Immunology 1986 **57** 281–289

The locomotor capacity of human lymphocytes and its enhancement by cell growth

P. C. WILKINSON *Bacteriology and Immunology Department, University of Glasgow (Western Infirmary), Glasgow*

Accepted for publication 7 October 1985

SUMMARY

The locomotor capacity of human blood lymphocytes taken directly from blood or cultured in various ways was measured by the change from a spherical to a polarized shape which occurs within minutes of adding locomotor stimulants. A minority of lymphocytes, either direct from blood or after culture in human serum albumin or fetal calf serum for up to 72 hr, responded rapidly to such stimulants, but most lymphocytes failed to show any shape change. Colchicine induced the highest proportion of polarized cells, though still below 50%, and deuterium oxide, which stabilizes microtubule assembly, inhibited shape-change, suggesting that microtubules have a regulatory function in the expression of lymphocyte locomotion. However culture in the presence of mitogens, namely, phytohaemagglutinin (PHA), PPD, mixed lymphocyte culture, or anti-T3 (OKT3 $\geqslant$ 25 pg/ml), caused a majority of lymphocytes to change shape slowly over a period of hours. In the presence of mitogens, a high proportion of cells was already polarized after 24 hr in culture without addition of further locomotor stimulants. It was concluded that locomotor capacity in lymphocytes is dependent on growth and synthesis for the following reasons. (i) There was a direct relationship between size and locomotor morphology in PHA-cultured lymphocytes. Those lymphocytes that increased in size also became polarized. (ii) Autoradiography showed that the polarized cells were more active in [^{3}H]uridine and [^{3}H]leucine uptake than the spherical cells. This relationship was obvious in PHA-cultured cells but was also evident even in cells direct from blood. The increase in locomotor morphology preceded detectable DNA synthesis ([^{3}H]thymidine uptake). (iii) Increase in locomotor capacity in culture was inhibited by cycloheximide but not by mitomycin *c*. These findings suggest that those cells most active in RNA and protein synthesis are also the most actively motile, and that, during culture with mitogens, locomotor capacity increases as G_1 phase progresses and prior to the commencement of DNA synthesis.

INTRODUCTION

When lymphocytes are first taken from blood, many of them are found to be immotile. It has frequently been reported (McCutcheon, 1924; review by Parrott & Wilkinson, 1981) that the proportion of locomotor cells among human blood lymphocytes increases with time of culture, and also that lymphoblasts are mostly motile (Russell *et al.*, 1975). Possibly then, there is a requirement for activation of growth for the cells to acquire locomotor capacity. On the other hand, an increase in locomotor capacity with time has been reported in lymphocytes cultured in the absence of known mitogens (O'Neill & Parrott, 1977) and even in serum-free medium (Wanger, Otteskog & Sundqvist, 1985), and may therefore result from some unknown property of the tissue culture environment unrelated to cell growth. In this paper, the relationship between locomotor capacity and growth of blood lymphocytes is examined directly. (The term 'growth' is used here to mean increase in cell size, not increase in population.)

A related question that needs to be settled is whether the paucity of locomotor cells among uncultured blood lymphocytes is because these cells lack intrinsic locomotor capacity, or whether they have such a capacity but it is not expressed until the cells are stimulated by appropriate ligands. Attempts to stimulate such cells have been made using stimulants such as LPS-activated serum or plasma, casein (Wilkinson *et al.*, 1976; El-Naggar, Van Epps & Williams, 1980, 1981), and a variety of other agents in micropore filter assays. Using these 'chemotactic' agents, only a minority of uncultured blood lymphocytes respond, suggesting that in short-term assays (up to 3 hr), many of the lymphocytes lack locomotor capacity. Locomotor capacity can be switched on during culture or by mitogenic activation over a period of many hours to yield a population, most of which are capable of a locomotor response. In this paper, therefore, a distinction is made between short-term assays, in which a chemical stimulus is added to activate the preexisting locomotor capacity of a cell population during a 30-

Correspondence: Prof. P. C. Wilkinson, Bacteriology and Immunology Department, University of Glasgow (Western Infirmary), Glasgow G11 6NT, U.K.

min incubation, and long-term assays, in which changes in the locomotor capacity of a cell population during culture *in vitro* over many hours are measured.

A simple and accurate way of measuring the response of locomotor cells to stimulants is to measure the change in shape from spherical to polarized which occurs upon stimulation. This has been studied in neutrophils (Keller, Zimmermann & Cottier, 1983; Shields & Haston, 1985; Haston & Shields, 1985) and monocytes (Cianciolo & Snyderman, 1981), and a similar assay was used some years ago to study mouse spleen lymphocytes (Schreiner & Unanue, 1975). Studies of neutrophils show that when unstimulated these cells remain rounded, but that, within a few minutes of adding a chemotactic factor, a change to locomotor morphology takes place which is easy to quantify and which allows accurate dose–response data to be obtained (Haston & Shields, 1985). Cells that change shape will, if placed on an appropriate substratum, move, and the nature of the locomotor response (chemotaxis, chemokinesis, etc.) then depends on the disposition of the attractant. Attachment to a substratum causes secondary shape changes, which may be unrelated to the locomotor stimulus, and the shape-change assay has the advantage that it is done in suspension and is therefore not affected by these secondary changes. In this paper, the shape-change assay has been used to measure the response of lymphocytes to a range of stimulants in short-term assays, and also to measure the change in numbers of locomotor cells in long-term cultures with and without activating agents. The paper does not address the question of the nature of the locomotor response (e.g. chemotaxis, chemokinesis) stimulated by particular agents.

