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Locomotor responses of human CD45 lymphocyte subsets: preferential locomotion of CD45RO$^+$ lymphocytes in response to attractants and mitogens

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SUMMARY

The CD45RO$^+$ population of lymphocytes from human blood contains a higher proportion of locomotor cells than the CD45RA$^+$ population. Direct from blood there were few locomotor lymphocytes ($< 15\%$), but, among these, a higher proportion of CD45RO$^+$ than of CD45RA$^+$ cells responded to the chemotactic stimuli, foetal calf serum (FCS) and interleukin-2 (IL-2) in polarization assays. Likewise, after overnight culture, a higher proportion of CD45RO$^+$ cells responded to IL-8. Culture for 24–72 hr in activators such as anti-CD3, purified protein derivative (PPD), phytohaemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM) or in an allogeneic mixed leucocyte reaction (AMLR) increased the proportion of locomotor lymphocytes to 20–60%, and the CD45RO$^+$ subset showed proportionately more polarized cells than the CD45RA$^+$ subset after culture with all the above activators. Preferential migration of CD45RO$^+$ cells into collagen gels was also seen after culture in antigenic stimuli (PPD or AMLR) but not with polyclonal activators (αCD3 or Con A). Double labelling showed that, within the CD4$^+$ and CD8$^+$ subsets, antigen-stimulated CD45RO$^+$ T cells invaded collagen gels in higher proportions than CD45RA$^+$ T cells. Clustering of lymphocytes with accessory cells is an essential prerequisite for locomotion and, after culture in αCD3, CD45RO$^+$ lymphocytes were found preferentially in clusters with monocytes. In all of the above populations, CD45RO$^+$ lymphocytes were larger in size. These findings suggest that, not only selective adhesion to vascular endothelium as reported earlier, but also selective locomotion recruits CD45RO$^+$lymphocytes into sites of inflammation.

INTRODUCTION

In contrast with small lymphocytes which recirculate between blood and lymph nodes under physiological conditions, activated lymphocytes migrate into peripheral tissues, such as the gut or skin, or chronic inflammatory sites where antigen is present.[1-4] These migrations require attachment to, and traversal of, endothelium and then a journey through tissue to the site where the cells are needed. CD45-related T-cell phenotypes are proving to be of special interest for lymphocyte migration. Cells of the CD45RO phenotype adhere better to non-specialized endothelium than CD45RA$^+$ cells[5] and are also found preferentially in inflammatory exudates.[6-8] CD45RO$^+$ cells express many adhesion molecules, including CD29, CD44 and lymphocyte function-associated antigen-1 (LFA-1) *inter alia* at higher levels than CD45RA$^+$ cells,[9] which may explain their preferential adhesion and migration into inflammatory sites. In contrast, L-selectin is well expressed on small, recirculating CD45RA$^+$ T lymphocytes.[10] The function of the CD45RO$^+$ and RA$^+$ phenotypes is controversial. They have been designated as memory and naive T cells respectively,[11] although other evidence suggests that the CD45RO$^+$ population also represents recently primed or effector T cells,[12,13] and that long-term memory cells may revert to a CD45RA$^+$ phenotype.

Lymphocyte recruitment whether into inflammatory sites or into lymphoid tissues requires locomotion as well as adhesion, and in this paper the question whether CD45 isoforms vary in their locomotor properties is addressed. There are few locomotor lymphocytes in human blood when studied by conventional *in vitro* assays directly after removal. Culture of mononuclear cells for 24–72 hr with antigen [purified protein derivative (PPD) or an allogeneic mixed lymphocyte reaction (MLR)] or polyclonal activators [αCD3, phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM)] increases the proportion of locomotor lymphocytes considerably.[14] This acquisition of locomotor properties can be divided into two stages.[15] Firstly cells stimulated to enter cell cycle acquire the capacity to show locomotor responses early in G_1. Secondly, once motile, these cells can respond rapidly by polarization and locomotion to attractants such as interleukin-8

Abbreviations: APAAP, alkaline phosphatase–antialkaline phosphatase; FACS, fluorescence activated cell sorter.

