

Lymphocyte adhesion to high endothelium is mediated by two β_1 integrin receptors for fibronectin, $\alpha_4\beta_1$ and $\alpha_5\beta_1$

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Summary

Using a rat model we have previously proposed a role for fibronectin as an adhesive ligand on high endothelial cells (HEC) for recirculating lymphocytes. Lymphocyte adhesion to high endothelial cells was blocked by CS1 peptide (from the type III connecting segment of fibronectin) and RGD-containing peptides using two different *in vitro* assays of lymphocyte-HEC recognition, the frozen section assay and cultured HEC. In order to study the receptors utilised by lymphocytes to bind to HEC we have developed a xenogeneic model in which the adhesion of human lymphocytes to HEC cultured from rat lymph nodes is measured. The basic properties of lymphocyte-HEC interaction were retained using human lymphocytes. CS1 peptide and RGD-containing peptides gave similar profiles of inhibition of lymphocyte adhesion as found previously using rat cells.

FACS analysis showed that the majority of peripheral blood lymphocytes expressed two β_1 integrin receptors, $\alpha_4\beta_1$ and $\alpha_5\beta_1$, which are known to recognise distinct adhesion domains in fibronectin. A subpopulation of lymphocytes also expressed $\alpha_3\beta_1$, which, like $\alpha_5\beta_1$, has been reported to be an RGD-dependent adhesion receptor for the central cell binding domain of fibronectin. Anti- α_4 and anti- α_5 subunit monoclonal antibodies

maximally inhibited adhesion to HEC by 60% and 65%, respectively. Monoclonal antibodies to the common β_1 subunit gave slightly higher inhibition at 70%. These results suggest that lymphocytes employ one or both of two different β_1 integrin fibronectin receptors to bind to HEC. The simultaneous or alternate engagement of two fibronectin receptors on the lymphocyte surface by immobilised fibronectin in the endothelial layer may contribute to the stabilisation of adhesive contacts or to the subsequent transendothelial migration of lymphocytes. In contrast to lymphocytes, peripheral blood neutrophils did not express any members of the β_1 integrin family. The selective expression of β_1 integrins by lymphocytes and not neutrophils contrasted with the widespread distribution of the other homing-associated adhesion molecules, LECAM-1, CD44 and LFA-1, on these two cell types. It is thus possible that the selective expression of β_1 integrins regulates the constitutive migration of lymphocytes but not neutrophils into organised lymphoid tissues.

Key words: β_1 integrins, lymphocytes, high endothelium, cell adhesion.

Introduction

The recirculation of lymphocytes between peripheral blood and lymphoid organs is a well-characterised process in which cells leave the blood stream by crossing the walls of specialised, post-capillary venules lined with high endothelial cells (HEC) (Duvestijn and Hamann, 1989). The tissue specificity of these events is thought to be controlled by unique cell adhesion molecules (termed "homing receptors") expressed by peripheral blood lymphocytes and complementary ligands ("vascular addressins") on HEC. Although a number of candidate molecules on the lymphocyte surface have been identified, none of the proposed

lymph node homing receptors is in fact specific to lymphocytes. The widespread distribution of LECAM-1, CD44 and LFA-1 on all leucocytes (Jutila et al., 1989) suggests that another receptor family may be responsible for the specificity of lymphocyte recirculation. Alternatively, a number of variably expressed molecules may either act in concert or be differentially activated, resulting in the extravasation of individual leucocyte populations at distinct sites.

Members of the β_1 integrin family of heterodimeric receptors (also known as the VLA family) have been reported to mediate the adhesion of different cell types to extracellular matrix components such as collagens, fibronectin and laminin (for review, see Hemler, 1990;

Albelda and Buck, 1990). In addition, $\alpha_4\beta_1$ is unique within this family as it also mediates various types of cell-cell adhesion such as T- to B-cell interactions (Clayberger et al., 1987; Takada et al., 1989), helper-suppressor cell interactions (Groux et al., 1989) and the homotypic aggregation of lymphocytes (Campanero et al., 1990). It has also been reported that peripheral blood lymphocytes express $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ at moderate levels and $\alpha_3\beta_1$ at low levels (Hemler, 1990). In the absence of activation, the α_1 and α_2 subunits are missing from these cells. In contrast, peripheral blood neutrophils do not express any detectable β_1 integrins (Hemler, 1990). It is therefore possible that in contrast to the widely distributed adhesion molecules LECAM-1, CD44 and LFA-1, some β_1 integrins might act as lymphocyte-specific homing receptors or play a crucial role in lymphocyte extravasation. In fact an α_4 subunit containing integrin has been shown to function as a Peyer's patch homing receptor in mice (Holzmann et al., 1989). A further unanswered question is whether lymphocyte homing occurs by direct lymphocyte-HEC interaction or by an indirect cell-cell adhesion mediated by intercellular matrix macromolecules (Hemler, 1990).

To address this possibility we have adopted an *in vitro* model of lymphocyte homing using HEC cultured from rat lymph nodes (Ager, 1987). Cultured HEC express several differentiated properties uniquely associated with HEV such as phenotypic markers (Ise et al., 1988) and secretion of HEC-specific sulphated macromolecule(s) (Andrews et al., 1983; Ager and Martin, unpublished). Cultured HEC express recognition molecules for recirculating lymphocytes but not for immature lymphocytes (Ise et al., 1988). Following adhesion to the surface of HEC a subpopulation of lymphocytes invade the HEC and occupy a position underneath the endothelial layer in a manner analogous to the diapedesis that occurs *in vivo* (Ager and Mistry, 1988). Cultured HEC therefore provide a novel *in vitro* assay for the properties of vascular endothelium that mediate not only the adhesion but also the subsequent transmigration of lymphocytes.

Using this and other models of lymphocyte homing we have previously proposed a role for HEC-associated fibronectin in the binding of unactivated lymphocytes (Ager and Humphries, 1990). Lymphocyte adhesion was blocked by both CS1 peptide (from the major cell adhesion site in the type III connecting segment) and RGD-containing peptides. In order to study the receptors used by lymphocytes to bind to HEC we have developed a xenogeneic model in which the adhesion of human lymphocytes to HEC cultured from rat lymph nodes is measured. CS1 peptide and RGD-containing peptides gave similar profiles of inhibition of human lymphocytes to those found previously using rat cells. Monoclonal antibodies to the α_4 and α_5 subunits of β_1 integrin receptors blocked adhesion by 60% and 65%, respectively. Simultaneous inhibition of all β_1 integrins gave slightly higher inhibition at 70%. These results suggest that lymphocytes employ one or both of two different β_1 integrin receptors for fibronectin to bind to HEC.

Materials and methods

Antibodies and peptides

The following monoclonal antibodies were obtained as gifts: TS2/7 (anti- α_1 , culture supernatant from M. E. Hemler, Dana-Farber Cancer Institute, Boston, MA, USA); 12F1 (anti- α_2 , 1 mg/ml from V. L. Woods, University of California, San Diego, USA); J143 (anti- α_3 , 2 mg/ml from A. P. