Lymphocyte Locomotion and Attachment on Two-dimensional Surfaces and in Three-dimensional Matrices

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ABSTRACT The adhesion and locomotion of mouse peripheral lymph node lymphocytes on 2-D protein-coated substrata and in 3-D matrices were compared. Lymphocytes did not adhere to, or migrate on, 2-D substrata such as serum- or fibronectin-coated glass. They did attach to and migrate in hydrated 3-D collagen lattices. When the collagen was dehydrated to form a 2-D surface, lymphocyte attachment to it was reduced. We propose that lymphocytes, which are poorly adhesive, are able to attach to and migrate in 3-D matrices by a nonadhesive mechanism such as the extension and expansion of pseudopodia through gaps in the matrix, which could provide purchase for movement in the absence of discrete intermolecular adhesions. This was supported by studies using serum-coated micropore filters, since lymphocytes attached to and migrated into filters with pore sizes large enough (3 or 8 

In early studies of lymphocyte movement, the polarized morphology was described, with (from front to back) a leading hyaline veil, a constriction ring, the cell body with the nucleus well forward, and the tail or uropod (8, 15, 16). This morphology was described as amoeboid, and comparisons with the locomotor behavior of amoebae were made. These lymphocytes were usually studied within the 3-D matrix of a fibrin clot (8, 18) but sometimes apparently on flat surfaces (5). Later work in which lymphocyte locomotion was studied in relation to other, larger cell types growing in culture, for example fibroblasts (12, 19) or lymph node reticular cells (14), showed that, whereas lymphocytes on the bare substratum hardly moved at all, lymphocytes that penetrated between the cultured cells and the substratum were highly motile. The requirement for close adhesion may be much less critical for cells moving in 3-D environments, as described by earlier workers, than for cells moving on plane surfaces. It is the major aim of this paper to explore this hypothesis. We compare the adhesion and locomotion of lymphocytes on 2-D substrata and
in 3-D matrices. These experiments lead us to suggest that lymphocytes may gain sufficient traction for locomotion within a 3-D framework by a pushing and pulling mechanism without making and breaking close, adhesive contacts.

We show that lymphocytes attach in larger numbers to 3-D collagen matrices than to 2-D collagen films. From this, it does not follow that lymphocytes are more adhesive to 3-D than to 2-D collagen, and, throughout this article, we use the word "attachment" to be distinguished from the word "adhesion," which implies particular molecular interactions between two surfaces.

MATERIALS AND METHODS

Materials

4- to 16-wk-old CBA mice from departmental stocks were used throughout. Human serum albumin (HSA) was obtained from Behringwerke, Marburg, FRG. Hanks' balanced salt solution (HBSS), RPMI 1640, newborn calf serum, and fetal calf serum (FCS) were purchased from Flow, Irvine, Scotland. HEPES and morpholinopropane sulfonate (MOPS) were obtained from Sigma Chemical Co. (St. Louis, MO). Casein and denatured HSA were prepared as described by Wilkinson (24) and Wilkinson and Allan (25). Na^14Cr was obtained from Radiochemical Centre, Amersham, Buckinghamshire, England. Labeling of cells and subsequent assays were performed in RPMI 1640 supplemented with 10% FCS for 30 min at 37°C. Cells were washed three times to remove excess unbound label.

Bovine fibronectin was purified by affinity chromatography as described by Yamada and Olden (27). The preparations of fibronectin were tested for their ability to promote adhesion and spreading of baby hamster kidney (BHK) fibroblasts and polymya-transformed BHK fibroblasts (our own unpublished data with Dr. J. Dysart and Dr. J. Edwards, Department of Cell Biology, Glasgow University) over the concentration ranges used in our experiments. Purity of the specimens was tested by PAGE (by J. Gilmour, Department of Bacteriology and Immunology, and Dr. J. Dysart, Department of Cell Biology) and showed two major bands of 220-240 kdaltons.

Methods

Small lymphocytes were obtained by gently teasing apart the brachial, axillary, and inguinal lymph nodes of CBA mice and preparing a cell suspension by standard methods.