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(IL-8)[16] or IL-2.[17] The whole lymphocyte population does not acquire locomotor capacity following such culture and it requires to be established whether there are phenotypic differences between the locomotor and the unresponsive cells. In this paper, blood lymphocytes were activated with a range of T-cell activators to establish the phenotype of the locomotor and non-locomotor cells in respect of CD45, CD29 (the β_1-integrin chain), CD4 and CD8. Locomotor activity was assessed by counting the proportion of cells with the head–tail polarity characteristic of locomotor lymphocytes, using a modified alkaline phosphatase–antialkaline phosphatase (APAAP) technique[18] to phenotype these cells, and also by measuring the capacity of the lymphocytes to invade collagen gels in the presence of an attractant. These assays were used to establish whether differences in locomotor properties of CD45RO$^+$ and CD45RA$^+$ lymphocytes could help to explain the differential accumulation of CD45RO$^+$ cells in inflammatory lesions as well as the already-studied differences in adhesion to endothelium.[5]

MATERIALS AND METHODS

Cell preparation

Normal human blood was collected in heparin and mononuclear cells separated using lymphocyte separation medium (ICN Flow, High Wycombe, U.K.). They were washed and then cultured at 37° in 24-well dishes (1·6 × 1·7 cm; ICN Flow) at 2–4 × 10^6 cells/ml in Hank's balanced salt solution (HBSS) (ICN Flow) buffered with morpholinopropane sulphonic acid (Sigma, Poole, U.K.) at pH 7·4 with added human serum albumin (HSA; 10 mg/ml; Behringwerke, Marburg, Germany) (HBSS–HSA). Appropriate activators were added to this medium. At the end of the culture period, non-adherent cells were removed for testing in locomotion assays.

Short-term polarization assays

To test the polarizing response to foetal calf serum (FCS), IL-2 and IL-8, cells were washed thrice in HBSS then resuspended in HBSS containing 20% FCS (ICN Flow), IL-2 (250 U/ml) or recombinant human (rh) IL-8 (100 ng/ml; Peprotech Inc., Rocky Hill, NJ). Cells were reincubated for 60 min at 37° then fixed by adding an equal volume of 2·5% glutaraldehyde (Sigma) for 15 min followed by washing. Polarization was assayed as previously described.[14] The response to FCS and IL-2 was tested on the day of blood collection. The response to IL-8 was tested on lymphocytes that had been cultured overnight in 10% FCS since this gives a better response.[15]

Cell culture

To evaluate the activation of lymphocyte locomotion during a 48–72 hr culture period, the following were added as appropriate: αCD3 (OKT3, Orthodiagnostics, High Wycombe, U.K.) at 10 ng/ml; PPD (Evans Medical, Horsham, U.K.) at 1000 U/ml; PHA (Wellcome Diagnostics, Beckenham, U.K.) at 1 μg/ml; Con A (Sigma) at 5 μg/ml; or PWM (Sigma) at 10 μg/ml. Allogeneic MLR were set up by mixing equal cell numbers from unrelated donors. PPD, which was dialysed against saline before use, was tested with cells from subjects known to give vigorous tuberculin reactions. Cells were either fixed *in situ*, then removed and washed for polarization assays or were removed unfixed for collagen gel assays.

Phenotype antibodies

UCHL1 (αCD45RO) was a gift from Professor P. C. L. Beverley (ICRF, London, U.K.) and was used at 1/2; 2H4 (αCD45RA), 4B4 (αCD29), phycoerythrin-conjugated αCD4 and phycoerythrin-labelled αCD8 were from Coulter (Luton, U.K.) and were used at a dilution of 1/10. The αCD3 and αCD22 used for T and B phenotyping were from the Scottish Antibody Production Unit (Carluke, U.K.).

Phenotyping of locomotor cells

Two methods were used both of which have recently been appraised.[18]

(1) *Phenotyping polarized cells.* APAAP staining can be done on glutaraldehyde-fixed cells using UCHL1 and 2H4, but not 4B4 or αCD4. Fixed cells were washed twice, then incubated at 4° for 15 min, in 0·05 M glycine. Cytocentrifuge preparations were then stained at room temperature using UCHL1 or 2H4 for 30 min. Slides were washed in 0·05 M Tris-buffered saline, pH 7·6. Rabbit anti-mouse immunoglobulin (Dako, High Wycombe, U.K.) was added at 1/25 for 30 min, then APAAP (Dako) at 1/50 for 30 min. The final two stages were repeated for 10 min each, then substrate added. This was naphthol AS-MX phosphate (Sigma) diluted 1 in 12·5 in water containing 0·24 mg/ml levamisole (Sigma) and 1 mg/ml Fast Red (Sigma). Slides were counterstained in 0·5% methyl green for 2 min. Preparations were randomly selected and at least 300 consecutive cells from each category classified. B cells were distinguished from T cells by APAAP using αCD22 and αCD3.

