and transformed cell types where we found that cell migration into the gel matrix did vary as a function of initial plating density in a manner characteristic of each cell type examined (reference 22 and unpublished observations).

TABLE II

Distribution of Lymphocytes within the Gel Matrix and in the Growth Medium

Day	No. of previous medium changes	Percentage of lymphocytes: Within gel S	1	2	3	4	In growth medium
Immediately after gel cast	—	0	24.6	26.9	23.9	24.6	0
1	0	1.9	23.8	25.4	22.1	24.4	2.4
2	1	1.5	25.1	22.9	24.4	24.0	2.1
3	2	2.0	26.0	24.1	23.7	22.4	1.8
3	0	1.8	25.4	24.8	24.3	21.8	1.9

10^6 lymphocytes were initially incorporated within the three-dimensional collagen gel matrix when the gel was cast. The number of cells floating in the medium and the distribution of cells within the gel matrix were determined as soon as possible after the gels had set (i.e., within 10 min). These measurements were made at 24-h intervals for the duration of the 3-d incubation period; medium was changed on these dishes on day 1 and 2 of the experiment. Approximately 2% of the cells were recovered in the growth medium after each 24-h incubation period and the lymphocytes remained homogeneously distributed within the gel matrix. Similar results were obtained in replicate cultures left without medium change for the entire 3-d incubation. Data are expressed as a percentage of the total lymphocytes present in the culture at the time the determinations were made.

TABLE III

Effects of Cell Density on Lymphocyte Migration

Cell density $\times 10^5$	Level within gel matrix: S	1	2	3	4
1.0	20.6	38.1	20.1	17.2	4.0
2.5	19.3	35.9	26.1	13.8	4.9
5.0	17.3	36.8	23.4	16.3	6.2
10.0	19.9	34.9	24.8	16.8	3.6
20.0	17.8	38.1	21.9	16.3	5.9

Lymphocytes were plated onto the collagen gels at densities ranging between 10^5 and 2×10^6. The distribution of lymphocytes at the gel surface (*S*) and within the three-dimensional matrix (levels *1*, *2*, *3*, and *4*) were determined after 3 d of culture. Results are expressed as percentages of "adherent" cells and represent the mean values of duplicate cultures.

The effects of serum on lymphocyte migration into the gel matrix are shown in Fig. 5. In this experiment lymphocytes were isolated in the usual manner by gradient centrifugation (Materials and Methods) but washed in serum-free medium rather than medium containing 15% calf serum. These cells were then plated onto gels containing either 1 ml of serum-free medium or 1 ml of serum-containing medium calculated to give a final concentration of 15% in the cultures. Lymphocytes cultured in serum-free medium in this fashion were not even transiently exposed to heterologous serum during their preparation. The results presented in Fig. 5 indicate that lymphocytes cultured in the absence of serum still migrate extensively into the collagen matrix, although their overall rate of penetration appears to be somewhat reduced compared to cells incubated in the presence of calf serum. Lymphocytes cultured in the presence of autologous serum migrated in a manner indistinguishable from that observed in the presence of calf serum (data not shown).

To determine whether lymphocyte migration into the gel matrix is associated with any lytic degradation of collagen fibers, we plated 10^6 lymphocytes in serum-free medium onto gels prepared from high specific activity ^{3}H-labeled collagen (18). As can be seen in Table IV, the migration of lymphocytes into the gel matrix was not accompanied by any detectable release of soluble radio-labeled collagen degradation products into the growth medium.

Lymphocyte Morphology Within the Collagen Matrix

Lymphocyte morphology within the collagen matrix was observed to be highly variable. Although apparently round cells were often observed, cells with rather extensive cytoplasmic extenstions were also common (Fig. 6). The dynamics of cell morphology change are sufficiently rapid not to necessitate the use of time-lapse cinematography to visualize cell movement.