MATERIALS AND METHODS

Reagents

RPMI-1640, Hanks' medium and fetal calf serum (FCS) were from Flow Labs, Irvine, Ayrshire. Morpholinopropane sulphonate (MOPS), colchicine, vinblastine, cycloheximide, mitomycin *c*, deuterium oxide (D_2O), formyl-methionyl-leucyl-phenylalanine (FMLP) were from Sigma, Poole, Dorset. Purified PHA was from Wellcome, Dartford, Kent. PPD (tuberculin) was from Evans Medical, Greenford, Middlesex. Leukotriene B_4 (LTB_4) was from Miles, Stoke Poges, Bucks. Human serum albumin (HSA) was from Behringwerke, Marburg, FRG. [5,6-^{3}H]Uridine (40 Ci/mmol), methyl-[^{3}H]thymidine (42 Ci/mmol) and L-[4,5-^{3}H] leucine (45 Ci/mmol) were from Amersham International, Aylesbury, Bucks. Anti-T3 (OKT3) was from Ortho Diagnostics, Raritan NJ. Pure interleukin-1 (IL-1) (catabolin, pI 5·1) was a gift from Dr J. Saklatvala, Strangeways Research Labs, Cambridge (Saklatvala *et al.*, 1984). Interleukin-2 (IL-2) was from Genzyme, Haverhill, Suffolk. Pure C5a was a gift from Dr B. Damerau, University of Göttingen, FRG (Zimmerman, Damerau & Vogt, 1980).

Preparation of reagents

Those of the above reagents that contained azide, phenol or other preservatives (e.g. PPD, anti-T3) were dialysed before use. The protein synthesis inhibitor cycloheximide was added to cultures of lymphocytes at 2×10^{-6} M, together with PHA (1 μg/ml). The proportion of locomotor cells was measured at 30 min, 24 and 48 hr. The DNA synthesis inhibitor mitomycin *c*, was incubated with lymphocytes at 25 μg/ml for 30 min at 37°. The cells were then washed, cultured with PHA and tested as above. D_2O, which stabilizes microtubule polymerization (Inoue & Sato, 1967), was used to make up RPMI to a final D_2O concentration of 70%. Lymphocytes were cultured in D_2O-RPMI and H_2O-RPMI. HSA was denatured by overnight incubation at pH 12 as detailed by Wilkinson & Bradley (1981). Other details of reagent preparation and dose are given where appropriate later in the paper.

Cells

Lymphocytes were obtained from heparinized human blood. The cells were separated by centrifugation through Ficoll-Hypaque (Flow) and the mononuclear fraction was removed and washed three times with Hanks' solution containing 10 mM MOPS, pH 7.4 (Hanks–MOPS). The locomotor capacity of the cells was tested immediately by resuspending an aliquot in Hanks–MOPS + HSA (10 mg/ml) (Hanks–HSA) with and without various stimulants in a short-term shape-change assay as described below. The remainder of the cells were plated out in RPMI–MOPS at 2×10^6/ml in tissue culture plastic petri-dishes (50 × 13 mm or 30 × 10 mm, Flow) for assay after a period of culture. The RPMI was supplemented with different media in different experiments as follows: (i) HSA, 10 mg/ml; (ii) heat-inactivated FCS, 20% (the same batch of FCS was used throughout); (iii) autologous plasma (heparinized) or heat-inactivated autologous serum from the same blood sample as the cells and used fresh; (iv) various activating agents were used as follows: purified PHA (0·5–1 μg/ml); PPD (1000 U/ml or 100 U/ml); anti-T3 (OKT3) 50 ng–2·5 pg/ml, either alone or with IL-2 (7 U/ml); a mixed lymphocyte culture from two normal donors. In most experiments, the whole mononuclear cell suspension was cultured without further purification. Aliquots of cells that were non-adherent at various times were removed for assessment of locomotor morphology. Such aliquots contain a mixture of lymphocytes together with (after 24 hr) some dendritic cells, and very few monocytes, most of which remain adherent to plastic for long periods (Van Voorhis *et al.*, 1982). The lymphocytes were easily identifiable as such by phase-contrast microscopy.

Shape-change assay

The shape-change assay depends on the observation that the majority of blood lymphocytes, resuspended in Hanks–HSA, remain spherical in morphology (Fig. 1a), more so in Hanks–HSA than in Hanks alone, and that when these cells are filmed, they are seen to remain immotile. Test agents were added to these cells, and the number of cells that changed from a spherical to an elongated, polarized shape was scored. Lymphocytes were tested immediately after separation from blood, and after culture for 24, 48 and 72 hr.

Assay of short-term effects of added stimulants on shape change. Lymphocytes were removed from culture and washed twice in Hanks–HSA. They were resuspended in conical tubes at 1×10^6 cells/ml in Hanks–HSA with the test agent and incubated at 37°. After 30 min, the cells were fixed, without being allowed to cool, by addition of 1 ml of 2·5% glutaraldehyde. After 10 min, they were centrifuged, the supernatant was decanted, and the cells were washed. After spinning, the supernatant was decanted and the cells were resuspended in the remaining droplet of fluid (approximately 100 μl). The proportion of cells