Cells were labelled with Na^14Cr at 50,000 cpm per 5 x 10^6 cells/ml in RPMI 1640 + 10% FCS for 30 min at 37°C. Cells were washed three times to remove excess unbound label.

RPMI 1640 + 10% FCS were allowed to attach to and infiltrate collagen gels for 1-24 h. Gels were fixed for 1 h in 2.5% glutaraldehyde in HBSS. The distance that the leading two cells had migrated into the gel was measured using the calibrated micrometer of the inverted microscope under 200 magnification. Six randomly chosen fields on duplicate gels were counted.

Lymphocytes were immobilized with a Nikon phase-contrast inverted microscope with an attached 16 mm movie camera using 10x and 20x objectives at 1 frame/4 s on Kodak Plus-X reversal film. Films were analyzed with a stop-action cine-projector that projected the film sequences onto drawing paper, thus allowing frame-by-frame analysis. Additional information was obtained by using high-powered (40x objective) phase-contrast and differential interference contrast optics. Still photographs were printed from time-lapse films.

RESULTS

2-D Substrata

We began by investigating lymphocyte adhesion to the surfaces of glass cover slips using both visual and distraction assays. The substrata were modified by coating with a variety of proteins known to influence locomotion and adhesion of other cell types. Results for these modified substrata were compared with those for clean glass, which will bind a substantial number of lymphocytes. Lymphocytes failed almost completely to attach to cover slips coated with serum, fibronectin (shown in Fig. 1), casein, or denatured HSA. Time-lapse cinematography of lymphocytes that had settled on the substrata showed that they were unable to move on them.

3-D Substrata

Collagen: Collagen can easily be made to form 3-D gels in vitro (10, 20). The gels can be air-dried to form a flat 2-D surface of collagen fibers that can, in turn, be rehydrated to regain their 3-D structure (9).

Five collagen substrata were used that should be chemically identical but topographically different. These were:

(a) Wet collagen coats. The collagen that coats clean glass rehydrates to form a 3-D gel upon immersion in physiological medium.

(b) Serum: The serum is coated and then rehydrated.
(b) Dried collagen coats. As for a but air-dried to form a 2-D surface after rehydration to a gel (as described in a).
(c) As for b but rehydrated again by immersing the air-dried coat in 2% acetic acid followed by physiological medium.
(d) As for c but dried again after rehydration.
(e) Hydrated collagen gels cast in tissue culture dishes.

We also prepared coats from collagen heated to 80°C for 30 min, since heating destroys its capacity to form a gel. Lymphocyte attachment was measured using distraction assays with 51Cr-labeled lymphocytes and by visual inspection.

Lymphocytes were not easily distracted from wet coats of collagen (a 3-D substratum) but they did not attach well to collagen coats that had been air-dried and thus made 2-D (Fig. 2, columns C and DC at 37°C). The numbers attaching were significantly increased when dried coats were rehydrated into 3-D gels. This was not a result of collagen dissolving and leaving bare glass because, when the process was repeated (i.e., the rehydrated gels were re-dried), the number of lymphocytes attaching was again reduced. Lymphocyte attachment to coats prepared from heated collagen was much lower than that to hydrated collagen (wet coats) although fibers of the heated collagen were clearly visible on staining the cover slips, indicating that heating had not converted the collagen to gelatin.

Attachment to hydrated coats was much lower at 4°C than at 37°C, indicating that it required an active cell metabolism and was not the result of a passive trapping by the gel surface. Visual counting of lymphocytes attached to hydrated and air-

**TABLE I**

| Temperature | Concentration of collagen in gel, mg/ml | 0.5 | 1.0 | 2.0 | 3.0 |
|-------------|----------------------------------------|-----|-----|-----|-----|
| 4°C         | + FC5indicates 10% FCS in the medium added to the gel. Each figure is the mean of six replicates. |
| 22°C        | 2.3                                    | 4.8 | 3.9 | 4.0 |
| 37°C + FCS* | 10.18                                  | 10.10 | 13.3 | 10.42 |
| 37°C - FCS  | 10.9                                   | 11.3 | 17.6 | 9.2 |

Effect of collagen concentration, temperature, and presence of serum on lymphocyte numbers in collagen gels, using 51Cr-labeled lymphocytes. The figures represent the radioactivity associated with washed collagen gels expressed as a percentage of the total radioactivity added, in this case 2,000 cpm/8 x 10^8 lymphocytes on each gel.

