Lymphocyte migration across high endothelium is associated with increases in $\alpha_4\beta_1$ integrin (VLA-4) affinity

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SUMMARY

The constitutive recirculation of lymphocytes between the widely distributed organs of the immune system is essential for host defence. We have developed an in vitro model of lymphocyte migration from the blood into lymph nodes which employs primary cultures of high endothelial cells (HEC). HEC-adherent lymphocytes adopt one of two distinct morphologies which correlates with their position in the endothelial layer; type I cells are bound to the surface of HEC and type II cells are underneath the endothelial layer. In a previous study we reported that the numbers of type I and type II cells are independently regulated, however the relationship between these two lymphocyte populations was not determined. In this study we have carried out detailed kinetic, phenotypic and functional analyses of type I and type II lymphocytes and determined their relationship. Using allotype marked lymphocytes from the PVG.RT7a and PVG.RT7b rat strains in a pulse-chase analysis, type I and type II lymphocytes were found to represent the same population of lymphocytes at different stages of interaction with the endothelial layer, rather than representing two independent lymphocyte populations. Migration was an irreversible event and the efficiency of migration (i.e. transition from type I to type II) was related to the concentration of lymphocytes plated on to the HEC layer. Following transmigration lymphocytes showed an increased ability to migrate across HEC layers and to bind to immobilised CS1 peptide. The increased binding to CS1 peptide was transient and fell to control levels over a 3 hour time period. The expression of $\alpha_4$ integrin subunit on lymphocytes was unchanged following migration which suggests that the affinity of the CS1 receptor, $\alpha_4\beta_1$, is upregulated by interaction with HEC. Together these results suggest that transendothelial migration is regulated by increases in the affinity of $\alpha_4\beta_1$ integrin on lymphocytes following contact with HEC.

Key words: lymphocyte, high endothelium, transendothelial migration, $\alpha_4\beta_1$ integrin

INTRODUCTION

The ability of lymphocytes to migrate into secondary lymphoid organs and sites of inflammation is essential for host defence (Ford, 1975). Recent studies have shown that the distinct migration pathways of lymphocytes are largely dependent on sorting by vascular endothelial cells (EC). It is proposed that combinations of “homing receptors” and other adhesion molecules on lymphocytes recognise “vascular addressins” (complementary ligands) on EC to sort lymphocytes according to subset, subclass and activation state (for reviews see Berg et al., 1989; Michl et al., 1991; Picker, 1992). The majority of unactivated lymphocytes constitutively recirculate through the spleen and lymph nodes for efficient antigen encounter (Duivestijn and Hamann, 1989; Mackay et al., 1990). Following activation, lymphocytes enter non-lymphoid tissues and sites of inflammation more efficiently than unactivated cells (Mackay et al., 1992) and, in so doing, ensure efficient elimination of antigen. Altered expression of adhesion molecules on lymphocytes and ligands on EC, particularly at sites of inflammation, is thought to underly the differential migration of activated lymphocytes (Picker et al., 1990; Munro et al., 1989).

Although adhesive interactions between lymphocytes and EC have been the subject of intense study, they are only the first stage in the process of extravasation. For successful tissue entry, adhesion must be followed by the translocation of lymphocytes from the luminal to abluminal surface of EC and subsequent migration across the basal lamina to enter the tissue proper. Antigen-activated lymphocytes adopt locomotor morphology in vitro and are able to migrate into collagen gels (Wilkinson, 1986). The migratory capacity of these cells may, in part, regulate their
increased entry into non-lymphoid tissues and sites of inflammation. In comparison, unactivated lymphocytes do not demonstrate locomotor morphology or migratory activity, which is difficult to reconcile with the fact that these cells are constitutively migrating through lymphoid organs. Unactivated lymphocytes may therefore use a different pathway to migrate from the blood. It is known that unactivated lymphocytes preferentially migrate across the walls of specialised high endothelial venules (HEV) found within lymph nodes (Gowans and Knight, 1964). Several distinct properties of these vessels contribute to the high levels of lymphocyte extravasation, including the constitutive expression of addressins by lining high endothelial cells (HEC) (Bjerknes et al., 1986; Michl et al., 1991), altered blood flow for efficient trapping of lymphocytes (Belisle and Sainte-Marie, 1985) and local release of soluble factors (Andrews et al., 1983) that stimulate lymphocyte motility (Harris, 1991) and may direct lymphocyte migration across the vessel wall. Studies of lymphocyte-HEV interactions have shown that the transendothelial migration of lymphocytes is not an inevitable consequence of binding to the luminal surface of HEC (Bjerknes et al., 1986) and is therefore an additional regulatory step in lymphocyte extravasation.

In vitro assays have been widely used to identify adhesion molecules on lymphocytes and EC that mediate the initial binding event. Of these, the frozen section assay is restricted to use at low temperatures (Stamper and Woodruff, 1976) and does not allow the events following adhesion to be studied. The availability of cultured EC allows the adhesion interactions of lymphocytes to be studied at physiological temperatures. Most of these studies have employed non-specialised large vessel EC (Shimizu et al., 1991), and lymphocyte migration across the endothelial layer has been documented using these models (Oppenheimer-Marks et al., 1990, 1991). However the relevance of these studies to the recirculation of lymphocytes through lymphoid organs is unclear. We have developed an in vitro model of lymphocyte migration to lymphoid organs which employs primary cultures derived from the specialised high endothelium found there (Ager, 1987). Cultured HEC constitutively express several properties normally only associated with HEV, including phenotypic markers (Ise et al., 1988), vascular addressins (Chin et al., 1990, 1991), synthesis of HEV-specific sulphated macromolecule(s) (Andrews et al., 1983; A. Ager, unpublished), the selective adhesion of mature but not immature lymphocytes (Ise et al., 1988; Chin et al., 1990) and the ability to bind large numbers of unactivated lymphocytes in comparison with non-specialised EC (Ager and Mistry, 1988). Previous studies have shown that HEC-adherent lymphocytes adopt one of two distinct morphologies and we have referred to these as type I and type II lymphocytes. A combination of light and electron microscopy has shown that type I cells are bound to the upper surface of HEC and type II cells are underneath the endothelial layer (Ager and Mistry, 1988).