The surface morphology of lymphocytes observed in the scanning electron microscope is shown in Fig. 7. Cells fixed in suspension at the same stage that they would have normally been plated onto gels have a microvillous surface morphology consistent with that previously reported (13) for normal pe-

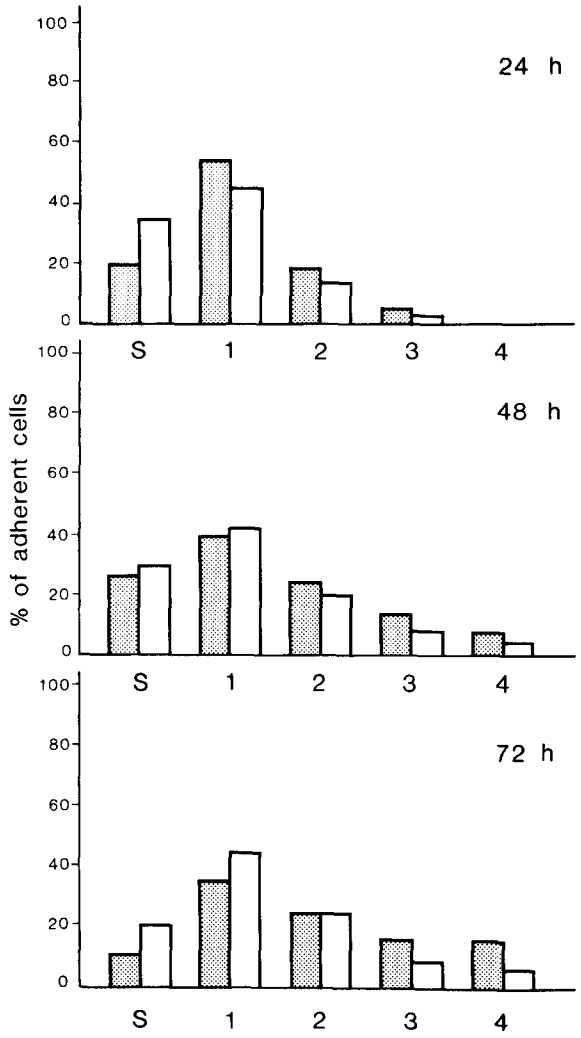


FIGURE 5 The effects of serum on lymphocyte migration into the collagen gel matrix. Cells were plated onto the collagen matrix either in serum-free medium (white bars) or in 15% serum-containing medium (stipled bars). The number of lymphocytes on the gel surface (S) and within the collagen matrix (levels *1*, *2*, *3*, and *4*) were determined as described in Materials and Methods after 1, 2, and 3 d of culture. The data presented are the means of duplicate cultures.

TABLE IV

Absence of Collagen Degradation during the Process of Lymphocyte Migration

Day	% of cells within gel matrix	CPM in growth medium: Gels with cells	CPM in growth medium: Gels without cells	Net CPM released by cells
1	21.8	2,150	2,090	60
2	63.9	2,095	1,895	200
3	82.1	1,870	1,920	−50

10^6 lymphocytes were plated onto gels made with radio-labeled collagen with a specific activity of 0.95×10^6 cpm/mg collagen. Each 2-ml gel (consisting of 4 mg collagen) therefore contained 3.8×10^6 cpm. The amount of radioactivity present in the growth medium was determined in replicate cultures after 1, 2, and 3 d of incubation as previously described (18). The radioactivity in the growth medium of control gels containing no cells was also measured on each of these days, and these values were subtracted from the above figures to ascertain the amount of net radioactivity released by the cells. No net radioactivity was detected in the medium after 3 d of incubation when >80% of the total cells are within the collagen matrix. Radioactive gels incubated with 0.05% trypsin for 1 h released 40,100 cpm into the medium (i.e., 1.06% of the total counts). The data presented are the means of three replicate cultures.