(2) *Recovery of cells after invasion of collagen gels.* Type I rat tail collagen[19] was gelled by restoration to physiological pH and osmolarity from a dilute acid solution and used at a final concentration of 2 mg/ml in 1-ml volumes in 24-well dishes. Culture supernates were incorporated into the gels as stimuli for locomotion. Cells were layered onto the gels and allowed to invade overnight at 37°. Non-motile cells were then gently washed from the gel surface and retained. Motile cells were released from the gel by incubating the gel with collagenase (BDH, Poole, U.K.) at 12·5 U/ml for 30 min at 37°. Overnight culture of lymphocytes in contact with collagen, and subsequent digestion of the collagen, has previously been shown to have no effect on lymphocyte surface expression of CD45RO, CD45RA, CD29, CD4 or CD8.[18] Counts of motile and non-motile cells were performed and phenotyping of each population was done by FACS analysis using a FACScan (Becton Dickinson, San Jose, CA). Conventional immunofluorescence was done using fluorescein isothiocyanate (FITC)-conjugated F(ab′)$_2$ rabbit anti-mouse immunoglobulins at 1/200 as a secondary antibody for UCHL1, 2H4 and 4B4. For each sample 10,000 events were recorded using the Consort 30 or Lysis II programs.

Cell sizing

(1) For cell area measurement of APAAP preparations, CD45RO$^+$ or CD45RA$^+$ cells were selected, classified as spherical or polarized, and their areas measured using a × 50 objective on a Leitz Ortholux microscope attached to a Hamamatsun Vidica C1000 camera. Cell images were digitized for input to the screen memory of a BBC Archimedes microcomputer using a Data Harvest video interface. Areas of 50 cells in each category were measured.[14,20]

(2) Cell sizes were also assessed using the FACS. Lymphocytes which had been cultured for 72 hr were phenotyped and

the forward light scatter of cells staining positively for CD45RO or CD45RA was measured.[21]

Distribution of CD45 phenotypes in lymphocyte–monocyte clusters

Cells were cultured in αCD3 as above on circular 16-mm glass coverslips in 12-well dishes (ICN Flow). The non-adherent cells were removed for studies of locomotion, leaving glass-adherent cells on the coverslip both in clusters and as single cells. These were stained by immunofluorescence with UCHL1 and 2H4 and the proportion of each phenotype in the clusters and as single cells was counted after mounting the coverslips on microscope slides. Lymphocytes could readily be distinguished from monocytes morphologically. B cells were distinguished from T cells using double labelling with αCD19 and αCD3 (Simultest, Becton Dickinson).

RESULTS

Polarization of CD45 subsets

Figure 1 shows the results of polarization assays of lymphocytes tested on the day of collection for response to FCS or IL-2, and after overnight culture for response to IL-8 in short-term (60 min) polarization assays. APAAP staining for CD45RO and CD45RA showed that, while the proportion of the whole population that polarized was low in each experiment, and with all three stimuli, a higher proportion of CD45RO$^+$ cells than of CD45RA$^+$ cells became polarized. Even amongst unstimulated cells left in HBSS alone for the short-term assay, more CD45RO$^+$ cells had polarized. Figure 2 shows the results obtained after APAAP staining of lymphocytes which had polarized during culture in PPD, αCD3, Con A, PHA or PWM in response to attractants released into the culture and which were fixed without addition of further stimuli. A higher proportion of these cells than of those direct from blood was polarized. Again the results were consistent. In all individual experiments and with all activators, a higher proportion of CD45RO$^+$ cells than of CD45RA$^+$ cells became polarized. Similar experiments using cells from allogeneic MLR gave the same consistently higher proportion of polarized CD45RO$^+$ cells (not shown). The proportions of T and B cells that polarized in response to these activators was checked. With all stimuli, 74–80% of the polarized cells were CD3$^+$; 8–13% were CD22$^+$. It was concluded that the predominant responding population is CD3$^+$, CD45RO$^+$.