There have been few quantitative studies of lymphocyte migration across cultured endothelial cell layers, thus the cell and molecular bases of this crucial event in lymphocyte homing are poorly understood. In a previous study of the effects of cytokines on cultured HEC, we reported that the numbers of type I and type II lymphocytes are independently regulated (May and Ager, 1992); however the relationship between these two lymphocyte populations was not determined. In this report we have performed detailed kinetic, phenotypic and functional analyses of type I and type II cells in order to gain further insight into the mechanism of lymphocyte extravasation.

**MATERIALS AND METHODS**

**High endothelial cell culture**

Primary cultures of high endothelial cells (HEC) were established from cervical lymph nodes of individual 8- to 12-week AO or (AOxDAF) male rats. Confluent cultures were serially passaged and plated at 50% of confluent density, as described previously (Ager, 1987). Three different strains of HEC were used between the 5th and 18th passage in this study. Previous studies have shown that the interactions between lymphocytes and cultured HEC are independent of passage number (Ager and Mistry, 1988).

**Lymphocyte-HEC adhesion assays**

In this study, the number of lymphocytes that bind to cultured HEC was determined by quantitative light microscopy. HEC were plated at 3×10⁵ cells/well in 8-well multichamber glass slides (Lab-Tek slide, ICN) and grown to confluence over 2-3 days. Lymphocytes were collected as suspensions from axillary, brachial and cervical lymph nodes of rats syngeneic to HEC, resuspended to 50×10⁶/ml in Hepes-buffered RPMI 1640 plus 5% FCS and incubated for 60 min at 37°C in tissue culture flasks (to remove plastic-adherent macrophages). Lymphocytes were resuspended at 10²/ml in assay medium (Hepes-buffered RPMI 1640 plus 1% FCS); 0.3 ml of the suspension was plated/well and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for up to 4 h. Unbound lymphocytes were removed by aspiration, the plastic gasket was removed from the glass slide and the HEC layer was washed five times in wash solution (Dulbecco’s PBS plus 1% FCS, pre-warmed to 37°C).

HEC layers were fixed using 3% glutaraldehyde in PBS for 30 min at 37°C, counterstained with 0.1% toluidine blue and examined by high power light microscopy. Previous studies have shown that HEC-adherent lymphocytes can be separated into two populations that are readily distinguished by differences in size and staining pattern. Type I lymphocytes are 4-5 µm in diameter and uniformly stained, whereas type II lymphocytes are larger (up to 10 µm diameter), flattened cells in which the nucleus and cytoplasm are seen. A combination of light and electron microscopy has shown that type I cells are bound to the upper surface of HEC and type II cells are underneath the endothelial layer (Ager and Mistry, 1988). For each sample, the numbers of type I and type II lymphocytes were counted at x1000 magnification in 25 fields using a 0.0272 mm² graticule. In some experiments, HEC nuclei were also counted. On average, 500 HEC and 250-1000 lymphocytes were counted in each sample. Results from individual experiments, which are representative of 3 or 4 performed, are presented either as the mean number of type I or type II lymphocytes per mm² or as the number of adherent lymphocytes per HEC ± s.e.m. For some studies the results from 3 or 4 experiments are pooled and expressed as mean ± s.d. In order to compare the abilities of different lymphocyte populations to migrate, the migration index was calculated as follows: type II cells per HEC/(type I + type II) cells per HEC.
Pulse-chase analysis of HEC-adherent lymphocytes

Lymphocytes from the congenic rat strains PVG.RT7a (RT7a) and PVG.RT7b (RT7b) which bear allelic variants of the CD45 molecule were used in this analysis. RT7b (pulse) lymphocytes were incubated with HEC in replicate slides for 20 min, unbound cells were removed by rinsing, and the HEC layer was either fixed immediately or incubated for a further 20 or 40 min with an equivalent concentration of RT7b (chase) lymphocytes. In these experiments lymphocytes were plated at the lower concentration of 3x10^6/ml. After removing unbound lymphocytes, the HEC layer was fixed for 10 min in ice-cold methanol, air dried and stored at -70°C for up to 4 weeks. HEC-adherent lymphocytes from the congenic rat strains were identified by indirect immunocytochemical staining, as described previously (Ager and Mistry, 1988), using monoclonal antibodies against the CD45 variants (Bell et al., 1989). RT7a and RT7b lymphocytes were identified and scored as either type I or type II. Results are expressed as the mean number of RT7b lymphocytes per mm^2 in the type I and type II fractions of HEC-adherent lymphocytes.