ripheral blood lymphocytes (Fig. 7 *A*). Lymphocytes examined at the surface of the gel 48 h after plating retain a similar microvillous surface morphology, although there is an apparent reduction in the density of microvilli along the under surface of the cell (Fig. 7 *B*). Cells which had migrated into the gel (but remain clearly visible just below the gel surface) have a greatly reduced density of a microvilli on their surface compared to cells in suspension (Fig. 7 *C*). Cells which had migrated 2–3 mm into the gel can be observed by "dissection" of the gel as described in Materials and Methods. These cells uniformly display a smooth surface topography (Fig. 7 *D*). A large proportion of lymphocytes within the gel matrix were observed to possess a single, large, veil-like cytoplasmic projection (Fig. 7 *E* and *F*). These cytoplasmic extensions were often in excess of the diameter of the cell body and appeared to be extremely thin, as there was clear penetration of the veil by the electron beam (at the relatively high accelerating voltage of 20 kV) allowing visualization of the collagen fibers behind. At no time did we observe evidence of collagen fiber lysis, even in the immediate vicinity of lymphocytes deep within the gel matrix.

DISCUSSION

Recently, Haston et al. (8) have presented data regarding the mechanism of lymphocyte translocation through three-dimensional collagen matrices. They conclude that lymphocyte movement into this substratum involves the penetration of cellular processes between the randomly enmeshed collagen fibers, with the resultant creation of transient anchorage points. Our observations presented here are consistent with this conclusion.

The movement of lymphocytes into the collagen matrix appears to be an active process requiring the expenditure of cellular energy. Furthermore, the data presented in Fig. 4 and Table II suggest that lymphocyte migration into the gel occurs by a "random walk" progress. Our observations with the SEM failed to reveal any evidence of collagen fiber lysis as a consequence of lymphocyte penetration into the gel matrix. The use of radio-labeled collagen gel matrices similarly failed to detect any evidence of gel dissolution. These findings suggest that lymphocyte migration into the gel does not depend upon the expression of collagenolytic activity by the cells. Similar conclusions regarding the ability of a number of normal and tumour (tissue) cell types to migrate into the gel matrix without detectable collagenolytic activity have been reported by us elsewhere (18).