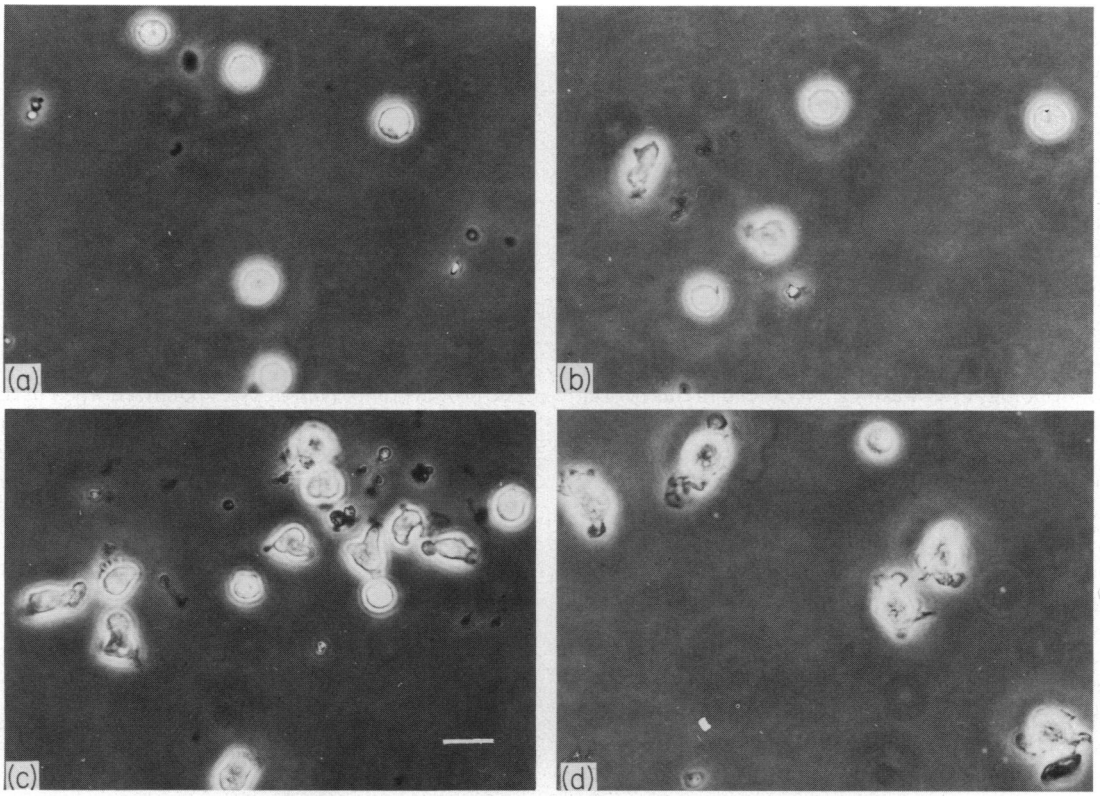


Figure 1. Phase-contrast photographs of lymphocytes (a) direct from blood in Hanks–HSA showing non-motile cells; (b) after 30 min in 20% FCS showing that a proportion of cells (Table 1) change to a typical, polarized, locomotor shape; (c) after culture for 24 hr in PPD (100 U/ml) showing many polarized cells; (d) after culture for 72 hr in PHA: lymphoblasts in locomotor morphology. Bar = 10 μm.

in locomotor morphology was estimated by making slide-and-coverslip preparations and enumerating morphologies on 200 or 300 cells using phase-contrast optics and a $\times 40$ objective. Cells that were spherical were scored as immotile. Cells that showed ruffling at one edge, or a polarized morphology with constriction ring and/or uropod, were scored as motile (Fig. 1a and b). In practice, this distinction was straightforward.

Assay of effects of long-term culture on shape change. For studies of locomotor morphology in lymphocytes cultured in media containing mitogens and other activators, an aliquot of cells was removed from the culture and, without washing, glutaraldehyde was added at 37° as above. Scoring of locomotor morphology was done as described above.

Viability. In all experiments, the proportion of motile cells was estimated from the total of *viable* cells. Since lymphocytes may die in substantial numbers in culture, especially in the presence of mitogens, this is important. Dying lymphocytes may not become permeable to trypan blue, but show the typical features of apoptosis (Moss *et al.*, 1985). Therefore, lymphocyte damage was assessed on morphological criteria, by phase-contrast microscopy, not by dye exclusion.

Collagen gel assays

The relationship between locomotor shape-change and locomotion itself was studied using the same batch of lymphocytes in the shape-change assay described above, and in a collagen gel assay in which lymphocytes were observed by time-lapse filming migrating through a 3D collagen gel. The preparation of collagen gels and the method of assessing locomotion were as described earlier (Haston, Shields & Wilkinson, 1982).

The relationship between cell size and locomotor morphology

Lymphocytes were cultured as described above, fixed with glutaraldehyde and washed. Sealed, wet slide-and-coverslip preparations were made and examined using the $\times 100$ oil-immersion objective of a Wild M20 microscope. Using the *camera lucida* attachment of this microscope, the outlines of lymphocytes were drawn onto heavy paper. The outlined shapes were cut out and weighed. The areas of the cell images as seen under the microscope were calculated in μm^2 by calibration from these weights. For a population of spherical cells, area is directly related to volume, but for cells in polarized morphology the relationship will be inexact, though it is reasonable to assume that if the areas of the images of polarized cells in a population increase, the volume of the cells is also increased. However, in view of this inaccuracy, no attempt has been made in presenting the results of these experiments to relate the area of the spherical group of cells directly to that of the polarized group of cells. Rather, changes in cell area within each group under different culture conditions were compared.

Uptake of [^{3}H]uridine, [^{3}H]leucine and [^{3}H]thymidine measured by autoradiography

Uridine. Incorporation of uridine by lymphocytes was studied by the addition of 5 μCi of [^{3}H]uridine per 10^6 lymphocytes in 1 ml. After 30 min at 37°, free uridine was

removed by centrifugation and washing (twice) of the lymphocytes. Since it was anticipated that most blood lymphocytes would incorporate uridine, a brief pulse of uridine was used to enhance differences in uptake between different cells. The following groups of cells were studied. (i) Cells pulsed with uridine, then washed and resuspended for 30 min at 37° in heat-inactivated autologous serum (20%); (ii) cells cultured overnight in autologous serum (20%), pulsed with uridine for 30 min at 37°, washed and recultured for 30 min at 37° in autologous serum; (iii) cells cultured overnight in autologous serum + PHA (1 μg/ml), pulsed with uridine for 30 min at 37°, washed and recultured for 30 min at 37° in autologous serum + PHA. The post-uridine culture period was to allow the cells to regain locomotor morphology after washing. At the end of this period, the cells were fixed with glutaraldehyde and washed. The fixed cells were then attached to glass slides coated with polylysine (2 mg/ml) to permit firm attachment during autoradiography. The slides were coated with Ilford F2 emulsion by dipping in a 1:1 emulsion of F2 with water at 40°, dried and exposed for 2 weeks. The autoradiographs were then developed using neat Kodak D19 developer for 5 min. After fixation and washing, the slides were stained with Giemsa (Gurr) 1:50 for 10 min. They were examined using a × 100 oil-immersion objective. The number of cells with superimposed silver grains was counted, and the number of grains over each cell was also counted. Cells were divided into spherical or polarized groups, and grain counts were done on both groups; 300–400 cells were counted on each slide.

Leucine. The procedures for [^{3}H]leucine uptake by lymphocytes and for autoradiography were exactly as described above for [^{3}H]uridine, using a 30-min pulse with leucine (5 μCi/10^6 cells/ml) and a 14-day exposure of the slides following dipping.

Thymidine. A similar procedure was used to measure [^{3}H]thymidine uptake by lymphocytes using 5 μCi thymidine per 10^6 cells. The cells were pulsed with thymidine for 2 hr and the remainder of the procedure was identical to that used for uridine.

RESULTS

In this section, the results for lymphocytes without added mitogens in the short-term shape-change assay are described first, followed by those of the effect of mitogens on long-term shape change, and of the relation between locomotor capacity, lymphocyte size, and uptake of trace labels.