Collection of type I and type II lymphocytes

HEC were plated at 5x10^5 per 80 cm^2 in a tissue culture flask and grown to confluence over 2-3 days. Lymphocytes were resuspended to 50x10^6 to 100x10^6 cells/ml in assay medium, 5 ml of the suspension plated/flask and then incubated for 2 h at 37°C. Non-adherent lymphocytes were collected by washing and HEC layers incubated for a further 60 min at 37°C with 10 ml of lymphocyte-free assay medium. Under these conditions, type I lymphocytes detached from HEC layers (see Results) and were collected by washing. HEC layers plus type II cells were incubated in PBS-0.025% EDTA for 5 min at 37°C, after which all cells were released from the tissue culture flask. In some experiments cell suspensions were filtered through 35 µm nylon mesh to remove large clumps of HEC from type II lymphocytes. Microscopic analysis showed that type II lymphocytes were no longer attached to HEC following this harvesting procedure. For comparison with type II lymphocytes, all other lymphocyte populations were incubated in PBS-EDTA under identical conditions. Equivalent numbers of HEC were added back to the control lymphocyte population for comparison with type II lymphocytes. In some experiments type II lymphocytes were incubated in assay medium for up to 3.5 h at 37°C before analysis. Lymphocyte numbers and viability were determined by haemocytometry following incubation with 0.1% trypan blue in PBS.

Adhesion of lymphocytes to immobilised CS1 peptide

Lymphocytes were resuspended to a concentration of 50x10^6/ml, labelled with 10 µCi/ml L-[4,5-3H]leucine (60 Ci/mmol, Amersham International UK) in leucine-free MEM supplemented with 5% dialysed FCS for 60 min at 37°C, and resuspended in assay medium as described previously (Ager and Humphries, 1990). 3H-leucine labelled lymphocytes were incubated with HEC in 80 cm^2 flasks and type I and type II lymphocytes collected as described above. Quadruplicate wells of 96-well cluster trays (Nunc, Gibco) were coated with CS1-IgG or CS1-Src-IgG conjugates (Komoriya et al., 1991; generously provided by Dr. M. Humphries, University of Manchester) in Dulbecco’s PBS for 1-3 h at room temperature. Non-specific binding sites were blocked for 1-2 h using 10 mg/ml heat-inactivated (85°C, 10 min) BSA in PBS and the wells washed twice in assay medium. Lymphocytes (3-10x10^6 cells/ml; 50 µl/well) were incubated with peptide conjugates for 20 min at 37°C; non-adherent cells were removed by aspiration and the wells washed twice with 100 µl Dulbecco’s medium-PBS containing 1% FCS. Adherent cells were examined by phase-contrast microscopy before being solubilised in 100 µl 1 M NH_4OH. Samples were counted on a Beckman 1801 scintillation counter. Results are expressed as a percentage of total d.p.m. plated/well and the means ± s.d. of quadruplicate samples were calculated.

FACS analysis of lymphocytes

50 µl of a lymphocyte suspension (10^7 cells/ml) was stained with 50 µl tissue culture supernatant of mAb HP2/1, a mouse anti-human α4 subunit antibody that cross-reacts on rat α4 (Yednock et al., 1992; generously provided by Dr F. Sanchez-Madrid, Madrid) or the control antibody MRC-OX2 (anti-human C3b inactivator, provided by Dr A. Williams, University of Oxford) for 60 min at 4°C. Cells were washed three times with Dulbecco’s PBS containing 1% FCS and stained with 50 µl of FITC-conjugated (Fab)_2 fragments of rabbit anti-mouse Ig (DAKO Ltd) for 60 min at 4°C. After washing, cells were fixed in 1% formaldehyde in PBS and analyzed on a FACSscan (Becton Dickinson, UK) using Consort 30 software.

Time-lapse video microscopy

Cellular interactions were monitored by time-lapse video microscopy described previously (Allen, 1987). Briefly, HEC were grown to confluence in modified 25 cm^2 tissue culture flasks, incubated for up to 4 h with lymphocytes (10^6 cells/ml; 5 ml/flask) and examined by phase-contrast microscopy using a x25 objective. Two frames were exposed at 20 s intervals on U-matic film. Using normal replay, the time-lapse factor was 250:1.

Statistical analysis

Student’s t-test was used to compare groups of data.

RESULTS

Lymphocytes which remain bound to cultured HEC after a standardised washing procedure (HEC-adherent lymphocytes) adopt one of two distinct morphologies. Type I cells are phase-bright and stain uniformly with toluidine blue, whereas type II cells are phase-dark, flattened, and the nucleus and cytoplasm are visible in counter-stained samples. Light and electron microscopic analyses have shown that type I cells are bound to the upper surface of HEC and type II cells are underneath the endothelial layer (Ager and Mistry, 1988; Pankonin et al., 1992). We have studied the regulation of lymphocyte binding to and migration across cultured HEC by determining whether HEC-adherent lymphocytes adopt either type I or type II morphology. In this paper we will refer to HEC-adherent lymphocytes as type I/II cells or as having type I/II morphology. Lymphocytes that are removed from HEC by washing will be referred to as non-adherent cells.

Time-lapse video analysis of lymphocyte-cultured HEC interactions

Lymphocytes were plated onto HEC layers and examined by phase-contrast microscopy. Time-lapse video recordings were collected for up to 4 h after addition of lymphocytes. During the first 15 min, lymphocytes gradually settled on top of the HEC layer by gravity. In these experiments, non-adherent lymphocytes were not removed by washing; it was therefore not possible to determine how many lymphocytes (which are phase-bright in suspension) actually became...
type I, HEC-adherent cells. However, the appearance of type II cells was clearly seen in this analysis. As early as 5 min after plating some lymphocytes were seen to crawl between HEC and adopt the morphology of type II cells, i.e. phase-dark and flattened. The number of type II lymphocytes increased in a linear manner for up to 120 min incubation (see next section). The average time taken for the transition of individual lymphocytes from type I to type II morphology (i.e. for migration underneath the HEC layer) was ~3 min. Type II cells did not remain underneath a single HEC but were seen to crawl underneath large areas of the HEC monolayer. Analysis of 30 type II cells showed that only one lymphocyte crawled out from underneath the endothelial layer and became phase-bright over a 4 h incubation period. Thus the migration of lymphocytes underneath HEC layers was essentially one-way in this model.