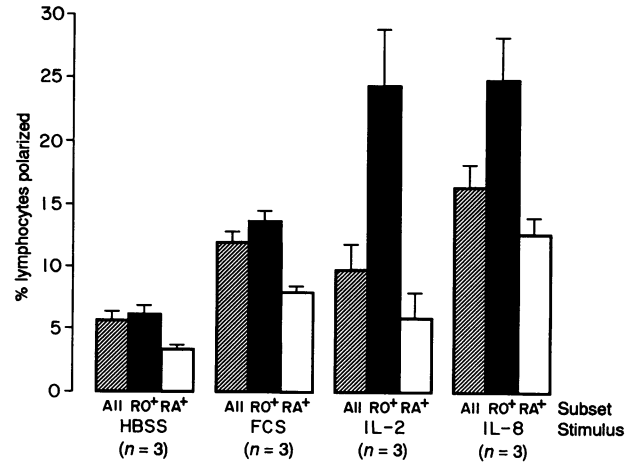

Figure 1. Proportion of CD45RO$^+$ and CD45RA$^+$ lymphocytes which polarize after short-term culture ($n = 3$). Cells were washed then exposed to HBSS, FCS, IL-2 or IL-8 for 60 min then phenotyped by APAAP. Significantly more CD45RO$^+$ cells were polarized than CD45RA$^+$ ($P < 0{\cdot}01$, ANOVA test). In this and subsequent figures, means and standard errors are shown. Groups of experiments were performed on various days, on lymphocytes from various donors, which were usually distributed in a quotient CD45RA : CD45RO of around 1·5 : 1·0, though this varied slightly from sample to sample.

Invasion of collagen gels by CD45 subsets

Figure 3 summarizes the results of the phenotyping by FACS analysis of cells recovered from collagen gels expressed as a percentage of all the cells layered onto the gel. The proportion of cells entering the gel after 3 hr was about the same as the proportion polarizing (see Fig. 2), but overnight a larger overall

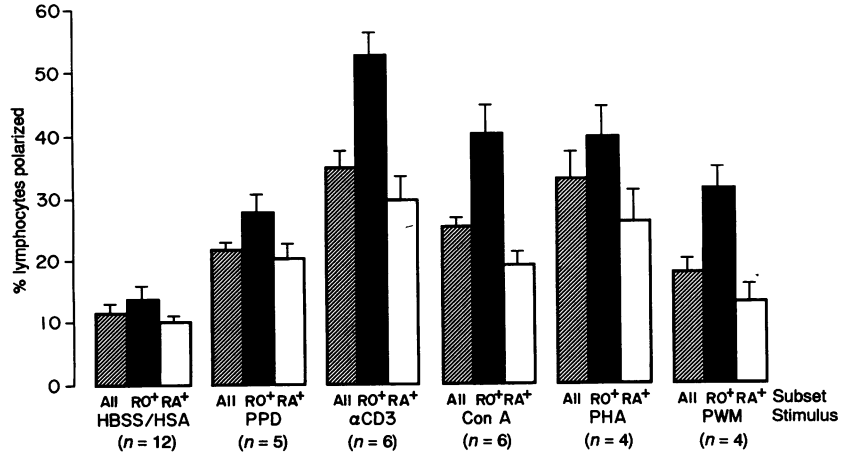

Figure 2. Proportion of CD45RO$^+$ and CD45RA$^+$ lymphocytes which polarize after 3-day culture. Cells were fixed *in situ* in their own culture supernatants then phenotyped by APAAP. Significantly more CD45RO$^+$ cells were polarized than CD45RA$^+$ ($P < 0{\cdot}01$, ANOVA test).