Relationship between shape change and locomotor capacity

The proportion of lymphocytes polarized when fixed at a single time-point was compared with the proportion of the same population showing locomotion during an extended time (15 min) in a collagen gel as measured by time-lapse filming. These cells were in 20% FCS in both cases. In time-lapse films 24·5% showed translocations through collagen gels. The cells were observed to move, sometimes to round up, then to change shape and move again. In the shape-change assay, 21% were polarized. Thus, polarized morphology is directly related to locomotion itself.

Studies of shape change in short-term assays on cells cultured under various conditions

In these experiments, the locomotor response of lymphocytes cultured in the absence of added mitogens was examined. Some results for cells taken direct from blood and after various times of culture are shown in Table 1. A very low proportion of unstimulated lymphocytes direct from blood showed locomotor morphology in Hanks–HSA, and this proportion increased only slightly with time of culture. FCS stimulated locomotion in the short-term assay. In neat FCS 30–45% of lymphocytes changed

Table 1. Percentages of lymphocytes in polarized morphology following 30 min exposure to various agents

Agent added to cells	Conditions of lymphocyte culture: Tested directly after separation from blood	24 hr culture in RPMI–HSA (10 mg/ml)	24 hr culture in RPMI–FCS (20%)	48 hr culture in RPMI–FCS (20%)
HSA 10 mg/ml	3·4 ± 1·0 (7)	10·6 ± 1·1 (8)	7·0 ± 1·3 (11)	13·2 ± 2·7 (3)
FCS 100%	32·5 ± 2·5 (3)		44·3 ± 3·2 (3)	
FCS 20%	23·3 ± 5·3 (6)	27·4 ± 1·8 (8)	26·1 ± 2·5 (10)	24·0 ± 2·4 (4)
FCS 20%: cells in D_2O–Hanks*	6		7	
FCS 20% Cells ex-D_2O–Hanks, replaced by H_2O-Hanks*			24	
Endotoxin-treated human serum (20%)	24·1 ± 5·4 (4)		25·0 ± 2·1 (5)	
Colchicine 10^{-5} M	45·9 ± 3·0 (5)			
Alkali-denatured HSA				
6 mg/ml			18·1 ± 1·0 (4)	
3 mg/ml			19·4 ± 2·2 (4)	
1·5 mg/ml			14·9 ± 1·8 (6)	

Figures are mean percentages ± SEM. The number of assays is given in parentheses. Where only a single figure appears, only a single test was done. Where no figure appears, no test was done.

* Cells were cultured and tested in D_2O–Hanks, then washed in H_2O–Hanks and retested after 30 min in H_2O–Hanks–FCS.

shape. In 20% FCS (the concentration used routinely as a control) 20–30% changed shape. FCS had an activity equal to that of LPS-activated human serum. During 24 hr of culture, it made little difference to the locomotor response of the lymphocytes to various agents, whether they had been cultured in FCS or in HSA alone. Several neutrophil chemotactic factors were studied. FMLP (10^{-6}–10^{-11} M), C5a (10^{-7}–10^{-9} M) and LTB_4 (10^{-6}–10^{-11} M) all had no effect (data not shown in table). However, alkali-denatured HSA, a neutrophil chemotactic factor, did induce shape change in lymphocytes (as in neutrophils; Haston & Shields, 1985). Interleukin-1 (0·01–10 ng/ml), interleukin-2 (0·1–20 U/ml) and a monoclonal anti-T3 antibody (OKT3 250 pg–250 ng/ml) were all without effect when each was used in isolation in short-term assays (data not shown). Likewise, PHA did not induce shape change in a 30-min assay.

The most effective agent for inducing polarization in lymphocytes direct from blood was colchicine (Table 1). Colchicine caused 40–50% of lymphocytes to change shape within 30 min, and vinblastine (not shown) had the same effect. Polarization of lymphocytes in colchicine was slow (> 15 min, Fig. 2), possibly because colchicine must enter the cytoplasm to

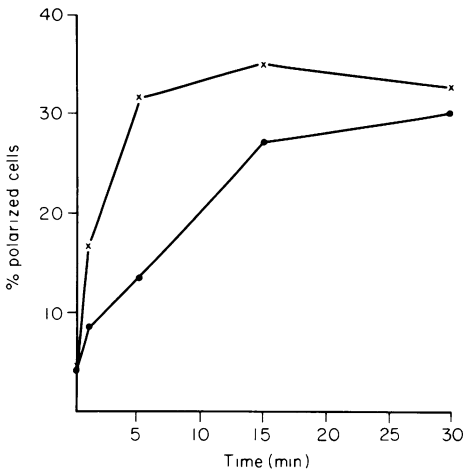


Figure 2. Time-course of polarization of lymphocytes direct from blood exposed to neat FCS (×) or colchicine, 2×10^{-5} M (●).

act, compared to polarization in FCS which was essentially complete in 5 min (Fig. 2). The morphology of the polarized colchicine-treated cells was very similar to that of locomotor lymphocytes in the absence of anti-tubulin drugs under phase-contrast, with the constriction ring typical of locomotor lymphocytes (Lewis, 1931). Lymphocytes treated with colchicine or vinblastine were actively motile and invaded collagen gels rapidly, showing typical locomotor morphology (Haston & Shields, 1984; Haston *et al.*, 1982) while doing so. Conversely, D_2O, which stabilizes microtubule polymers, completely inhibited polarization and locomotion in lymphocytes (Table 1) as in neutrophils (Zimmermann & Keller, 1984). This was rapidly reversed on replacing D_2O with H_2O.

In all of the above short-term assays, a high proportion of lymphocytes remained round and unresponsive. Since these cells may have lacked locomotor capacity, further experiments, described below, were carried out to define the conditions under which the locomotor capacity of blood lymphocytes could be increased by culture in the presence of mitogens, and the relationship of increased locomotor capacity to growth.

The effect of mitogens on the locomotor capacity of lymphocytes

In the short-term assay, PHA had no effect on lymphocyte locomotion. Lymphocytes were then placed in longer-term culture in the presence of PHA and other activators. Aliquots were taken from the cultures and the cells were fixed without washing. The proportion of polarized cells was then measured. Figure 3 shows shape change with time for lymphocytes cultured in FCS (20%) alone or in FCS + PHA. FCS alone did not induce polarization in more than 30% of lymphocytes. However, almost all of the viable lymphocytes assumed locomotor morphology over a period of 72 hr in PHA (Fig. 1d) in all of eight experiments (Chi-squared; $P < 0{\cdot}001$). A high proportion of these cells became polarized during the first 24 hr, and large numbers of locomotor cells were present before any cells of typical lymphoblast morphology were present. The protein-synthesis inhibitor cycloheximide (2×10^{-6} M) incorporated into the culture prevented this shape change. After 24 hr exposure to PHA, 45% of the cells were polarized in the absence of cycloheximide, 14% in its presence. This inhibitory effect of cycloheximide was not seen in short-term assays, suggesting that protein synthesis is not necessary for stimulation of locomotion in cells that already possess locomotor capacity, but is necessary for the induction of locomotor capacity. The DNA-synthesis inhibitor mitomycin *c* had no effect on PHA-induced shape change in lymphocytes. Several activators other than PHA were studied. These included PPD (in two experiments using cells from a tuberculin-positive subject) (Fig. 1c), mixed lymphocyte culture (one experiment) and culture in the presence of anti-T3 (25 pg–50 ng/ml) with or without IL-2 (7 U/ml) (three experiments). All of these induced an increase in polarized cells within 24 hr (Fig. 3).

The dose–response of lymphocytes cultured for 24 hr in the presence of anti-T3 is shown in Table 2. Anti-T3 at concentrations down to 25 pg/ml induced polarization above control levels. Many of the polarized cells were large. Culture in IL-2 alone had no appreciable effect, either on polarization or cell size, though IL-2 did have some enhancing effect on the polarization response to low concentrations (25–250 pg/ml) of anti-T3 (Table 2).

Relationship between lymphocyte size and locomotion

Lymphocyte size was determined from the measured areas of cells using a drawing tube. The cells were divided into 'spherical' and 'polarized' groups. Figure 4 shows the sizes of cells in these groups, direct from blood and after culture. Culture in autologous plasma alone did not induce cells to enlarge, and the size distribution was similar to that of cells straight from blood. In PHA there were already many large cells by 24 hr. Most of the large cells were in the polarized group. The size distribution of the immotile cells was still not much different from that of cells straight from blood. After 48 hr in PHA, it was still more obvious that most of the large cells were in locomotor morphology. These results strongly suggest that cells that increase in size in PHA also acquire locomotor capacity.

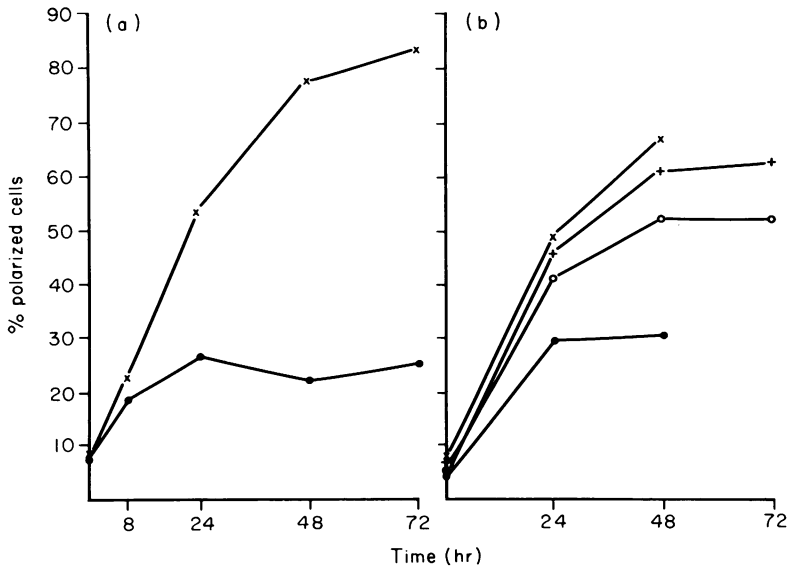


Figure 3. (a) Increase in proportion of polarized cells with time of culture in 20% FCS alone (●) and in 20% FCS + PHA 1 μg/ml (×). (b) Increase in proportion of polarized cells with time of culture in 20% FCS alone (●); an MLR mixing 10^6 lymphocytes/ml from each of two donors (○); 20% FCS + PPD (1000 U/ml) (+); and a mixture of anti-T3 (50 ng/ml) + IL-2 (7 U/ml) in 20% FCS (×). Experiments (a) and (b) were done on different cell batches, and the experiment with anti-T3 + IL-2 was carried out on a separate batch from the others.

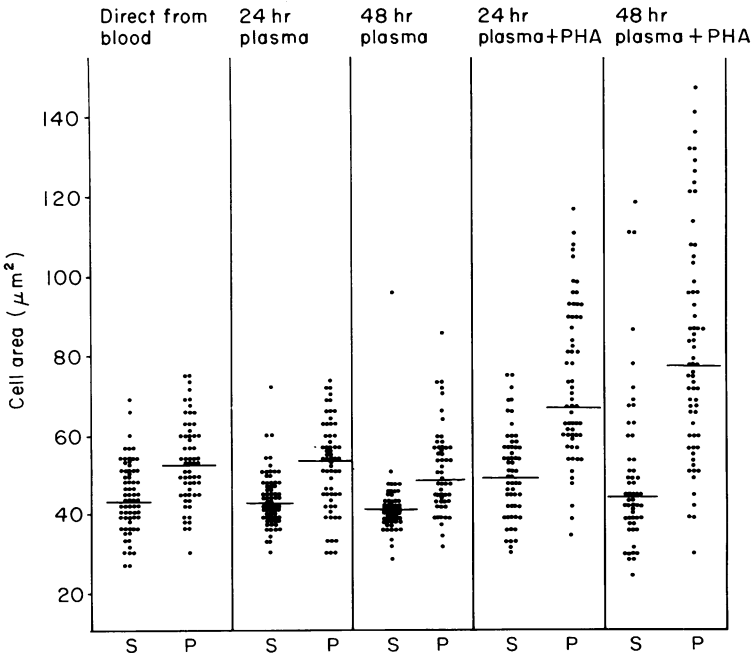


Figure 4. The size distribution of individual cells from groups of lymphocytes cultured in different ways. Each group has been subdivided into spherical cells (left) and polarized cells (right). The numbers in each group do not reflect the original proportion of polarized cells in each group, since cell images were selected for drawing to give approximately equal numbers of spherical and polarized cells. Note that size increase with time in culture is more evident in the polarized than in the spherical group (i.e. as cells grow, they also assume locomotor morphology). Bars = median values. S, spherical cells. P, polarized cells.