Phase-bright lymphocytes on top of the HEC layer were not stationary over this incubation period but underwent very rapid shape changes. Some of these lymphocytes appeared to “swim” over the surface of HEC without obvious direction. Shape changes in individual lymphocytes were extremely transient, persisting for not more than 60 s. These ranged from protruding veils and spikes to full polarised or locomotor morphology, as described by Wilkinson et al. (1988). In a single frame, ~25% of all phase-bright cells were non-round. Most phase-bright lymphocytes (>90%) underwent shape changes and it was therefore not possible to identify which cells were actually type I, HEC-adherent lymphocytes.

**Kinetic analyses of type I and type II lymphocytes**

Using the differences in size and staining patterns to identify these two populations, type I and type II lymphocytes accumulated onto HEC layers at different rates (Fig. 1A). The number of type I cells saturated after 45 min at ~2 lymphocytes/HEC and remained relatively constant over a 4 h incubation period. The number of type II cells increased linearly for up to 4 h to reach ~7 lymphocytes/HEC. There was a lag period of 15 min before significant numbers of lymphocytes became bound to HEC, during which time the cells were settling onto HEC layers (see above).

The number of type I lymphocytes did not change significantly if the lymphocyte concentration was increased up to 10-fold. As shown in Fig. 1B, the number of type I cells remained constant at ~2 lymphocytes/HEC when the lymphocyte concentration plated was varied between 10-100×10^6 cells/ml. In contrast, the number of type II cells increased in relation to the lymphocyte concentration plated. At the highest lymphocyte concentration, the number of type II cells reached ~9 lymphocytes/HEC after 180 min.

**The binding and migration of lymphocytes is independent of HEC density**

The accumulation of lymphocytes underneath HEC layers could simply result from the use of subconfluent HEC in which there are few interendothelial junctions to restrict lymphocyte passage. Although confluent HEC were used throughout this study, it is not possible to ensure identical HEC densities between individual experiments. The relationship between lymphocyte migration and HEC density was therefore studied. Lymphocytes were incubated with HEC cultured over a 5-fold range in density up to confluence, when proliferation ceases (Ager, 1990). As shown in Table 1, the number of lymphocytes that had bound to and migrated underneath individual HEC at 60 min was completely independent of the density of the endothelial layer. At confluence (720 HEC/mm²), HEC formed a continuous layer of tightly apposed cells with few obvious free cell edges, whereas at subconfluence (140 HEC/mm²), individual HEC were singly dispersed with few cells in visible contact with each other.

We have previously shown that cultured HEC bind ~50-fold more lymphocytes than non-specialised aortic EC (Ager and Mistry, 1988). A direct comparison between HEC and aortic EC showed that of the few lymphocytes bound to aortic EC, none had adopted the morphology of type II cells (Ager, 1987, and data not shown). Other stud-
ies have documented the ability of lymphocytes to migrate into 3-dimensional collagen gels (Wilkinson, 1986). We therefore asked whether cultured HEC simply represented a 3-D matrix which supported lymphocyte migration. Using type I collagen gels prepared in multichamber slides, gel-associated lymphocytes were not detectable either by high power light microscopy or using [3H]leucine-labelled lymphocytes after 60 min incubation, whereas type I lymphocytes bound to the surface of HEC and type II lymphocytes underneath the HEC layer were readily detectable in adjacent wells coated with HEC (data not shown). Thus the high rate of lymphocyte migration across cultured HEC that we have demonstrated here provides a biologically relevant model with which to study the regulation of this event.

Transition of HEC-adherent lymphocytes from type I to type II morphology

The kinetic analysis described above did not indicate a direct relationship between type I and type II lymphocytes; we therefore determined this directly by performing the following pulse-chase analysis. A pulse of RT7a lymphocytes was collected onto HEC layers and chased with RT7b cells. The numbers of RT7a cells in the type I and type II fractions of HEC-adherent lymphocytes were determined by indirect immunostaining. In preliminary experiments, type I RT7a cells stained uniformly using mAb NDS58, however staining of type II RT7a cells was too weak for light microscope analysis (data not shown). In contrast, the staining of RT7b cells using mAb 8G6.1 was strong enough to be able to detect both type I and type II lymphocytes. RT7a lymphocytes and HEC did not stain using 8G6.1 (Fig. 1 and data not shown). RT7a (unstained) and RT7b (stained) lymphocytes in the type I and type II fractions of HEC-adherent lymphocytes were therefore determined following staining with mAb 8G6.1.

The accumulation of RT7a and RT7b lymphocytes on HEC layers followed similar time courses to those shown in Fig. 1A. As shown in Fig. 2, there was a time-dependent disappearance of RT7a cells from the type I fraction. Between 20 and 60 min, the number of RT7a cells in the type I fraction fell by 50% from 260 to 130 cells/mm². Over the same time period, the majority of these cells (~80%) appeared in the type II fraction, increasing the number of type II RT7a cells from 280 to 390 cell/mm². The remainder were lost from HEC-adherent fractions. Similar results were obtained when RT7b lymphocytes were pulsed onto HEC layers and chased with RT7a cells (data not shown). These results show that type I and type II lymphocytes represent the same population of cells at different stages of interaction with the endothelial layer, rather than representing two different lymphocyte populations.