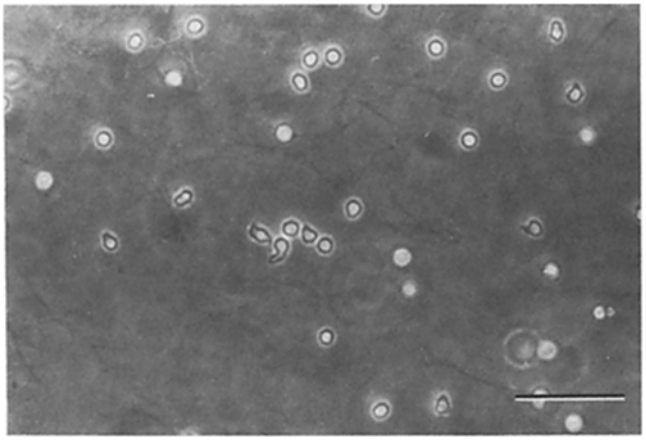
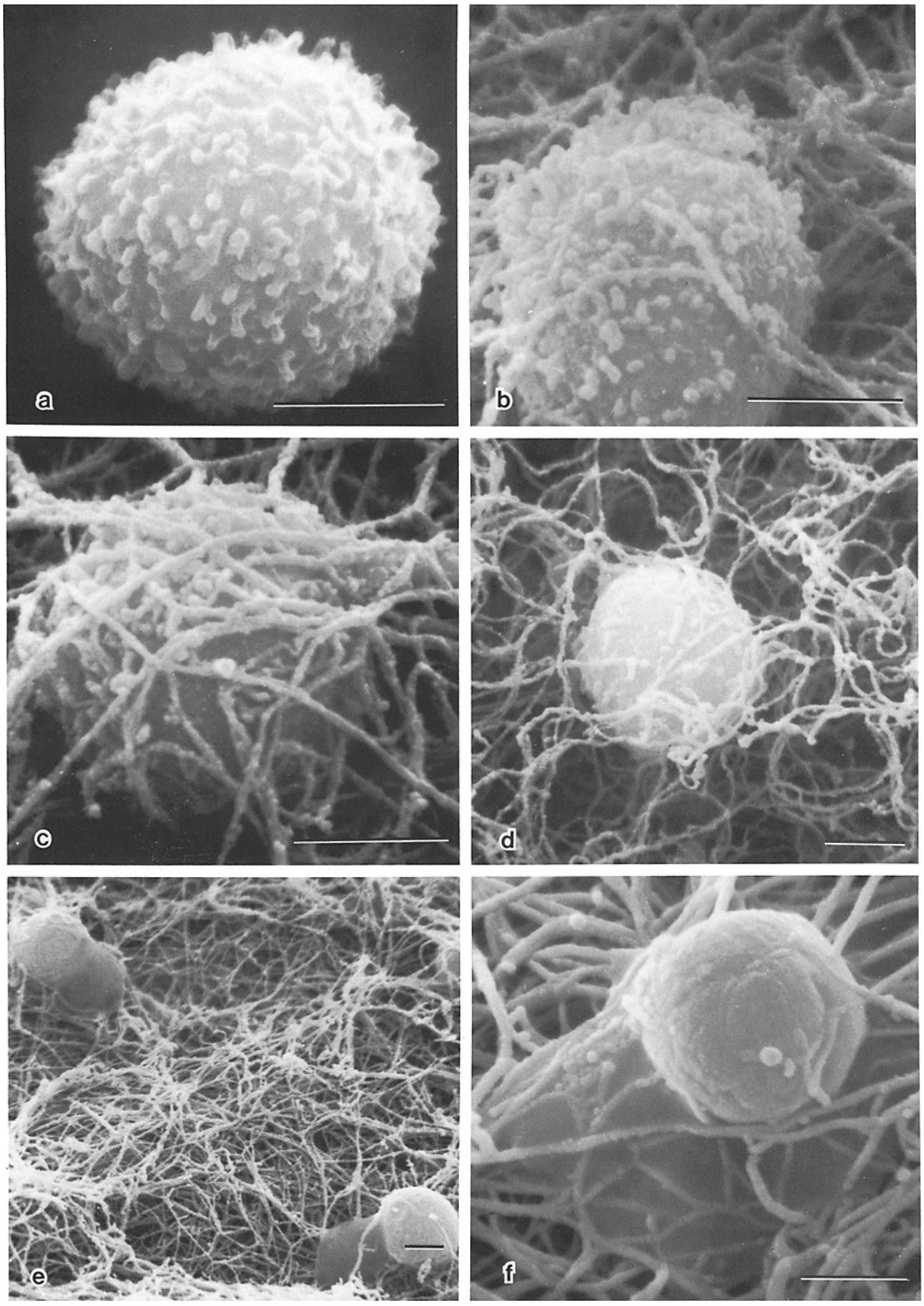


FIGURE 6 The morphology of lymphocytes within the collagen gel matrix. Living cells were observed and photographed using phase contrast optics 48 h after plating. Bar, 50 μm. × 330.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The lymphocytes within the collagen gel matrix were observed to have a significantly smoother surface morphology compared to cells in suspension. This observation in consistent with the adoption of a smooth surface morphology by peripheral blood lymphocytes when they adhere to and migrate through the endothelial cell lining of blood vessels in vivo (1, 26). The functional significance of these observations is not known.

Quantitative data are presented in this communication regarding the migration of human peripheral blood lymphocytes into three-dimensional collagen gel matrices. The compiled results of 20 individual experiments indicate that normal peripheral blood lymphocytes migrate in a consistent manner in this experimental system and that results with a high degree of reproducibility may be easily obtained. The approach which we have adopted in this study (i.e., determining the number of cells present at different levels within the gel matrix) makes it possible to obtain information about the migratory behavior of the total number of applied cells. A similar approach has been employed by Parrott (15) and Ward et al. (27) in a study regarding the migration of lymphocytes into micropore filters. In contrast, data regarding cell migration has been commonly analysed by the "leading front" method of analysis, in which the distance travelled by the fastest moving cells in the population is determined. One difficulty with this approach is that the data which are collected may pertain to only a small subset of the total cells present. This is a potentially serious problem when dealing with peripheral blood lymphocytes, considering the known heterogeneity of this cell population.