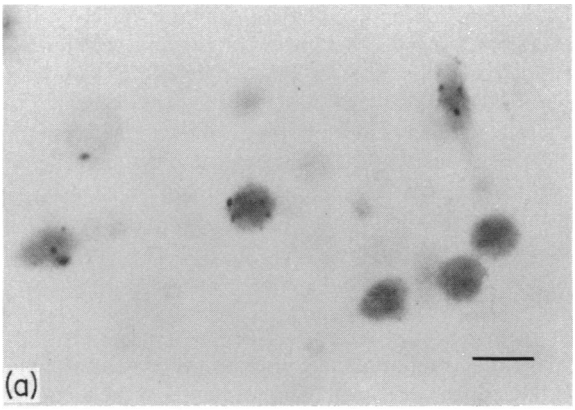
Table 2. Percentage of polarized lymphocytes after 24 hr culture in anti-T3 with or without IL-2

Lymphocytes in FCS (8%) plus:	No. IL-2 in culture	IL-2 (7 U/ml)
No anti-T3	19	22·5
Anti-T3 (OKT3) 2·5 pg/ml	19·5	19·5
Anti-T3 (OKT3) 25 pg/ml	37·5	56
Anti-T3 (OKT3) 250 pg/ml	59	66·5
Anti-T3 (OKT3) 2·5 ng/ml	65·5	63·5
Anti-T3 (OKT3) 25 ng/ml	65·5	69

Autoradiography using tritiated uridine, leucine and thymidine

Results of autoradiography after [^{3}H]uridine incorporation are shown in Table 3. Most cells in all preparations showed some uridine labelling, and a distinction was therefore made between lightly labelled cells ($\leqslant 3$ grains per cell) and heavily labelled cells (>3 grains and >10 grains per cell). The PHA-cultured cells, as expected, took up more uridine than those direct from blood or cultured in autologous serum alone, and also showed a higher proportion of polarized forms (Fig. 5). In all groups, including cells direct from blood, polarized cells took up more uridine than spherical cells (Table 3) suggesting that, even without a known stimulus, the locomotor cells are more active in RNA synthesis than those that remain immotile. A second experiment using lymphocytes from a different donor gave almost identical results (data not shown).

The findings for [^{3}H]leucine were very similar to those for [^{3}H]uridine, except that the PHA-induced increase in uptake was smaller in this experiment. Direct from blood, the percentage of cells with >10 grains was 4·6 for spherical cells and 16·9 for polarized cells. After 24 hr culture in PHA, the percentages were 7·1 for spherical cells and 22·9 for polarized cells. Thus, in both groups polarized cells incorporated more leucine.



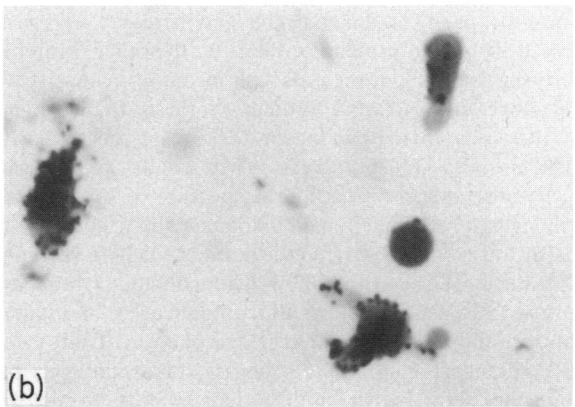


Figure 5. Autoradiographs of lymphocytes pulsed with [^{3}H]uridine: (a) lymphocytes direct from blood; (b) lymphocytes following 24 hr culture in PHA, 1 μg/ml. Note increased uridine uptake after exposure to PHA when most of the cells are in locomotor morphology, but also that cells direct from blood, particularly polarized cells, also take up uridine. (bar = 10 μm).

Table 3. Autoradiography: [^{3}H]uridine grain counts for spherical and polarized lymphocytes

Provenance of cells	Percent cells showing: >3 grains per cell	>10 grains per cell
Taken direct from blood (in autologous serum, 20%)		
Spherical	21·8	3·2
Polarized	50	12
Cultured for 24 hr in autologous serum (20%)		
Spherical	24·2	4·6
Polarized	37·2	18·6
Cultured for 24 hr in autologous serum (20%) +PHA (1 μg/ml)		
Spherical	49·6	32·2
Polarized	72	56·4

The figures in the first column are inclusive of those in the second column.

There was little uptake of [^{3}H]thymidine in PHA-cultured cells before 48 hr or in other groups at any time, and few of the large polarized lymphocytes present at 24 hr showed any evidence of DNA synthesis.

These experiments suggest that, following activation, the lymphocyte population that is growing and synthesizing RNA and protein rapidly also acquires locomotor capacity. The acquisition of locomotor capacity is an early event which precedes DNA synthesis.

DISCUSSION

The experiments reported here point to the conclusion that the locomotor capacity of small lymphocytes from the blood is not fully expressed until the cells commence growth. There was a positive correlation between the proportion of cells with locomotor capacity on the one hand, and increased cell size and increased RNA and protein synthesis on the other. Moreover, even direct from blood, the cells with locomotor capacity were more active in RNA and protein synthesis than the non-locomotor cells. Increase in locomotor capacity occurs early in growth, prior to DNA synthesis. These results are consistent with the observation that a high proportion of large granular lymphocytes direct from human blood respond to chemoattractants (Bottazzi *et al.*, 1985), since these are likely to be actively synthetic cells. We have not followed human blood lymphocytes in culture to a post-blast stage, though our earlier observations on antigen-induced locomotion in lymphocytes from primed mice (Wilkinson *et al.*, 1977) suggest that small post-blast lymphocytes show higher motility than small, unprimed lymphocytes.