Fate of HEC-adherent lymphocytes in lymphocyte-free media

Since the number of lymphocytes that migrated across HEC layers was directly related to the concentration plated, we determined the fate of HEC-adherent lymphocytes following the removal of non-adherent lymphocytes. As shown in Fig. 3, incubation for a further 60 min in lymphocyte-free media reduced the number of type I cells by 95%, from ~3 lymphocytes/HEC to ~0.2. The number of type II cells did not change over this period and remained constant at ~2 lymphocytes/HEC. Time-lapse video analysis showed that there was no exchange of individual lymphocytes between type I and type II fractions during incubation in lymphocyte-free media (data not shown). The selective detachment of type I lymphocytes from HEC layers in lymphocyte-free media allowed these cells to be collected in isolation from type II cells. Type II lymphocytes trapped underneath HEC were subsequently collected following detachment from HEC layers using PBS-EDTA (see Materials and methods section). The ability to harvest type I and type II lymphocytes independently allowed a direct comparison of these two populations.

Table 1. Effect of HEC density on lymphocyte binding and migration

<table>
<thead>
<tr>
<th>HEC/mm²</th>
<th>Type I lymphocytes</th>
<th>Type II lymphocytes</th>
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<tbody>
<tr>
<td>720a</td>
<td>1.15 ± 0.11</td>
<td>1.54 ± 0.09</td>
</tr>
<tr>
<td>290b</td>
<td>0.91 ± 0.12</td>
<td>1.68 ± 0.14</td>
</tr>
<tr>
<td>140c</td>
<td>1.15 ± 0.15</td>
<td>1.57 ± 0.18</td>
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HEC were plated at 3 different densities in multichamber glass slides and grown for 3 days. Lymphocytes were incubated with HEC for 60 min and HEC-adherent lymphocytes were identified as either type I, surface-bound or type II, migrated lymphocytes. The number of lymphocytes bound to the upper surface of HEC and the number that had migrated were both independent of the density of the HEC layer.

a Results are mean number of type I lymphocytes/HEC ± s.d. (n=3).
b Results are mean number of type II lymphocytes/HEC ± s.d. (n=3).
c Confluent HEC.
d Sub-confluent HEC.

Fig. 2. Transition of type I lymphocytes to type II lymphocytes. PVG.RT7a lymphocytes were pulsed onto HEC layers for 20 min and chased for 40 min with PVG.RT7b cells. RT7a cells in the type I (open symbols) and type II (filled symbols) fractions were determined by indirect immunocytochemical staining after 20, 40 and 60 min. There was a time-dependent disappearance of RT7a cells from the type I fraction and an appearance of RT7a cells in the type II fraction. Results from one representative experiment are presented and are the mean number of lymphocytes/mm² ± s.e.m. (n=25).
Transfer of types I and II lymphocytes to a second HEC layer

Several adhesion molecules on the lymphocyte surface are dependent on divalent cations for function, therefore the effect of the PBS-EDTA treatment used to harvest type II lymphocytes was first determined. Lymphocytes were pre-incubated in PBS-EDTA or assay medium (control) for 10 min, resuspended in assay medium and plated onto HEC for 60 min. The total number of lymphocytes that bound to HEC following brief incubation in PBS-EDTA was 3.14 ± 0.14 lymphocytes/HEC. This level of binding was slightly lower than the level of binding of control lymphocytes (3.55 ± 0.23). Thus, for comparison with type II lymphocytes, control and type I lymphocytes were pre-incubated in PBS-EDTA under identical conditions.

When type I cells were plated onto a second HEC layer, the total number of lymphocytes (type I + type II) that adhered to HEC was not significantly different from control lymphocytes (Fig. 4). Type I cells migrated across the endothelial layer as efficiently as control cells to give similar migration indices of 0.66 and 0.67 respectively (Fig. 4). On transfer to a second HEC layer, type II cells adhered between 1.7- and 2.5-fold better than control lymphocytes. Analysis of the numbers of “surface bound” and “migrated” lymphocytes showed clearly that the increased binding of type II cells was accounted for by a selective increase in the number of lymphocytes that migrated across second HEC layers, which ranged between 2- and 3.5-fold that of control cells (Fig. 4). Consequently the migration index of type II lymphocytes was higher than that of control cells at 0.75 and above. Type II lymphocytes were “contaminated” with HEC which were also detached by PBS-EDTA. Although large clumps of HEC were removed by filtration, small clumps (1-3 cells) of HEC remained in this population. Some HEC were found attached to second HEC layers, but they were readily distinguishable from adherent lymphocytes due to their large size and distinct morphology.

To determine what fraction of lymphocytes is able to bind to HEC, non-adherent cells were transferred to a second HEC layer. As shown in Fig. 4, these cells adhered as well as control lymphocytes, demonstrating that HEC-adherent cells do not represent a minor subpopulation of lymphocytes. However their migration across HEC was slightly reduced in comparison with control lymphocytes, as shown by the lower migration index of 0.49.

