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Reversible stimulation of lymphocyte motility by cultured high endothelial cells: mediation by L-selectin

H. HARRIS & M. MIYASAKA*† *Department of Immunology, Babraham Institute, Babraham, Cambridge, UK and* **Department of Immunology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*

SUMMARY

Lymphocyte emigration from blood into peripheral lymph nodes is mediated by specialized high endothelial cells (HEC) lining the postcapillary venules. A current model for this process postulates that it occurs in three steps: weak, selectin-mediated interactions tether lymphocytes to the blood vessel wall; the lymphocytes are activated to increase the affinity of integrin-dependent adhesion and enhance motility; and finally the lymphocytes migrate actively across the endothelial cell layer. Some features of this model are simulated *in vitro* by cultured HEC, which support the adhesion and transmigration of lymphocytes. In particular, cultured HEC stimulate lymphocytes to change shape from spherical to polar. This shape change provides a convenient assay of the motility activation of lymphocytes. In this paper it is shown that this occurs without the lymphocytes becoming tightly adherent, but depends on contact with the endothelial cell surface. The shape change is labile: non-adherent polar lymphocytes removed from HEC revert to round with a half-time of less than 8 min. Reagents which block the interaction of L-selectin with its ligands inhibit the HEC-induced shape change; these include mannose-6-phosphate, fucoidan, polyphosphomannan ester, treatment of HEC with sialidases and an anti-L-selectin monoclonal antibody known to block its lectin function. The change in shape is partially inhibited by antisera to the L-selectin ligand GlyCAM-1. Thus it is concluded that in this *in vitro* system, L-selectin-mediated binding of lymphocytes to HEC is essential for optimal induction of the shape change. Lymphocytes change shape in response to cultured HEC without loss of surface L-selectin, although activation stimuli are known to promote shedding of neutrophil L-selectin as well as motility and increased adhesiveness. However, the lymphocyte change in shape is a reversible process, and this may have implications for the nature and sequence of the signals transmitted from endothelium to lymphocytes during homing to peripheral lymph nodes.

INTRODUCTION

The capacity for blood-borne leucocytes to be recruited to specific sites in the body is owing to the ability of certain populations of these cells to interact in a tissue-dependent manner with endothelium. Models for the mechanism of homing suggest a minimum of three steps for the leucocyte–endothelial interaction, each of which contributes to the specificity of the overall process.[1–3] The cells first bind weakly, through interactions between the lectin domains of selectin molecules and carbohydrate ligands; such interactions promote leucocyte rolling along the endothelial surface. Leucocyte activation then induces higher-affinity adhesion through an additional class of adhesion molecules, the integrins, and the leucocytes become motile. Some activating ligands have been identified, for example, for neutrophil entry into inflamed endothelium, but the molecules responsible for the analogous stimulation of lymphocytes, particularly for constitutive homing to lymphatic tissue, have not been identified. Signals from endothelium to lymphocytes may be transmitted through adhesion molecules or from cytokines located on endothelial cell-surface carbohydrates.[4] Finally the combination of adhesion and motility allows the leucocytes to migrate actively across the endothelial cell layer.

An *in vitro* model for lymphocyte homing to the high-walled endothelial cells of peripheral lymph nodes has been developed,[5] in which cultured high endothelial cells (HEC) from rat cervical lymph nodes specifically support the high-affinity binding of lymphocytes and their subsequent migration across a monolayer of endothelial cells.[6] This binding has been shown to be integrin mediated[7,8] so the interaction

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Abbreviations: DAB, Dulbecco's A + B solution; FCS, fetal calf serum; HEC, high endothelial cells; PPME, polyphosphomannan ester.

†Present address: Biomedical and Educational Centre, Osaka University, Osaka, Japan.

Correspondence: Dr H. Harris, Department of Immunology, Babraham Institute, Babraham, Cambridge CB2 4AT, UK.

corresponds to the third step of the general model outlined above. In addition, similar lines of HEC were shown to stimulate lymphocytes to become motile[9] by a simple shape-change assay which quantifies locomotory activation.[10] This motility stimulation was specific for HEC as compared with fibroblasts and both tightly adherent and non-adherent lymphocytes were equally susceptible. These observations suggested firstly that induction of lymphocyte motility is dependent on interaction with HEC-derived ligands and secondly that this process is independent of high-affinity adhesion of lymphocytes to HEC.

This paper describes the requirements for induction of motility in lymphocytes which are not tightly adherent to HEC, using the shape-change assay. The non-adherent lymphocytes constitute the bulk of the population over the time–course of the shape change.[5] A variety of potential inhibitors of weak binding between lymphocytes and endothelium was used to characterize ligand–receptor interactions participating in motility activation. This approach identified L-selectin as an important mediator of the shape change. Motility induction is shown to be a readily reversible phenomenon.

MATERIALS AND METHODS

Materials

HEC were isolated from cervical lymph nodes of female DA rats (Harlan Olac, Bicester, UK) and cultured as described by Ager.[5] Lines used were between the third and 25th passage. At subconfluent densities about 50% of the cultured cells supported migration of lymphocytes which distinguishes the lines used from fibroblasts. In addition, and in contrast to the fibroblast line Rat 2, HEC stain for GlyCAM-1 with anti-GlyCAM-1 antiserum CAMO-2 (A. Whyte & H. Harris, unpublished data). Rat cervical lymph node lymphocytes were prepared as described previously.[9] Lymphocytes were washed in Dulbecco's A + B solution [DAB, i.e. phosphate-buffered saline (PBS) containing calcium and magnesium] containing 50 U/ml penicillin, 50 μg/ml streptomycin and 1% fetal calf serum (FCS) (DAB-1). Cells were incubated in RPMI-1640 (Gibco, Paisley, UK) containing 12 mM $NaHCO_3$, 1 mM L-glutamine, penicillin and streptomycin as above and FCS at 1% (RPMI-1) or 5% (RPMI-5). All incubations were performed at 37°. FCS was heat treated for 30 min at 56°.

Monosaccharides, chondroitin sulphate A (Sigma catalogue no. C8529), heparin (H5640), heparan sulphate (H5393), hyaluronic acid (H4015) and fucoidan were obtained from Sigma Chemical Co. (Poole, UK). Polyphosphomannan ester (PPME) was prepared by acid hydrolysis of *Hansenula holstii* phosphomannan.[11,12] *Arthrobacter ureafaciens* and *Salmonella typhimurium* sialidases were obtained from Oxford GlycoSystems (Oxford, UK).

CAMO-1 and CAMO-2 rabbit antisera to mouse GlyCAM-1 peptides 1 and 2 were a gift from Professor S. Rosen.[13] Hamster monoclonal antibodies to rat L-selectin, HRL-1 and HRL-2, were as described previously.[14] Fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster immunoglobulin was purchased from Cappel Research Products (Durham, NC).

Methods

Lymphocyte shape change assay. The proportion of non-adherent lymphocytes changing shape upon incubation with HEC was determined essentially as described previously,[9] except that most assays were performed in 96-well MicroWell plates (Nunclon, Roskilde, Denmark), using 100 μl lymphocyte suspension per well rather than 24-well multiwell dishes. Plates were set up containing HEC, medium and additions as indicated and the incubations started with 10 μl concentrated lymphocyte suspension (usually about 2×10^7/ml) to give a final concentration 10-fold less. Exact conditions for each experiment are given in figure legends. Incubations were terminated by 100 μl 4% glutaraldehyde in DAB, at 37°. Non-adherent lymphocytes were removed by pipette, centrifuged and resuspended in 20 μl PBS. The proportion of round and polar lymphocytes in each sample was determined by haemocytometry, as described, counting at least 300 cells/sample.

The proportion of lymphocytes changing shape in response to HEC varies up to twofold between experiments. Differences between lymphocyte populations as well as differences between HEC cultures contribute to this variability (H. Harris, unpublished observations). Quantitative comparisons between experiments were therefore not made, but the phenomena illustrated in Figs 1–7 were all reproduced in at least two independent experiments. Within experiments, reproducibility is high; error bars show range of two determinations.

Fluorescent antibody staining. Lymphocytes were incubated in primary antibody at 4° for at least 30 min and washed three times with DAB-1. FITC-conjugated second antibody was added at an appropriate dilution (determined by titration) in PBS containing 5% normal rat serum. The cells were incubated 30 min at 4° in the dark, washed three times with DAB-1 and fixed with 1% paraformaldehyde in DAB. Staining was analysed with a FACScan fluorescence activated cell analyser (Becton Dickinson, San Jose, CA). Primary antibodies were titrated to ensure that they were used at saturating concentrations.

RESULTS

The induction and maintenance of the shape change requires contact between the lymphocytes and HEC

When lymphocytes are cocultured with HEC, those which are not tightly adherent but are easily removed from unfixed cultures, contain a substantial proportion of polar cells (34% in Fig. 1). Continuing the incubation in the absence of HEC results in a rapid fall in the proportion of polar lymphocytes. The motile state is therefore labile, with a half-life of less than 8 min in the absence of further contact with HEC (Fig. 1).

Lymphocytes change shape only when allowed to settle on HEC in static culture, and not when the cultures are shaken (Titertek microplate shaker speed 4). A 50-fold change in lymphocyte density (2×10^5 to 10^7/ml) had little effect on the proportion of cells changing shape. These results suggest that the motility activation is not induced by a soluble molecule produced either by HEC or lymphocytes. This was confirmed by the failure of high-speed supernatants (100 000 ***g***) of conditioned medium from HEC (30 min), lymphocytes or incubations of lymphocytes with HEC to induce the shape change. Thus it is probable that lymphocytes are stimulated to change shape through direct contact with HEC. Low-speed (10 000 ***g***) supernatant of the same 30-min-conditioned medium from HEC increased the per cent polar lymphocytes by 7% above background.

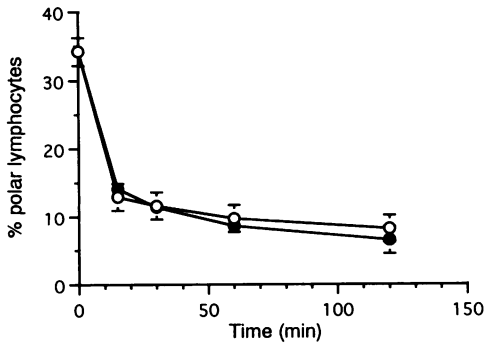

Figure 1. Persistence of the HEC-induced lymphocyte shape change. Lymphocytes were suspended at 4·4 × 10^6/ml in 5 ml of either RPMI-1 (○) or RPMI-5 (●) and incubated on confluent layers of HEC in 50-ml tissue culture flasks for 1 hr at 37°. The non-adherent lymphocytes were then withdrawn and 0·5 ml aliquots of this suspension were incubated in 24-well Multidish plates with sufficient agitation to prevent the cells settling. The lymphocytes were fixed at the times indicated and scored for shape.

Inhibition of the shape change by monosaccharides

In order to obtain information about which molecular interactions between HEC and lymphocytes are important for inducing the shape change, various compounds were tested for their ability to inhibit the change. Of a range of monosaccharides tested, only mannose-6-phosphate and glucose-6-phosphate were effective inhibitors (Fig. 2). Mannose and mannose-1-phosphate (not shown) and glucose and glucose-1-phosphate were not significantly inhibitory. In the experiment of Fig. 2, 3% of unstimulated lymphocytes were polar (zero control) as were 13% of those incubated without added sugar (100% control). A very similar inhibition profile was obtained with 60-min incubation, when 27% were polar without added sugar. The extents of inhibition by 50 mM monosaccharides at 60 min in the experiment of Fig. 2 were as follows: glucose-1-phosphate, 18%; glucose-6-phosphate, 53%; mannose, 8%; mannose-6-phosphate, 51%. In an independent experiment, glucose-6-phosphate and mannose-6-phosphate inhibited 91% and 100% respectively in a 1-hr incubation.

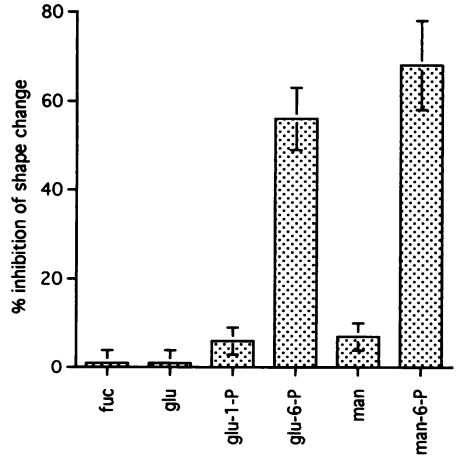

Figure 2. Inhibition of the HEC-induced shape change by monosaccharides. Lymphocytes at 5 × 10^6/ml in RPMI-1 were incubated with HEC for 30 min, in the presence of 50 mM monosaccharides: fuc, fucose; glu, glucose; glu-1-P, glucose-1-phosphate; glu-6-P, glucose-6-phosphate; man, mannose; man-6-P, mannose-6-phosphate.

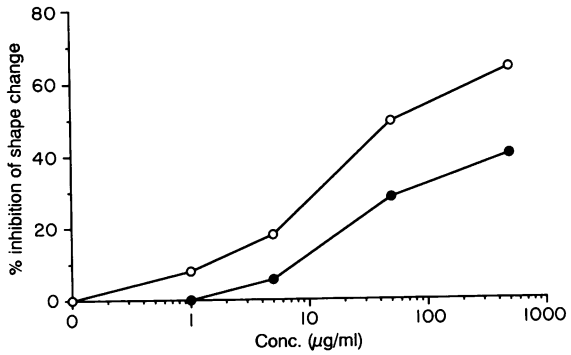

Figure 3. Dose dependence of shape change inhibition by fucoidan and PPME. Lymphocytes at 4 × 10^6/ml were incubated in RPMI-5 for 15 min with HEC. Unstimulated lymphocytes were 6% polar and in the absence of inhibitor, 32% of the cells were polar after 15 min. Fucoidan (○); PPME (●).