* + FCS indicates 10% FCS in the medium added to the gel. Each figure is the mean of six replicates.

Lymphocytes actively invaded collagen gels prepared over the limited concentration range that we found workable. The presence of serum had no effect on either attachment to or locomotion of the cells in collagen gels (Table I).

**FIGURE 2** Lymphocyte adhesion to glass cover slips coated with collagen (3 mg/ml). The columns represent radioactivity associated with collagen-coated cover slips expressed as a percentage of that associated with clean glass cover slips, which is taken as 100%. C, wet collagen coats; DC, dried collagen coat; DCR, dried coat rehydrated with 2% acetic acid; DCRD, dried coat rehydrated and then re-dried; HC, heated collagen. P values between • and ▲ = 0.0004 and between ■ and ▲ = 0.002.

**FIGURE 3** Lymphocyte migration into collagen gels. This shows the distance (measured in micrometers) migrated by lymphocytes into collagen gels over 24 h. X, 3.0 mg/ml collagen; ●, 1.5 mg/ml collagen; ◆, 1.0 mg/ml collagen. Bars, 1 SD from the mean of five fields counted. The leading-front method (24) was used to measure distance, i.e., the farthest distance from the surface reached by at least two lymphocytes in one field.

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graph of the time-course of lymphocyte movement into gels at three different collagen concentrations. Gels of 1.0 mg/ml were more quickly infiltrated than those made at 1.5 mg/ml, and appreciable migration into the more dense gel (3.0 mg/ml) took much longer, with a lag period of >4 h. Once inside the gel, the rate of invasion was similar in the dense gel to that at the lower concentrations, suggesting that the gel surface at higher concentrations is less easily penetrated. Time-lapse cinematography of individual cells within gels of different concentrations indicated that invasion was an active process, with the lymphocytes pushing their way through the filter network. Lymphocytes were never observed to translocate on the upper surface of the gel although they frequently had locomotor morphology.

There are various possible explanations for the greater attachment of lymphocytes to 3-D collagen gels than to 2-D collagen surfaces.

(a) Lymphocytes may adhere better to 3-D than to 2-D collagen. However, molecular adhesion forces should be similar for both since the two proteins are chemically identical, though a reversible denaturation on drying, followed by renaturation on rehydration, cannot be excluded.

(b) Lymphocytes may be passively trapped by 3-D collagen. This seems unlikely to provide an adequate explanation since attachment is reduced at 4°C.

(c) Lymphocytes may actively attach to the 3-D collagen by protruding pseudopods into gaps in the collagen lattice, which are no longer present once the collagen is dried. Thus, the increased attachment may be related not to differences in adhesion but to the capacity of lymphocytes to migrate into collagen gels. We explored possibilities a and c in two ways. Firstly, we used a different type of 3-D matrix, namely a serum-coated micropore filter. If explanation c is correct, lymphocytes should attach better to filters with pores wide enough for pseudopod insertion than to filters with pores too narrow for pseudopod insertion. Furthermore, the possibility of denaturation can be excluded in such an experiment. If explanation a were correct, lymphocytes should adhere equally well to all filters made of the same material whatever the pore size. Secondly, we examined the morphological changes occurring as lymphocytes moved in 3-D collagen gels. These experiments are described in turn below.