Adhesion of type I and type II lymphocytes to immobilised CS1 peptide

We have previously identified a CS1-peptide inhibitable ligand expressed by HEC which plays a major role in lymphocyte adhesion. To determine whether the enhanced binding of type II lymphocytes to HEC was mediated via altered recognition of the CS1 adhesion domain, we compared the adhesion of type I and type II cells to immobilised CS1 peptide. As shown in Fig. 5, the adhesion of control lymphocytes (that had been incubated in the absence of HEC) and type I lymphocytes were similar at ~10%. The adhe-

Fig. 3. Lymphocyte transmigration is not an automatic consequence of binding to the surface of HEC. HEC-adherent lymphocytes were collected for 60 min and the incubation was continued for a further 2 h in the presence and absence of excess lymphocytes. The numbers of type I (open symbols) and type II (filled symbols) lymphocytes during culture in lymphocyte-free media (△, ▲) were compared with parallel samples which were not washed (○, ●). In the absence of excess lymphocytes, the majority of type I cells detached and were released into the supernatant. Results from separate experiments were pooled and are mean numbers of lymphocytes/HEC ± s.d. (n=3) for type I and type II cells.

Fig. 4. Transfer of HEC-adherent lymphocytes to a second HEC layer. Lymphocytes were incubated in the absence (control) and presence of HEC grown in 80 cm² flasks for 120 min. Non-adherent lymphocytes were collected by washing and HEC-adherent lymphocytes were separated into type I and type II populations (see Materials and Methods section). All lymphocyte populations were treated with PBS-EDTA, resuspended to the same concentration and incubated with HEC grown to confluence in multichamber glass slides. After 60 min, adherent lymphocytes were identified as either “surface bound” or “migrated” following counterstaining with toluidine blue and high power light microscopy. Results are mean numbers of lymphocytes/HEC ± s.e.m. for total (filled bars), surface-bound (open bars) and migrated (hatched bars) lymphocytes, with migration indices in parentheses. Significance values with respect to control are: **, P<0.001; *, P<0.05.
Lymphocyte migration across high endothelium

Table 2. Increased binding of type II lymphocytes to CS1 peptide is reversible

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<tr>
<th>Incubation time (h)</th>
<th>Fold increase in adhesion</th>
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<tr>
<td>0</td>
<td>4.0</td>
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<tr>
<td>1</td>
<td>3.3</td>
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<tr>
<td>3.5</td>
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Lymphocytes were labelled with 3H-leucine, incubated with cultured HEC and type II lymphocytes collected and resuspended to 10^5 cells/ml in assay medium (see Materials and Methods section). Type II cells were incubated at 37°C for up to 3.5 h, adhesion to CS1 and CS1-Scr peptides measured and compared with a control population of lymphocytes that had been incubated in the absence of HEC and treated identically to type II cells. Background adhesion to CS1-Scr was subtracted from specific adhesion to CS1 peptide for each lymphocyte population and the ratio of adhesion of type II lymphocytes to control lymphocytes calculated to give fold increase in adhesion.

**Expression of α4 integrin subunit by type I and type II lymphocytes**

The reversible increase in binding of type II cells to CS1 peptide suggests that the activity of α4β1, the lymphocyte receptor for CS1 (Wayner et al., 1989), may be transiently upregulated following interaction with HEC. To determine whether increased binding to CS1 is simply due to higher levels of receptor we have measured the expression of the α4 integrin subunit on type I and type II lymphocytes.

As shown in Fig. 6, analysis of control, type I and type II lymphocytes by 2-dimensional forward versus side scatter analysis on the FACScan showed that all 3 populations comprised mainly small, unactivated lymphocytes (gate 1). Large lymphoid cells (gate 2) were enriched in HEC-adherent lymphocytes, from 8% in the starting population to 14% in type I and 23% in type II fractions. Single staining FACS profiles of unactivated lymphocytes showed that the percentages of α4-positive lymphocytes were similar in control, type I and type II lymphocytes at 77, 86 and 81% respectively. The levels of α4 integrin subunit on all three populations were similar, with mean fluorescence intensities of 9. Lymphoblasts expressing the α4 integrin subunit were slightly enriched in HEC-adherent fractions, from 45% in control cells to ~60% (Fig. 6). However the number of α4 integrin subunit-positive cells and the level of α4 subunit expression in the type I and type II fractions were similar (Fig. 6). HEC in the type II fraction were analysed separately from lymphocytes using different gain settings. HEC did not express the α4 integrin subunit, as assessed using mAb HP2/1 (data not shown).

**Discussion**

The constitutive migration of lymphocytes into lymph nodes via HEV is an excellent example of efficient cell sorting by vascular EC. Following binding to the luminal surface of HEV, the time taken to reach the basal lamina is 5-10 min (Fossum et al., 1983), thus the maximal rate of extravasation via these specialised vessels is between 10 and 20 lymphocytes per HEC per hour. Other leucocyte populations, such as neutrophils, do not normally migrate across these vessels (Gowans and Knight, 1964) although they are as abundant as lymphocytes in the blood. The majority of lymphocytes in the blood are able to migrate...
across HEV (Ford, 1975; Picker, 1992) although some sub-populations such as recently activated cells also migrate to non-lymphoid organs (Mackay et al., 1990). A number of different adhesion molecules have been implicated in binding to HEV; however the molecular basis of lymphocyte migration across the vessel wall is poorly understood.

The migration of lymphocytes across non-specialised, large vessel endothelial cells in culture has been reported previously using umbilical vein EC (Oppenheimer-Marks et al., 1990). A recent study using this model demonstrated that migration is restricted to recently activated T cells (Masuyama et al., 1992). The use of non-specialised EC may therefore be relevant to the study of lymphocyte migration across the vessel wall. However, the molecular basis of lymphocyte migration is poorly understood.

Our demonstration of the ability of cultured HEC to support high levels of lymphocyte migration in vitro provides a biologically relevant model with which to study the regulation of this event.