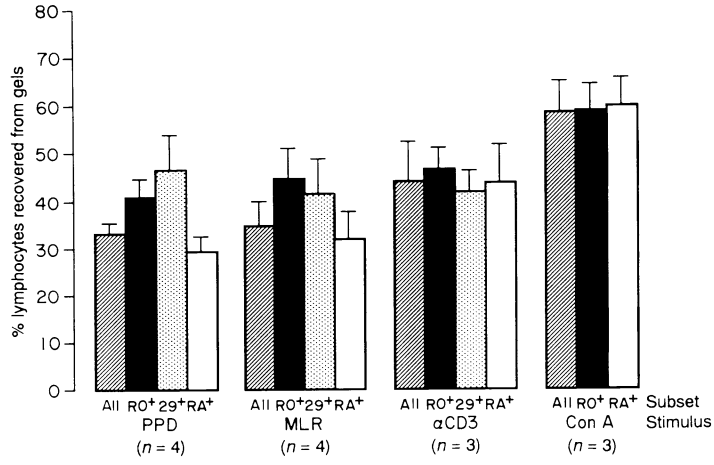

Figure 3. Proportion of CD45RO$^+$, CD29$^+$ and CD45RA$^+$ lymphocytes recovered from collagen gels. Cells were allowed to invade overnight, then the populations washed from the gels, or recovered after collagenase digestion of the gels, were phenotyped by immunofluorescence on FACS. In each individual experiment, PPD or MLR stimulation resulted in a higher proportion of CD45RO$^+$ and CD29$^+$ cells being recovered than of CD45RA$^+$, although this was not statistically significant. No preferential recovery occurred after αCD3 or Con A stimulation.

number of cells had entered the gels. Figure 3 shows the results for overnight assays but the patterns were the same at 3 hr. The pattern of results was different from that obtained in polarization assays. In each experiment with PPD- and allogeneic MLR-stimulated cells, a higher proportion of CD45RO$^+$ and CD29$^+$ cells entered the gels than of the reciprocal CD45RA$^+$ subset.

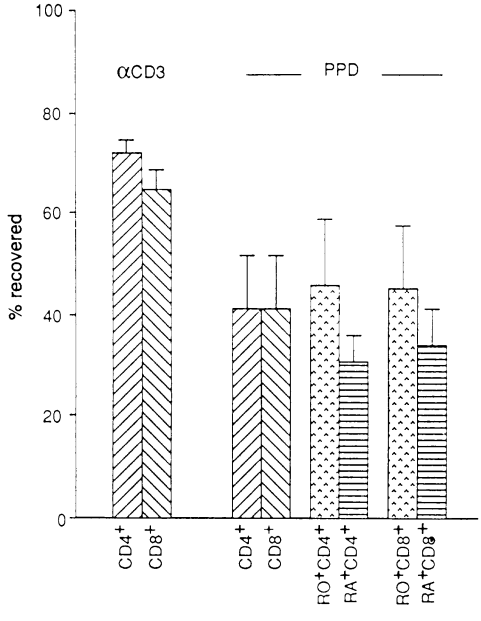

Figure 4. Proportion of CD4$^+$ and CD8$^+$ lymphocytes recovered from collagen cells. Cells cultured in αCD3 ($n = 4$) or PPD ($n = 3$) were allowed to invade for 3·5 hr, then the populations washed from the gels or recovered after collagenase digestion of the gels were phenotyped by immunofluorescence on FACS. In each experiment, more CD4$^+$ than CD8$^+$ cells were recovered after culture in αCD3, but not PPD. Results were not statistically significant. After culture in PPD more CD45RO$^+$ than CD45RA$^+$ cells were recovered among both CD4$^+$ and CD8$^+$ cells.

However after αCD3 or Con A stimulation, no preferential locomotion of either subset was evident.

Experiments were also carried out to see whether CD4$^+$ and CD8$^+$ cells entered gels in similar proportions (Fig. 4). It was consistently found tha slightly more CD4$^+$ cells entered the gels after αCD3 but not after PPD. Differences were not statistically significant. These experiments were extended by double-labelling cells for CD4 and CD8 with UCHL1 or 2H4. A higher proportion of PPD-activated CD4$^+$, CD45RO$^+$ cells than of CD4$^+$, CD45RA$^+$ cells entered and also a higher proportion of CD8$^+$, CD45RO$^+$ cells than of CD8$^+$, CD45RA$^+$ cells entered the gels. A similar pattern was seen with allogeneic MLR-stimulated cells. These experiments confirm that the differences between CD45 subsets were differences within the T-lymphocyte population and also that they were not confined to either of the CD4 and CD8 T-cell subsets.