Inhibition of the shape change by fucoidan and PPME

Chondroitin sulphate, heparin, heparan sulphate, fucoidan, PPME and hyaluronic acid did not induce lymphocytes to change shape. Both the fucose- and sulphate-containing polysaccharide fucoidan and the mannose- and phosphate-containing polysaccharide PPME inhibited the HEC-induced shape change in a dose-dependent manner, with fucoidan being about fivefold more potent on a weight basis than PPME (Fig. 3). Fucoidan was significantly inhibitory at as little as 5 μg/ml. By contrast, chondroitin sulphate, heparin and heparan sulphate were about 200-fold less potent on a weight basis, giving substantial inhibition (55 and 77% for chondroitin sulphate and heparin respectively) only at 10 mg/ml. Hyaluronic acid similarly was only effective at about 5 mg/ml or greater.

In a preliminary experiment fucoidan at 0·05, 0·5 and 5·0 mg/ml inhibited shape change by 79, 95 and 91% after 15 min but 56, 65 and 61% after 30 min. Thus the potency of fucoidan as an inhibitor of the shape change was not maintained at longer incubations. This effect was demonstrated in five independent experiments (using lymphocytes from different animals) and on three different HEC lines within one experiment. Pooled data for inhibition by 50 and 500 μg/ml fucoidan gave mean ± SD per cent inhibitions as follows: 15 min, 60 ± 20, n = 6, range 46–84; 30 min, 35 ± 24, n = 10, range 4–77; 60 min, 9 ± 6, n = 6, range 0–17. The effects of fucoidan and PPME on the HEC-induced shape change for five inhibitor concentrations and two incubation times are shown in Fig. 4. Both polysaccharides inhibited strongly in a 15-min incubation, but not significantly after a 60-min incubation (fucoidan) or 30-min incubation (PPME). In the experiment of Fig. 4, data for 30 min with fucoidan were intermediate between 15 and 60 min; data for 60 min (PPME) and 90 min (PPME or fucoidan) showed no inhibition.

The diminished effectiveness of fucoidan might be owing to either a detoxifying modification of the fucoidan, or to the lymphocytes becoming refractory. Extra fucoidan added to

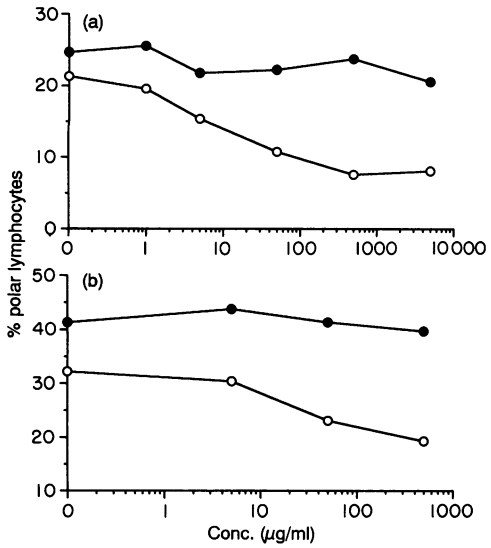

Figure 4. Time dependence of inhibition of the HEC-induced shape change by (a) fucoidan and (b) PPME. (a) Lymphocytes at 5×10^6/ml incubated with HEC in RPMI-5, range of fucoidan concentrations as shown, for 15 min (○) or 60 min (●). (b) Lymphocytes at 4×10^6/ml incubated with HEC in RPMI-5, range of PPME concentrations as shown, for 15 min (○) or 30 min (●).

lymphocytes 15 min before terminating a long incubation failed to inhibit as potently as at the beginning. Thus 0·5 mg/ml fucoidan was > 80% inhibitory over the first 15 min, but when added 15 min before the end of a 60- or 90-min incubation in the continuous presence of 0·05 mg/ml fucoidan, it only inhibited by 45%. This shows that fucoidan (or its impurities) does not reverse the shape change by, for example, acting as a metabolic inhibitor. The motility–stimulating interaction of HEC with lymphocytes is less sensitive to fucoidan inhibition upon prolonged (> 15min) incubation.

Inhibition of the lymphocyte shape change by pretreatment of HEC with sialidases

Confluent HEC were preincubated for 2 hr at 37° with either *A. ureafaciens* sialidase at 2 U/ml or *S. typhimurium* sialidase at 2 U/ml. This pretreatment reduced the ability of the HEC to induce the lymphocyte shape change by about 60% in a 15-min incubation and 55% in a 30-min incubation. There was no significant inhibition of the shape change induced by a 60-min incubation (Fig. 5). The extent of inhibition was dose dependent. In separate experiments, *S. typhimurium* sialidase inhibited the shape change by 74, 36, 4 and 0% at 2, 0·4, 0·2 and 0·1 U/ml respectively (30 min incubation) while *A. ureafaciens* sialidase inhibited by 37·4 and 9·1% at 1 and 0·2 U/ml respectively (15 min incubation).

Pretreatment of HEC with another enzyme, hyaluronidase (7500 U/ml), which might affect its interaction with lymphocytes had no effect on the induction of lymphocyte motility.