A novel finding from this study was that following migration, lymphocytes showed a 3- to 4-fold increase in binding to CS1 peptide, which correlated with an increased ability to migrate across HEC layers. The level of α4 integrin subunit on type II lymphocytes was similar to that on type I cells, which suggests that the affinity of the CS1 receptor, α4β1 integrin (Mould et al., 1990), is upregulated on these cells. The demonstration that the increased binding of type II cells to CS1 peptide was reversible over a 3 h period also supports this proposal. These results suggest that lymphocyte migration may be regulated by increased affinities of adhesion receptors for ligands in the endothelial layer following interaction with HEC. This proposal provides an explanation for the one-way traffic of lymphocytes across the HEC layer reported in this study, in that type II cells are trapped underneath via altered affinities of adhesion receptors for ligands in the HEC layer and/or the extracellular matrix. We have already identified an important role for another β1 integrin, VLA-5, in the adhesion of lymphocytes to cultured HEC (Szekanecz et al., 1992) and, although we only demonstrate increases in VLA-4 activity.

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**Fig. 6.** Morphology and expression of α4 integrin subunit by type I and type II lymphocytes. Lymphocytes were incubated with cultured HEC for 120 min, type I and type II lymphocytes were harvested as described in the Materials and methods section and compared with a control population of lymphocytes that had been incubated in the absence of HEC. All populations were pretreated with PBS-EDTA, stained using mAb HP2/1 and analysed on the FACScan. (A) Cell size. Samples were analysed for cell size using forward versus side scatter analysis. Electronic gating was used to separate small lymphocytes (Gate 1) from lymphoblasts (Gate 2) and the percentage large cells is given in the top right hand corner. (B) Expression of α4 subunit. Samples were analysed for expression of α4 integrin subunit following staining with mAb HP2/1 (solid lines) and compared with an irrelevant control antibody MRC OX-21 (dashed lines). Figures in top right hand corner are percentage positive cells with mean fluorescence intensities in parentheses for both small (Gate 1) and large (Gate 2) lymphocytes. Profiles show log fluorescence (0 to 10^4 channels) on the x-axis and cell number (gate 1, 0 to 500; gate 2, 0 to 100) on the y-axis.
here, we predict that other $\beta_1$ integrins may be similarly affected. Studies of neutrophil migration across cytokine-activated umbilical vein EC have demonstrated that migration is regulated by increases in affinity of $\beta_2$ integrins on the neutrophil surface, following engagement of receptors for PAF and IL-8 on the neutrophil by these ligands presented on the EC surface (for review see Zimmerman et al., 1992). This provides an efficient mechanism for stimulation of only those neutrophils in close or direct contact with the endothelial layer. Our results suggest that the migration of lymphocytes across high endothelium is also regulated by increased affinities of integrins on the lymphocyte surface following contact with the EC layer. However, the binding of unactivated lymphocytes to cultured HEC is not mediated by $\beta_2$ integrins (Tamatani et al., 1991, Pankonin et al., 1992) but, in contrast to neutrophil-EC interactions, is largely mediated by $\beta_1$ integrins (Szekanecz et al., 1992).

Although the integrin stimulus was not identified in this study, our results clearly show that it is associated with the endothelial layer rather than with a secreted factor. The adhesive behaviour of non-adherent lymphocytes was unaltered and we have been unable to detect effects of media conditioned by cultured HEC on the binding of lymphocytes to CS1 peptide (May and Ager, unpublished). It is therefore unlikely to be related to a previously described soluble factor from cultured HEC that induces locomotor morphology in lymphocytes (Harris, 1991). We have reported that lymphocyte migration across cultured HEC is upregulated following treatment of the endothelial layer with interferon-$\gamma$ and tumour necrosis factor-$\alpha$ but not IL-1$\beta$ (May and Ager, 1992). We would therefore predict that the integrin stimulus is upregulated by the former cytokines but not by IL-1.

The recent demonstration that integrin activity on lymphocytes can be upregulated using antibodies against the $\beta_1$ subunit (van de Wiel-van Kemenade et al., 1992; Kovach et al., 1992) suggests that ligand engagement may be sufficient. It was also demonstrated in these studies that antibodies to $\beta_1$ integrins on lymphocytes stimulated adhesion to cultured umbilical vein EC. However, lymphocyte migration across HEC in our model was unaffected by prior adhesion of lymphocytes to CS1 peptide (May and Ager, unpublished). It is unlikely that integrin activation is upregulated by mitogenic antibodies to CD2 and CD3 on T-cells (Shimizu et al., 1990). It is unlikely that integrin activation is mediated by antigenic stimulation in our model, since we have used syngeneic lymphocytes in the adhesion assays and we have shown in this study that the majority of lymphocytes that bind to HEC are unactivated. In fact, previous studies of lymphocyte recirculation have shown clearly that B-lymphocytes as well as T-cells constitutively migrate into lymphoid organs (Fossum et al., 1983) and that their entry is not dependent on antigen (Ford, 1975). It will be interesting to determine the role of CD31 in this model, since antibodies to CD31 have recently been shown to preferentially upregulate $\beta_1$ and not $\beta_2$ activity in human T-cell subsets (Tanaka et al., 1992).