The nature of the substratum plays an important role in controlling the migratory behavior of a number of cell types (9). Furthermore, cells may display quite different migratory activity on the artificial substrata commonly employed in tissue culture compared with macromolecular matrices, such as collagen gels (17). To obtain information regarding the factors which influence cell migration in vivo, it is therefore important to provide cells with a substratum which begins to approximate the physical and chemical properties of their natural environment. Collagen is a major component of the extracellular tissue matrix through which lymphocytes migrate in vivo and we believe that three-dimensional gels of collagen fibers are therefore an appropriate substratum on which to examine lymphocyte migration.

The data presented in Fig. 5 reveal that lymphocyte migration into the collagen gel matrix is not dependent upon the presence of serum, although serum does appear to have a measurable stimulatory effect. It should also be noted that the serum-free medium used in these experiments is devoid of any exogenous proteinaceous material. We have observed (20, 21) that the migration of a number of normal and transformed cell lines into the collagen matrix is similarly not dependent upon the presence of serum. There has been a certain amount of speculation in the literature regarding the spontaneous migratory activity of freshly isolated lymphocytes. Previous studies have indicated that the migration of lymphocytes into Millipore and related filters is significantly enhanced by the prior incubation of cells in serum-containing medium, as well as by the presence of serum (or other proteinaceous material) during the migration experiment (14, 15, 28). In view of the artificial nature of the filter substratum, it is possible that the serum requirements manifest under these experimental conditions are not representative of lymphocyte migratory behavior within a more physiological environment. It is, however, difficult to draw definitive conclusions at this time regarding the possible differential requirements for serum on these substrata in view of the different time scales in the respective experiments (i.e., hours with the filters and days with the collagen gels).

Our objective is to use collagen gel substrata specifically to examine the factors which influence the migration and sorting out of lymphocyte subsets within the stroma of relevant organs under both physiological and pathological situations. In this context, the collagen gels provide a particularly useful substratum since other matrix macromolecules (e.g., fibronectin) and relevant cell types (e.g., interdigitating and other accessory cells, see references 23, 25) may be incorporated within the collagen matrix prior to the addition of the lymphocytes. Previous studies have demonstrated that various tissue cells so incorporated into the three-dimensional collagen matrix maintain a more differentiated phenotype compared to cells cultured on the usual two-dimensional plastic surfaces (10, 11, 30, 31). It may, therefore, be possible to study specific aspects of lymphocyte interaction with relevant tissue cells in a controlled manner in vitro under conditions which begin to approximate their physiological state in vivo. Soluble cell-produced factors believed to influence lymphocyte migration (3) could also be examined in this system.

The first step in the process of lymphocyte recirculation (i.e., the adherence of lymphocytes to an endothelial cell layer and their subsequent migration through this layer) may similarly be examined by plating the lymphocytes on an endothelial cell monolayer growing on the surface of the collagen gel. Data relating to this interaction of lymphocytes with endothelial cells will be presented elsewhere and should provide a generally useful model system for examining lymphocyte extravasation.

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FIGURE 7 The morphology of lymphocytes observed with the scanning electron microscope. (*a*) Lymphocytes fixed in suspension (harvested onto poly-L-lysine-coated coverslip) display a typically highly microvillous surface morphology. Bar, 2 μm. × 15,500. (*b*) Lymphocytes on the surface of the collagen gel, 48 h after plating. Microvilli are apparent on the uppermost cell surface but are more sparse where the cell is in close apposition to the collagen fibers. Bar, 2 μm, × 14,500. (*c*) A lymphocyte just below the gel surface, 48 h after plating. Only occasional microvilli are visible. Bar, 2 μm. × 14,250. (*d*) Lymphocytes approximately 2–3 mm below the gel surface, exposed by "dissection" of fixed gel 48 h after plating. Cells display a basically smooth surface morphology compared to cells fixed in suspension. Bar, 22 μm. × 3,250. (*e*) Lymphocytes within gel matrix 48 h after plating, showing veil-like cytoplasmic extension characteristic of migrating cell. Bar, 2 μm. × 3,400. (*f*) Detail of lymphocyte within the gel matrix 48 h after plating showing insertion of cytoplasmic projection among collagen fibers. Bar, 2 μm. × 9,600.

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