**Microspore Filters:** We used cellulose ester filters of different pore sizes (from 0.22 to 8.0 µm) as an alternative 3-D fibrous matrix. Table II shows the results of experiments designed to investigate lymphocyte attachment to filter surfaces in the presence and absence of serum. Lymphocytes did attach to the surfaces of all filters in the absence of serum (a nonphysiological form of adhesion comparable to that on clean glass), but, when filters were pretreated with medium containing 10% FCS, attachment to the 0.22- and 0.45-µm (pore size) filters (which are too small to permit penetration by lymphocytes) was almost abolished. However, substantial numbers attached to 3- and 8-µm filters. We interpret this as meaning that, for lymphocytes, the 0.22- and 0.45-µm filters are essentially 2-D, in that the surface topography prevents insertion of pseudopodia. They can, however, use the 3- and 8-µm filters as 3-D matrices. Lymphocytes did not enter the 0.22- and 0.45-µm filters, but a small number (3% of the total adherent cells) entered the 3.0-µm filters, and over half of the cells attaching to the 8.0-µm filters penetrated the filter. These results, together with the collagen data, indicate that a surface that is nonadhesive in two dimensions will allow lymphocytes to attach and then migrate if the shape allows them to obtain traction by inserting and expanding pseudopodia.

**Lymphocyte Locomotion in Collagen Gels**

Frame-by-frame analysis of time-lapse films of lymphocytes moving in gels indicated that they were using a novel method of locomotion. Pseudopodia were extended and then rapidly expanded, with a marked constriction between the original pseudopod and the cell body. This constriction was apparently used as an anchor for exerting either a pulling or pushing force that could work only if the anchor was held firmly by the substratum, which in this case appeared to be small gaps in the

**TABLE II**

| Filter pore size (µm) | No. of cells adhering | % Cells migrating into filter | Distance migrated (mean ± 1 SD) µm |
|----------------------|-----------------------|-----------------------------|-----------------------------|
| 0.22                 | 27 ± 8                | 0                           | —                          |
| 0.45                 | 37 ± 11               | 0                           | —                          |
| 3.0                  | 378 ± 161             | 3.2                         | 36 ± 12                    |
| 8.0                  | 451 ± 112             | 5.2                         | 25 ± 10                    |
| 0.45                 | 1,340 ± 129           | 0                           | —                          |
| 3.0                  | 1,245 ± 171           | 0                           | —                          |
| 8.0                  | 1,242 ± 66            | 10                          | 10 ± 6                     |

This shows the number of lymphocytes attached to, or within, the matrix of micropore filters either pretreated with medium containing 10% FCS or with medium alone. The figures are means of numbers in four microscopic fields from two replicate filters, counted at ×400 magnification. The distance migrated was measured by the leading-front method (24).
FIGURE 5  A sequence from a time-lapse film of a lymphocyte moving through a collagen gel enlarged to show the "anchoring" pseudopodia. The time interval (in seconds) is shown in each frame. The lymphocyte moved from right to left past a fixed object. The large extension indicated by arrowheads remains fixed while the lymphocyte moves laterally past it in the direction of the fine arrow. These photographs were printed directly from a positive image time-lapse film and are therefore negative images.

fibrous gel matrix. On occasion, the cell moved through the constriction ring but the ring itself remained quite clearly related to a gap in the gel structure. This is shown in Fig. 4, a series of cell outlines traced from a cine film. The important features are described in the legend to Fig. 4. Further evidence is provided by the sequential photographs in Fig. 5. These are 12 frames printed directly from a time-lapse film showing the morphological changes as the lymphocyte moved away from a fixed point.

DISCUSSION

We wish to suggest on the basis of the results presented above that lymphocyte locomotion is largely independent of adhesion to a substratum. Lymphocyte adhesion to a 2-D protein-coated surface is so poor that the cells have no means of generating locomotor force. For a cell supported by a 3-D protein matrix, locomotion may be possible even though the cell lacks the capacity to make strong adhesions with that matrix. This was shown using collagen, which as a 2-D surface supported neither adhesion nor locomotion of lymphocytes. The proposal that lymphocyte locomotion does not require an adhesive interaction is based partly on their behavior on plane substrata. On such substrata, areas of close contact, or adhesion, must be broken and reformed during locomotion. Lymphocytes make no effective contacts on glass coated with serum, fibronectin, and denatured proteins but they can attach to the surface of a 3-D matrix such as a collagen gel or the surface of a serum-coated cellulose ester filter, provided the filter pore size is large enough to admit pseudopods such as those shown in Figs. 4 and 5. That these surfaces are basically nonadhesive for lymphocytes has been demonstrated by drying down the collagen gel to remove the 3-D matrix without inducing a chemical change, by using coats of collagen heated to prevent gel formation, or by reducing the pore size of filters so that the pores are too small (0.22 and 0.45 μm) for insertion of sizable pseudopodia, in which case the lymphocyte behaves as if it were on a 2-D rather than 3-D surface. As a result, the surface will not support lymphocyte attachment.