Effect of anti-GlyCAM 1 antisera on the HEC-induced lymphocyte shape change

In order to test whether reagents binding to a physiological L-selectin ligand interfered with shape change induction,

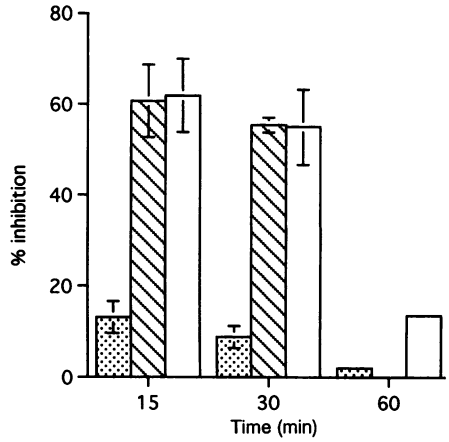

Figure 5. Inhibition of shape change induction by pretreatment of HEC with sialidases. Lymphocytes at 2×10^6/ml were incubated in RPMI-1 on HEC pretreated with buffer (DAB) (▨), *A. ureafaciens* sialidase in DAB (▧) or *S. typhimurium* sialidase in DAB (□) for 15, 30 or 60 min. After these times, the lymphocytes on untreated HEC were 12·2, 23·1 or 38·6% polar respectively (100% activity).

lymphocytes were incubated with HEC in the presence of serial dilutions of the antisera CAMO-1 and CAMO-2 to peptides of the L-selectin ligand GlyCAM-1 (Fig. 6). Both antisera partially inhibited the shape change (and were equally effective), while preimmune serum had no effect. The effect was dose dependent, saturating at about 1:60 dilution, but at this and higher concentrations the inhibition was incomplete and about 15% of the lymphocytes were polar.

Inhibition of the HEC-induced shape change by anti-L-selectin monoclonal antibodies

Two anti-rat-L-selectin monoclonal antibodies were tested for their ability to affect the HEC-induced shape change in lymphocytes. Antibody HRL-1 blocks ligand binding to L-selectin whereas HRL-2 does not.[14,15] The antibodies were tested at 20 and 40 μg/ml, at which concentrations binding was saturating as shown by FACS analysis. Neither antibody affected lymphocyte morphology when tested on lymphocytes

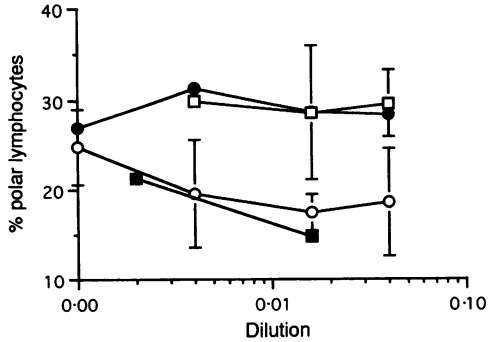

Figure 6. Effect of anti-GlyCAM 1 antisera on the induction of the lymphocyte shape change. Lymphocytes at 2×10^6/ml in RPMI-1 were incubated with confluent HEC for 30 min. Control sample without additions contained 29% polar lymphocytes. CAMO1 (■); CAMO2 (○); preimmune serum (●); antisera buffer (□).

Table 1. Effect of anti-L-selectins on HEC-induced lymphocyte shape change. Results for three different HEC lines at seventh, fifth and third passage respectively are shown. Data for 26CLN and 27CLN were obtained with the same preparation of lymphocytes; data for 24CLN in an independent experiment. Lymphocytes at 2 × 10^6/ml were incubated in RPMI-1 with or without antibodies for 15, 30 or 60 min before fixation. About 4% of the unstimulated lymphocytes were polar

	% polar lymphocytes		
HEC line	26CLN7	27CLN5	24CLN3
15 min incubation			
Control medium	17·2 ± 1	17·9 ± 1·5	23 ± 2·1
HRL1			
20 μg/ml	11·9 ± 2·0*	11·0 ± 1·1*	12·7 ± 2·1
40 μg/ml	10·1 ± 0·3	11·1 ± 0·9	—
HRL2			
20 μg/ml	16·6 ± 1·6	17·3 ± 3·4	26·6 ± 1·2
40 μg/ml	16·0 ± 0·7	17·8 ± 0·2	
30 min incubation			
Control medium	22·5 ± 1·4	21·2 ± 2·5	
HRL1 20 μg/ml	12·5 ± 1·6	13·8 ± 1·7	
HRL2 20 μg/ml	21·3 ± 2·2	17·3 ± 0·3	
60 min incubation			
Control medium	33·8 ± 2·7	36·2 ± 0·4	
HRL1 20 μg/ml	31·1 ± 3·8	30·0 ± 1·6	
HRL2 20 μg/ml	32·7 ± 0·5	28·6 ± 0·8	

*n = 4; otherwise n = 2.

in suspension in the absence of HEC, over a range of times from 10 seconds to 60 min. The effects of including HRL-1 or HRL-2 in the incubation buffer with lymphocytes allowed to settle on HEC are shown in Table 1 for three different HEC lines. After 15 min incubation HRL-1 inhibited the shape change by about 50% but HRL-2 had no significant effect. The inhibitory effect of HRL-1 was clearly saturating (no effect of increasing antibody concentration) but partial.