The molecular basis of upregulated integrin function in this and other models remains to be determined. Previous studies of the $\alpha_5\beta_1$ integrin (VLA-5) have shown that ligand occupancy results in redistribution of the receptor to focal contacts and this was thought to be regulated by extracellular changes that increase its ability to interact with cytoskeletal proteins, also resulting in receptor redistribution (La Flamme et al., 1992). The increased binding of lymphocytes to CS1 peptide that we have reported may be due to a stable association of VLA-4 with the lymphocyte cytoskeleton, which results in strong cell attachment to ligand rather than a conformational change in the extracellular domain that results in higher affinity binding of ligand. Studies of the distribution of VLA-4 on the lymphocyte surface and its association with the cytoskeleton on migrating lymphocytes may address this question.

The ability to conclude that VLA-4 affinity on lymphocytes is upregulated following migration across high endothelium was absolutely dependent on the demonstration that type I and type II lymphocytes represent the same, rather than different, populations. This conclusion was supported by the observation that type I and type II lymphocytes gave rise to mixtures of "surface bound" and "migrated" lymphocytes on transfer to second HEC layers. The use of lymphocytes from the congenic rat strains PVG.RT1$^a$ and PVG.RT1$^b$, which express allelic variants of CD45, was crucial to determining the relationship between type I and type II lymphocytes. Although these lymphocytes are allogeneic to cultured HEC used, previous studies have shown that the adhesion of lymphocytes to HEC both in vitro and in vivo is independent of antigen and is not restricted by MHC (May and Ager, 1992; Ager et al., 1988).

The results presented here show clearly that lymphocyte interactions with vascular endothelium are complex and regulated at several levels. Lymphocyte binding is regulated by HEC, in that there are a limited number of sites available on the upper (luminal) endothelial surface. Not all lymphocytes that bind to HEC go on to migrate across the layer; however, when it occurred, migration was a one-way event. The number of lymphocytes that migrated was directly related to the concentration of lymphocytes plated onto HEC layers. The curious effect of lymphocyte number on migration may reflect the probability of a binding site on the upper endothelial surface being re-occupied once it has been vacated by a lymphocyte which is about to migrate. However, lymphocytes that had already bound to the surface of HEC were found to detach rather than migrate across the endothelial layer when incubated in lymphocyte-free media. This result suggests an alternative explanation for the cell number effect in that HEC may synthesise an inhibitor of lymphocyte migration. Removal or inactivation of this inhibitor by the addition of excess lymphocytes would then allow migration to take place. This would ensure that only a fraction of lymphocytes in the blood will be extracted by any one lymphoid organ, to allow widespread dissemination of lymphocytes to all organs of the
immune system. Although the mechanism of lymphocyte migration was not studied further, these results suggest that the minimal requirements are lymphocytes and HEC, and that additional cells or factors from the lymph node are not required, as has been suggested previously (Hendriks and Estermans, 1983).

As shown here, the interactions between lymphocytes and cultured HEC are dynamic. The number of lymphocytes that will bind to and migrate across HEC (i.e. the proportion of type I and type II cells) depends on a combination of factors, including the number of HEC, the concentration of lymphocytes plated and the incubation time. In individual experiments, the number of HEC-adherent lymphocytes after 60 min incubation varied between 5% and 20% of the total plated. These studies did not support the conclusion that HEC-adherent lymphocytes simply comprised mainly unactivated cells, and there was little evidence for enrichment of α4 integrin subunit-expressing lymphocytes even though adhesion is largely mediated by α4β1/CS1 recognition in this model (Ager and Humphries, 1990; Szekanecz et al., 1992).

Further work is required to understand the precise role of the HEC layer in lymphocyte extravasation. The migration of bound lymphocytes was not simply due to the HEC layer functioning as a 3-D matrix which allows lymphocytes to migrate, since lymphocytes did not migrate into collagen gels under identical assay conditions. In addition, the accumulation of lymphocytes underneath the endothelial layer was independent of HEC density. A previous study of lymphocyte interactions with non-specialised EC cultured from pig aorta found that the number of lymphocytes penetrating the monolayer was inversely related to EC density (de Bono, 1976). Previous studies of HEC have shown that the endothelial lining is more permeable than in other types of blood vessel (Anderson and Anderson, 1976), which suggests that high endothelium may contain fewer adherens-type junctions. If so, this could explain the lack of effect of endothelial density on lymphocyte migration that we report in this study. Further work is required to identify such junctions in high endothelium as well as in other types of endothelium, and to determine their role, if any, in regulating lymphocyte extravasation.

Our results suggest a model in which the extravasation or successful “homing” of lymphocytes is regulated by a ‘migration stimulus’ emanating from HEC which upregulates β1 integrin affinity on cells in close contact with the endothelial layer. If this proposed stimulus were specific for lymphocytes, rather than for other populations of leukocytes, it would regulate the selective migration of lymphocytes. Using other models of lymphocyte homing, an α4 subunit containing integrin, α4β1, has been shown to mediate the migration of lymphocytes to some lymphoid organs as have other molecules such as l-selectin, CD44 and LFA-1 (for a review, see Picker, 1992). The precise roles of these other molecules in the binding and migration of lymphocytes across endothelium are as yet undefined, although a recent report demonstrated that l-selectin supported low affinity interactions between lymphocyte and umbilical vein EC and did not mediate lymphocyte adhesion under the assay conditions described here (Sperini et al., 1991). The distribution of l-selectin, CD44 and LFA-1, as well as α4β1 integrin on leukocytes other than lymphocytes suggests that a lymphocyte-specific migration stimulus could play an important role in the selective homing of lymphocytes to lymph nodes via HEV. Cultured HEC will be useful in identifying the β1 integrin stimulus that we have reported and in determining its precise role in lymphocyte extravasation.

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Lymphocyte migration across high endothelium


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