Our morphological observations on lymphocyte locomotion are very similar to those made several decades ago by workers who described lymphocyte locomotion in cell cultures, in plasma clots, and on plane glass (8, 13, 16, 18). Lewis (15) observed that lymphocytes moving in liquid medium on a plane surface had a very tenuous hold on the substratum, and Harris (13) reported that even the few lymphocytes moving on a plane substratum could be removed by inverting the slide chamber. Lymphocytes can, however, be induced to adhere and to move, although poorly, if the substratum is coated with a ligand. For example, Fc-positive lymphocytes adhere and translocate on surface-bound antigen-antibody complexes (2), and phytohemagglutinin can be used to induce adhesion in the same way (26). A distinctive morphological feature of moving lymphocytes observed by workers cited above was the constriction ring, which remained fixed with respect to the environment as the cell moved through it. Lewis (15) suggested that the presence and position of the constriction was independent of the environment because it was also present in lymphocytes in fluid medium. However, de Bruyn (8) reported that two lymphocytes following an identical path produced constriction rings at identical points, e.g., a narrow gap in a network of fibrin. He concluded that in this case the constrictions were "indentations caused by external factors." This type of locomotion and the observations described in this paper have little in common with the classical description of fibroblast translocation over a plane substratum such as serum-coated glass (1). Comparable studies of fibroblasts moving in situ (4, 22) and in hydrated collagen lattices (4, 6, 7, 10) have demonstrated that adhesion of the leading part of the cell is probably important in gaining traction and that forward movement is accomplished by a flow of cytoplasm into the anterior margin of the cell or by a shortening of the extended process thus pulling the cell body forward. In fact, Grinnell and Bennett (11) have shown
by electron microscopy that fibroblasts can form adhesion plaques with collagen fibers in the absence of fibronectin. We would suggest, however, that lymphocytes, rather than gaining traction by adhering to individual collagen fibers, make use of the constriction points as anchors for forward movement. It could still be argued that very low affinity adhesions might provide traction, given a large enough area of contact. However, the morphological changes observed during locomotion through a 3-D matrix would suggest that the extending and particularly the expanding of the pseudopodia through gaps in the gel framework are instrumental in providing anchorage points during locomotion.

It should be pointed out here that we have found lymphocyte morphology to be a very unreliable guide as to the direction of locomotion in 3-D matrices. Many of the protrusions shown in Figs. 4 and 5 would be taken for uropodia. In fact, such protrusions may be either at the front or at the back of the cell. As observed on 2-D surfaces, the uropod is a rather permanent feature of the morphology of moving lymphocytes. In collagen gels, lymphocytes form protrusions that are much more transient.

The highly efficient locomotion of lymphocytes through 3-D lattices in vitro is likely to reflect their ability to migrate through complex tissues in vivo. It is possible that the architecture of such lattices might lead to accumulation in, or avoidance of, particular sites in tissues. For example, lymphocytes might move well through a lattice with apertures of an optimal size for a cell to squeeze through. However, if the pores become too wide, the cells might lose purchase and stop moving. In other words, the availability of anchorage points would control lymphocyte speed. This could lead to cell accumulation in lacunae. We are at present attempting to test this hypothesis by comparing lymphocyte behavior, i.e., speed and turning angles in collagen and fibrin gels of different densities. Furthermore, cells that respond to the spatial configuration of their surroundings may be particularly responsive to guidance in the axis of aligned fibrous tissue matrices (9, 10). The contribution of these factors remains to be explored.

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