Observations that the proportion of locomotor lymphocytes increases with time in culture have been made in the absence of known added mitogens, e.g. in FCS alone (O'Neill & Parrott, 1977). FCS may itself be mitogenic, though the batch used in the present experiments was not, and did not change the proportion of locomotor cells with time in culture. Another possible reason for shape change is adhesion to substrata, especially serum-free substrata. Adhesion to HSA-coated plastic caused a small amount of shape change. It is by no means clear that adhesion-induced shape change is directly related to locomotor capacity, and in any case the assays reported here were carried out using cells in suspension.

Although increased locomotor capacity is related to increased cell size and synthetic activity, it has not been shown that the relationship is causal. One possible explanation is that signals that activate growth also activate locomotion through a common transduction pathway. This seems unlikely. Locomotion in leucocytes, including lymphocytes, is activated within seconds of stimulation by external ligands (Table 2; Zigmond & Sullivan, 1979; Keller, Naef & Zimmermann, 1984), yet blood lymphocytes take hours to respond by shape change to PHA or anti-T3. This suggests either that some cytoplasmic or cell-membrane change has to be induced before the cells acquired locomotor capacity, possibly requiring protein synthesis, or that the cells are responding not to the mitogen itself but to a cell product released slowly in response to the mitogen. Another possibility is that small lymphocytes, the cytoplasms of which are scanty, lack some cytoplasmic component essential for locomotion, though 40–50% of these small cells (possibly those most active in RNA and protein synthesis) can be induced to polarize in neat FCS or colchicine. The results with colchicine and D_2O should perhaps be interpreted cautiously, since these are unphysiological agents, but they do suggest that locomotion is permitted by microtubule disassembly. Whereas over 80% of human blood neutrophils can be induced to polarize in colchicine (Keller *et al.*, 1984), under 50% of lymphocytes do so. Do the rest have extremely stable colchicine-resistant microtubules, or is there some other unrelated reason for their lack of locomotor capacity? Does microtubule disassembly become easier to activate as cells grow, so that the cells are more easily stimulated to move?

Another possible reason for lack of locomotor responsiveness in small lymphocytes is that they lack receptors for chemoattractants, and that these receptors require to be synthesized before the cells can respond. This assumes that locomotion (and not only chemotaxis) is generally a sensory response to external signals in lymphocytes as in neutrophils (Shields & Haston, 1985), as is suggested by the unpolarized morphology of unstimulated lymphocytes (Fig. 1a). Over 95% of blood neutrophils have locomotor capacity and respond to chemotactic stimuli, and studies of the promyelocyte line HL-60 suggest that, as neutrophils mature, they synthesize chemotactic receptors at about the same time as they begin to acquire locomotor capacity (Niedel *et al.*, 1980; Fontana *et al.*, 1980). This may also be the case for lymphocytes. Other possibilities are that unactivated small lymphocytes lack some component of the transduction machinery that links the membrane signal to the locomotor response, or that their microfilaments are incompletely organized for locomotion. Further progress in unravelling the phenomenon at the molecular level is hampered by the present lack of clearly defined, purified lymphocyte chemoattractants to use as ligands, and the lack of information about receptors for such attractants. In the present study, lymphocytes did not respond to classical neutrophil chemotactic factors, with the exception of denatured proteins, which have not been shown to act by binding to defined receptors. They failed to respond to complement components including pure C5a, and Van Epps & Chenoweth (1984) failed to show C5a receptors on human lymphocytes. This raises the question as to what it is in serum that induces locomotor activity in lymphocytes: FCS is as active as LPS-activated human serum. It was hoped that interleukins might act as locomotor activators, but they were not shown to do so. Possibly, the physiological activator of lymphocyte locomotion is antigen (Wilkinson *et al.*, 1977). Anti-immunoglobulin induced good shape change in B cells (Schreiner & Unanue, 1975), and was reported by Ward et al. (1977) to be chemotactic for rat lymphocytes. It might therefore be a useful ligand for study and might act analogously with antigen. Anti-T3 might be expected to act in the same way on T cells. Low concentrations of the OKT3 antibody stimulated shape change in lymphocytes on overnight culture, but not in a short-term assay. It would be worth exploring a range of antibodies against T-cell receptors for such activity.

Mitogens such as PHA, Con A or anti-T3 have no effect on lymphocyte locomotion in short-term shape-change assays, and the increased locomotor capacity of lymphocytes in long-term culture with such agents may therefore not be a direct response to the mitogen, but a response to products which mitogen-stimulated cells released into the medium. If this were so, the amount of polarization seen would be expected to be cell-density dependent, as suggested in the report of Wanger *et al.* (1985)

using cells cultured in FCS. Van Epps, Potter & Durant (1983) reported that human mononuclear cells cultured in Con A released a lymphocyte chemotactic factor, not detectable before 3 hr of culture but detectable optimally after 24–48 hr. A similar factor was reported in a study by Center & Cruikshank (1982) of mitogen-stimulated lymphocytes, and by El-Naggar, Van Epps & Williams (1982) in mixed lymphocyte reactions. The factor is a protein, the nature of which has not yet been established. Delayed release of such a factor during culture might account for the lack of immediate locomotor response of lymphocytes to mitogens, the delayed response being induced not by the mitogen itself but by a cell-release product. This is under investigation at present.

ACKNOWLEDGMENTS

This work was supported by an MRC project grant. I thank Dr Wendy Haston for many useful suggestions.

REFERENCES

BOTTAZZI B., INTRONA M., ALLAVERA P., VILLA A. & MANTOVANI A. (1985) *In vitro* migration of human large granular lymphocytes, *J. Immunol.* **134,** 2316.

CENTER D.M. & CRUIKSHANK W. (1982) Modulation of lymphocyte migration by human lymphokines. I. Identification and characterization of chemoattractant activity for lymphocytes from mitogen-stimulated mononuclear cells. *J. Immunol.* **128,** 2563.

CIANCIOLO G.J. & SNYDERMAN R. (1981) Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemoattractants. *J. clin. Invest.* **67,** 60.

EL-NAGGAR A.L., VAN EPPS D.E. & WILLIAMS R.C. (1980) Human blood B and T lymphocyte locomotion in response to casein, C5a and f-met-leu-phe. *Cell. Immunol.* **56,** 365.