Analysis of cell size

Under the conditions reported here, increase in cell size has been found to correlate well with RNA and protein synethesis[14] and thus to be a useful measure of lymphocyte entry into cell cycle. Figure 5 shows the areas of cell images in APAAP-stained preparations from polarization assays. After culture in HBSS–HSA, PDD, αCD3 or Con A, CD45RO$^+$ cells were significantly larger than CD45RA$^+$ cells. This was true of both the spherical and the polarized cells. In the case of spherical cells, area is a good correlate of volume, though the correlation is less valid for polarized cells which have a variety of shapes. However, the differences in Fig. 5 for polarized cells are very marked and are unlikely to be determined by shape alone. A second approach was used, namely to measure cell volume by measuring forward light scatter of defined populations after FACS analysis, although this measure is less exact technically than visual measurement of cell area.[22] As above, with all stimuli tested, viz. HBSS–HSA, PPD, allogeneic MLR, αCD3, Con A, PHA and PWM, CD45RO$^+$ cells were significantly larger than CD45RA$^+$ cells (details not shown).

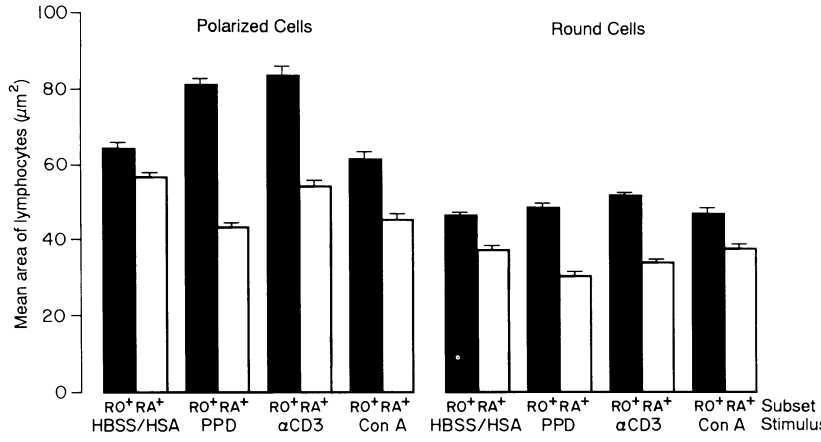

Figure 5. Cells areas of CD45RA$^+$ and CD45RO$^+$ lymphocytes after 3-day culture. APAAP-stained preparations were classified as polarized or spherical, then the areas of CD45RO$^+$ or CD45RA$^+$ cells were measured after the images were digitized using computer software. In all cases, CD45RO$^+$ cells were larger ($P < 0{\cdot}01$, Mann Whitney U-test).

CD45 lymphocyte isoforms in clusters with monocytes

Clustering of lymphocytes with monocytes precedes and is necessary for activation of lymphocyte locomotion by αCD3.[23,24] Table 1 shows the distribution of CD45 isoforms in clustered and single lymphocytes adherent to glass after 24 hr culture in αCD3. Both CD45RO$^+$ and CD45RA$^+$ cells were well represented among the clustered cells, but the single cells were almost all CD45RA$^+$. Thus the proportion of the total population of CD45RO$^+$ cells in clusters was higher than the proportion of CD45RA$^+$ cells. The possibility that B cells accounted for the single CD45RA$^+$ cells was checked. Only 20% of these cells were B cells, but 90% were CD45RA$^+$. Thus CD45RO$^+$ cells are enriched not only in the locomotor population but also in the clustered population. Previous observations indicated that once stimulated by clustering, motile cells could subsequently move away.[24] Thus clustered cells would later be available for locomotion assays.

Change in CD45R phenotype after activation

It is established that after activation, CD45RA$^+$ T cells progressively become CD45RO$^+$. The proportions of lymphocytes staining positive for CD45RO or CD45RA on day 0, after 3 days of culture in HBSS–HSA, PPD, αCD3, Con A, PHA or PWM was assessed by immunofluorescence and FACS analysis. No changes in proportions of CD45RO$^+$ cells were seen. Hence the CD45RO$^+$ cells examined for locomotion and clustering are unlikely to have switched phenotype. A slight decrease in the proportion of CD45RA$^+$ cells was observed by day 3. The quotient of CD45RA$^+$:CD45RO$^+$ cells was approximately 1·5:1·0. Most reports of phenotype shift at day 3, which generally use PHA as an activator, indicate a more dramatic shift in phenotype. However, these use richer growth media (e.g. RPMI–FCS). Preliminary experiments suggest that culturing lymphocytes in such media, rather than HBSS–HSA, alters the time-course of cell activation and phenotype shift (I. Newman and P. C. Wilkinson, unpublished observations).