HRL-1 was effective as an inhibitor at 15 and 30 min but at 60 min the proportion of polar lymphocytes was similar to controls without antibody (Table 1). HRL-2 had little effect at 15, 30 or 60 min (Table 1).

Effects of incubation with fucoidan or HEC on expression of lymphocyte surface L-selectin

Because L-selectin is known to be lost from leucocyte surfaces in response to some stimuli, its expression was monitored with time under the incubation conditions routinely used for the experiments in this paper. Lymphocytes incubated in RPMI-1 medium in suspension showed only a 5% decrease from 90 to 85% positive cells over 1 hr, as determined by FACS analysis using HRL-2 as primary antibody. The presence of the L-selectin ligand fucoidan (0·5 mg/ml) had little effect over the same time–course. L-selectin was completely down-regulated during an additional hour of incubation (with or without fucoidan), which may be owing to the mitogenic effect of FCS.

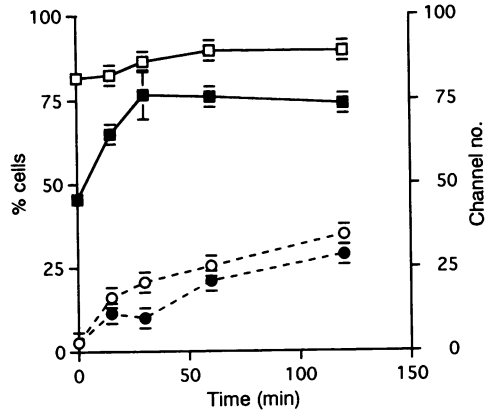

Figure 7. Changes in lymphocyte shape and surface L-selectin on incubation with HEC. Lymphocytes were incubated in RPMI-1 in three equivalent microwell plates of confluent HEC at a high enough concentration (6 × 10^6/ml) to provide sufficient cells for FACS analysis. Samples in one plate were fixed *in situ*, as usual. Non-adherent, unfixed lymphocytes from the second plate were removed in suspension into prepared aliquots of fixative and samples from the third were similarly removed into primary antibody (HRL-2) for fluorescence staining. Thus identical lymphocyte populations were assayed for shape and fluorescence. HRL-2 (L-selectin)-positive cells as per cent total (□); level of HRL-2 binding expressed as mean fluorescence of positive cells, channel number (■); per cent polar lymphocytes fixed *in situ* (○); per cent polar lymphocytes removed into fixative (●).

Under static conditions, lymphocytes allowed to settle onto bovine serum albumin (BSA)-coated plastic (which does not support adhesion) showed some loss of surface L-selectin from 86% to 74% positive cells over 2 hr. By contrast lymphocytes incubated with HEC showed a slight increase to 90% L-selectin positive.

Using a modified assay to follow induction of shape change in the same population of lymphocytes as L-selectin expression (Fig. 7), the proportion of L-selectin positive lymphocytes was found to increase slightly upon incubation with HEC from about 80% to 90%. This might be owing to selective removal of L-selectin negatives by tight adhesion, rather than to up-regulation of L-selectin. However, the per cent polar lymphocytes increased gradually to about 30% during the incubation period. At least two thirds (and possibly all) of these polar lymphocytes were therefore L-selectin positive. In addition, the mean surface density of L-selectin showed a marked increase in response to HEC. Thus lymphocyte surface L-selectin was up-regulated on many of the cells during incubation with HEC. However the intensity of fluorescence staining with HRL-2 showed a broad distribution and it is still unclear whether the lymphocytes which change shape are the same as those with increased surface L-selectin. Lymphocytes fixed *in situ* contain a slightly higher proportion of polar cells than those pipetted out into fixative, a result consistent with the lability of the motile state demonstrated in Fig. 1.

DISCUSSION

A high proportion of lymph node lymphocytes, up to 30% in the experiments described in this paper, may be stimulated to become motile by cultured HEC. Most of these change shape without adhering tightly. Motility activation therefore does not

necessarily activate high-affinity binding. Once removed from the HEC, the lymphocytes rapidly revert to their round shape (Fig. 1). This implies that, at least in the non-attached or weakly attached state, repeated contact with a stimulus is necessary to maintain the shape change. It is likely that direct contact with the HEC surface is required, because soluble motility-activating factors would be removed with the lymphocytes into the medium in the experiment of Fig. 1. Such a requirement for physical contact between lymphocytes and HEC is further supported by the need for lymphocytes to settle on HEC and for the absence of any evidence for HEC or lymphocyte-derived soluble factors sufficient to account for the shape change. The small stimulation obtained using low-speed conditioned medium supernatant is very probable owing to the high molecular weight material detected in 24-hr-conditioned medium.[9] Higher concentrations of this material localized on the HEC surface may well be important in stimulation of shape change but the rate of its appearance in medium is too slow for the soluble form alone to be effective.