EL-NAGGAR A.L., VAN EPPS D.E. & WILLIAMS R.C. (1981) Effect of culturing on the human lymphocyte locomotor response to casein, C5a and f-met-leu-phe. *Cell. Immunol.* **60,** 43.

EL-NAGGAR A.L., VAN EPPS D.E. & WILLIAMS R.C. (1982) A human lymphocyte chemotactic factor produced by the mixed lymphocyte reaction. *J. Lab. clin. Med.* **100,** 558.

FONTANA J.A., WRIGHT D.G., SCHIFFMANN E., CORCORAN B.A. & DIESSEROTH A.B. (1980) Development of chemotactic responsiveness in myeloid precursor cells: studies with a human leukaemia cell line. *Proc. natl. Acad. Sci. U.S.A.* **77,** 3664.

HASTON W.S. & SHIELDS J.M. (1984) Contraction waves in lymphocyte locomotion. *J. Cell Sci.* **68,** 227.

HASTON W.S. & SHIELDS J.M. (1985) Neutrophil leucocyte chemotaxis: a simplified assay for measuring polarising responses to chemotactic factors. *J. immunol. Meth.* **81,** 229.

HASTON W.S., SHIELDS J.M. & WILKINSON P.C. (1982) Lymphocyte locomotion and attachment on 2-dimensional surfaces and in 3-dimensional matrices. *J. Cell Biol.* **92,** 747.

INOUE S. & SATO H. (1967) Cell motility by labile association of molecules. *J. gen. Physiol.* **50** (supp.) 259.

KELLER H.-U., NAEF A. & ZIMMERMANN A. (1984) Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. *Exp. Cell Res.* **153,** 173.

KELLER H.-U., ZIMMERMANN A. & COTTIER H. (1983) Crawling-like movements, adhesion to solid substrata and chemokinesis of neutrophil granulocytes. *J. Cell Sci.* **64,** 89.

LEWIS W.H. (1931) Locomotion of lymphocytes. *Bull. Johns Hopkins Hosp.* **49,** 26.

MCCUTCHEON M. (1924) Studies on the locomotion of leucocytes. III. The normal rate of locomotion of human lymphocytes *in vitro*. *Am. J. Physiol.* **69,** 279.

MOSS D.J., BISHOP C.J., BURROWS S.R. & RYAN J.M. (1985) T lymphocytes in infectious mononucleosis. I. T cell death *in vitro*. *Clin. exp. Immunol.* **60,** 61.

NIEDEL J., KAHANE I., LACHMAN L. & CUATRECASAS P. (1980) A subpopulation of cultured human promyelocytic leukaemia cells (HL-60) displays the formyl peptide chemotactic receptor. *Proc. natl. Acad. Sci. U.S.A.* **77,** 1000.

O'NEILL G.J. & PARROTT D.M.V. (1977) Locomotion of human lymphoid cells. I. Effect of culture and Con A on T and non-T lymphocytes. *Cell. Immunol.* **33,** 257.

PARROTT D.M.V. & WILKINSON P.C. (1981) Lymphocyte locomotion and migration. *Prog. Allergy*, **28,** 193.

RUSSELL R.J., WILKINSON P.C., SLESS F. & PARROTT D.M.V. (1975) Chemotaxis of lymphoblasts. *Nature (Lond.)*, **256,** 646.

SAKLATVALA J., PILSWORTH L.M.C., SARSFIELD S.J., GAVRILOVIC J. & HEATH J.K. (1984) Pig catabolin is a form of interleukin 1. *Biochem. J.* **224.** 461.

SCHREINER G. & UNANUE E.R. (1975) The modulation of spontaneous and anti-Ig stimulated motility of lymphocytes by cyclic nucleotides and adrenergic and cholinergic agents. *J. Immunol.* **114,** 802.

SHIELDS J.M. & HASTON W.S. (1985) Behaviour of neutrophil leucocytes in uniform concentrations of chemotactic factors: contraction waves, cell polarity and persistence. *J. Cell Sci.* **74,** 75.

VAN EPPS D.E. & CHENOWETH D.E. (1984) Analysis of the binding of fluorescent C5a and C3a to human peripheral blood leukocytes. *J. Immunol.* **132,** 2862.

VAN EPPS D.E., POTTER J.W. & DURANT D.A. (1983) Production of a human T lymphocyte chemotactic factor by T cell subpopulations. *J. Immunol.* **130,** 2727.

VAN VOORHIS W.C., HAIR L.S., STEINMAN R.M. & KAPLAN G. (1982) Human dendritic cells: enrichment and characterization from peripheral blood. *J. exp. Med.* **155,** 1172.

WANGER L., OTTESKOG P. & SUNDQVIST K.-G. (1985) Anchorage and lymphocyte function. Acquisition of spontaneous motile behaviour by human blood lymphocytes and its modulation by concanavalin A. *Immunology*, **56,** 425.

WARD P.A., UNANUE E.R., GORALNICK S.J. & SCHREINER G.F. (1977) Chemotaxis of rat lymphocytes. *J. Immunol.* **119,** 416.

WILKINSON P.C. & BRADLEY G.R. (1981) Chemotactic and enzyme-releasing activity of amphipathic proteins for neutrophils. *Immunology*, **42,** 637.

WILKINSON P.C., PARROTT D.M.V., RUSSELL R.J. & SLESS F. (1977) Antigen-induced locomotor responses in lymphocytes. *J. exp. Med.* **145,** 1158.

WILKINSON P.C., ROBERTS J.A., RUSSELL R.J. & MCLOUGHLIN M. (1976) Chemotaxis of mitogen-activated human lymphocytes and the effects of membrane-active enzymes. *Clin. exp. Immunol.* **25,** 280.

ZIGMOND S.H. & SULLIVAN S.J. (1979) Sensory adaptation of leukocytes to chemotactic peptides. *J. Cell Biol.* **82,** 517.

ZIMMERMANN A. & KELLER H.-U. (1984) Effects of heavy water (D_2O) on shape, structure and motility of neutrophil granulocytes. *J. submicroscop. Cytol.* **16,** 201.

ZIMMERMANN B., DAMERAU B. & VOGT W. (1980) Purification and partial amino-acid sequence of classical anaphylatoxin from pig serum: identification with des-Arg C5a. *Hoppe-Seyler's Z. physiol Chem.* **361,** 915.