Table 1 Distribution of adherent lymphocytes as single cells or clustered with monocytes after 24 hr culture in αCD3 (mean ± SEM: four experiments)

	% staining for marker		
Marker	Single cells	Clustered cells	Proportion of total population
CD45RO	10 ± 4	53 ± 2	90 ± 4
CD45RA	77 ± 14	44 ± 2	64 ± 3

DISCUSSION

Although it is established that lymphocyte subsets redistribute non-randomly throughout the body, in particular at sites of inflammation, the role of differential locomotion in this process has not been established. One of the major problems has been finding an assay system in which adhesive interactions do not complicate the measurement of locomotion, especially since CD45RO$^+$ and CD29$^+$ cells are more adhesive than CD45RA$^+$ cells. A recent report suggested that the cytokine RANTES was selectively chemotactic for CD45RO$^+$ lymphocytes using polycarbonate filters.[25] Moving lymphocytes adopt polarized morphology and the proportion polarizing equates well with the proportion entering collagen gels over a 3–4 hr period.[23] These results indicate that a higher proportion of CD45RO$^+$ lymphocytes than of CD45RA$^+$ lymphocytes became polarized in response to short-term stimuli (FCS, IL-2, IL-8). Such stimuli are probably chemotactic factors for lymphocytes and it was shown that lymphocytes orient in a gradient of IL-8.[26] CD45RO$^+$ lymphocytes also showed preferential polarization after longer-term culture in growth activators. In this case the locomotor response is to material released into the supernates by the cultured cells themselves and not to the activators added at the beginning of the culture. It was recently shown that αIL-8 inhibited the locomotor response of αCD3 or PPD-cultured cells to their own supernates.[26]

When the study was extended to cells invading collagen gels, a slightly different pattern emerged. Contact with collagen gels incorporating the cells' own culture supernatant led to invasion

by a higher proportion of CD45RO$^+$ or CD29$^+$ cells than of CD45RA$^+$ cells if they had been cultured in antigen, i.e. PPD or an allogeneic MLR, and the same preference for CD45RO$^+$ invasion was seen in both CD4$^+$ and CD8$^+$ populations. However no preferential invasion of CD45RO$^+$ or CD29$^+$ cells occurred if the cells had been cultured in the polyclonal activators, αCD3 or Con A. The reasons for this are not clear. Adoption of polarized morphology indicates locomotor capacity, but collagen itself can affect lymphocyte locomotion and adhesion.[27] Adhesion may not be obligatory for lymphocyte locomotion in gels,[28] but activated lymphocytes express increased levels of activity of adhesion molecules for extracellular matrix proteins,[29] although rat tail collagen preparations appear to be free of fibronectin.[30] This was confirmed by SDS–PAGE electrophoresis of the collagen used in this study (not shown).

Acquisition of locomotor capacity is associated with cell growth.[14] These results indicate that CD45RO$^+$ cells are larger in area and volume than CD45RA$^+$ cells after activation, a finding that is consistent with previous findings of a similar difference in sizes of cells direct from blood.[21,31]

Overall it was concluded that CD45RO$^+$ and CD29$^+$ lymphocyte populations contain a greater proportion of locomotor cells than do CD45RA$^+$ populations. CD45RO$^+$ cells were earlier shown to adhere selectively to non-specialized vascular endothelium,[5] although, as with these findings, the difference between the CD45 subsets was not absolute. Selective adhesion of CD45RO$^+$ cells in monocyte–lymphocyte clusters during culture was also observed. Lymphocytes acquire locomotor capacity during clustering[24] and the selective clustering of the CD45RO subset may account for their subsequent selective locomotion. These findings are compatible with the suggestion that the CD45RO$^+$ subset contains a population of effector cells which have recently encountered antigen.[12] Such cells would need to be mobilized in sites of antigen deposition, and it is not obvious why a population consisting only of memory cells should be so mobilized. It may be that a series of differential enrichments after endothelial adhesion, response to cytokines and locomotion, all contribute to the eventual phenotype of cells found in sites of inflammation. Further understanding of such mechanisms may contribute to our knowledge of the establishment, maintenance and possible manipulation of inflammation.

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