Reagents which might block the induction of the lymphocyte shape change by HEC were tested to obtain information on specific ligand–receptor pairs required for motility stimulation. Particular attention was paid to substances likely to block the interaction of the peripheral lymph node homing receptor,[16,17] L-selectin, with its endothelial ligand. It is shown here that the shape change is blocked by mannose-6-phosphate and glucose-6-phosphate, but not by the corresponding 1-phosphates or unphosphorylated sugars. Lymphocyte binding to high endothelial venules (HEV) or to PPME-derivatized beads is similarly blocked by mannose-6-phosphate.[18] The polysaccharides fucoidan and PPME are also inhibitors of lymphocyte binding to high endothelial venules,[19] with fucoidan being the more potent.[18] The carbohydrate binding specificity of isolated L-selectin can account for the effects of mannose-6-phosphate, fucoidan and PPME while the polysaccharides are also recognized by a soluble L-selectin–IgG chimeric molecule.[20] In a preliminary experiment, fucoidan was observed to block lymphocyte migration across a monolayer of cultured HEC (H. Harris, unpublished data) and PPME and fucoidan inhibit the lymphocyte shape change (Fig. 3) at concentrations consistent with blocking of L-selectin.[18] Other anionic carbohydrate derivatives, and specifically chondroitin sulphate and heparin, do not compete with PPME binding to L-selectin–IgG[20] and inhibit the shape change only at 200-fold higher concentrations than fucoidan.

Hyaluronic acid was of interest because its receptor is a form of CD44,[21] a molecule implicated in the interactions of lymphocytes with other cells. Neither blocking of shape change nor hyaluronidase pretreatment of HEC indicated a role for hyaluronic acid in motility stimulation.

Sialic acid on the endothelial cell surface is required for binding of lymphocytes[22] and L-selectin–IgG chimera[23] to HEV, and L-selectin has been shown to bind to the sialyl Lewis^x epitope.[24] Binding of purified L-selectin ligand, GlyCAM-1, to L-selectin is impaired by treatment with both broad spectrum and α2-3 linkage specific sialidases.[25] In this paper, pretreatment of cultured HEC with sialidases was shown to inhibit substantially (about 60%) the capacity of these cells to induce lymphocytes to change shape (Fig. 5). *Arthrobacter ureafaciens* and *S. typhimurium* sialidases were chosen because both are active at physiological pH and are therefore suitable for treatment of viable HEC. The *A. ureafaciens* enzyme has a broad specificity while that from *S. typhimurium* prefers α2-3-linked terminal sialic acid, which is the linkage found in sialyl Lewis^x. Sialic acid therefore contributes to the HEC ligand responsible for optimal motility activation of lymphocytes.

The physiological ligand for L-selectin on high endothelium was first defined by the monoclonal antibody MECA-79 [26,27] which interacts with several components by molecular weight, probably because it identifies a carbohydrate epitope. The polypeptide chains of two L-selectin ligands which bind MECA-79 have been determined; one is identical with CD34[28] and the other is a novel mucin-like molecule known as GlyCAM-1.[13] This latter molecule is easily shed from the endothelial cell surface, and a relationship is probable with the heat-resistant, high molecular weight material found in conditioned medium from HEC which stimulates lymphocytes to change shape.[9] Antisera prepared against peptides 1 and 2 from the N-terminal and middle regions of mouse GlyCAM-1, respectively, cross-react with rat (S. Rosen & M. Singer, personal communication) and are here shown to inhibit the ability of HEC to induce the lymphocyte shape change (Fig. 6). The antisera do not block lymphocyte adhesion to HEV (S. Rosen & M. Singer, personal communication) so they may inhibit by sequestering GlyCAM-1 in a manner which obstructs motility activation. At saturation, a significant proportion of lymphocytes still change shape (15– 20%) consistent either with partial steric blocking of GlyCAM-1, or with the participation of different L-selectin ligands which, as indicated above, are certainly present *in vivo*.

The monoclonal antibodies to rat L-selectin, HRL-1 and HRL-2,[15] bind to different epitopes. HRL-1 blocks lymphocyte binding to HEV in the frozen section assay,[14] and also PPME binding to lymphocytes,[15] whereas HRL-2 does not. Consistent with this behaviour, HRL-1 inhibits the motility activation of lymphocytes by HEC, but HRL-2, at saturating concentrations, does not (Table 1). Thus the ability of anti-L-selectin antibodies to inhibit the HEC-induced lymphocyte shape change correlates with their ability to block L-selectin function.

From the effects of mannose-6-phosphate, fucoidan, PPME, sialidases and L-selectin antibodies it is inferred that the rapid induction of the shape change depends on a weak interaction between L-selectin on lymphocytes and ligands on cultured HEC. Antisera to one of those ligands, GlyCAM-1, also interferes with induction of shape change, probably by interfering with its interaction with L-selectin. The related molecules P-selectin and E-selectin also bind sialyl Lewis^x [24] but are endothelial lectins binding sialylated leucocyte surface ligands. In addition, human P-selectin does not bind PPME[29,30] nor human E-selectin fucoidan.[31] Although the carbohydrate specificities of E- and P-selectins have not been determined for rat it is likely that L-selectin is the relevant adhesion molecule for shape change. The other selectins may contribute to the proportion of shape change not inhibitable by HRL-1.

A role for L-selectin is an important addition to present understanding of this *in vitro* model of lymphocyte interactions with lymph node high endothelium. Although the HEV-derived line, Ax, expresses L-selectin ligand,[14] a functional role for L-selectin in the interactions of cultured HEC with lymphocytes has not previously been demonstrated. Instead, a binding assay has shown that the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins make a major contribution to lymphocyte adhesion to cultured HEC.[8] Thus,

lymphocytes first interact weakly with HEC lines, through L-selectin, within the few minutes it takes them to become motile, and subsequently bind tightly through activated integrins and migrate.[32] This sequence of events is that proposed for leucocyte homing *in vivo* and gives confidence that the *in vitro* model may give additional insights into physiological processes.

Molecules which have been identified at present do not explain fully the experimental data. The inhibition by glucose-6-phosphate, which is not characteristic of L-selectin, may depend on an alternative receptor–ligand pair. Furthermore, monosaccharide inhibition does not diminish with time, suggestive of an alternative mechanism. Inhibition by sialidases and antibody to L-selectin was always less than 100%, suggesting that the interaction through L-selectin may be supplemented by others. An unexpected feature of the motility-blocking inhibitors fucoidan, PPME, sialidases and L-selectin antibody was that their action was transient and slowed but did not abolish the shape change (Figs 4, 5 and Table 1), even when extra fucoidan was added. Recovery from sialidase inhibition might be owing to re-expression of sialic acid on the HEC surface, but an alternative mechanism is required for recovery from other inhibitors. The lymphocytes therefore appear able to use L-selectin-independent mechanisms also, although these require more time to be effective.

The rapid, L-selectin-dependent induction of the lymphocyte shape change corresponds to the pertussis toxin-sensitive phase previously described.[9] It is therefore probable that motility is activated through binding of a specific ligand to a G-protein-linked receptor. L-selectin ligation may present the lymphocytes to a stimulant on the endothelium, possibly to cytokines bound to the endothelial glycocalyx.[33,34] It is also possible that L-selectin itself contributes to signal transmission.[35] However, L-selectin is not sufficient for motility stimulation: 90% of the lymphocytes are L-selectin positive but only about 30% change shape. Time-lapse video microscopy shows than many motile lymphocytes alternate rapidly between round and polar forms, so the 30% polar detected after fixation is an underestimate; however a high proportion show no sign of movement and remain round. Thus some other property of the lymphocytes also governs their capacity to respond to HEC, and this may be the presence of an appropriate cytokine receptor. Preliminary data show that subsets of both T lymphocytes and major histocompatibility complex (MHC) class II-positive (putative B) lymphocytes change shape (H. Harris & C. Jolley, unpublished data). Although it has not been shown directly whether those lymphocytes which become tightly adhesive and migrate are derived from the larger population which changes shape, actively migrating lymphocytes are polarized[5,36] and must therefore be responsive to motility-inducing stimuli.

The three-step model for leucocyte extravasation postulates that activation switches on three events: a change in cell shape from a spherical form to elongated and motile, an increase in the adhesivity of surface integrins; and, in the case of neutrophils, the shedding of surface L-selectin.[1–3] Much of the initial work in this field was performed on chemotactic factors acting on neutrophils, which may activate all these functions simultaneously. In the experiments described here, activation' of lymphocytes by HEC is not a single event, as adhesion and L-selectin shedding are not inevitable consequences of motility induction. Lymphocyte surface L-selectin is slightly up-regulated by HEC although lymphocytes have the capacity to down-regulate their surface L-selectin in response to phorbol myristate acetate[37] or cross-linkage.[38]

In conclusion, the results presented here suggest that the initial consequences of lymphocyte interaction with HEC are wholly reversible: the cells change shape in a manner which reverses when the stimulus is removed; they are bound reversibly at this stage, predominantly through L-selectin, and they retain their surface L-selectin, so permitting reattachment. For the cells to proceed through the subsequent stages of adhesion and migration may require a succession of stimuli, allowing fine tuning of the necessary activation steps and additional checks on the specificity of eventual entry into the lymph node.

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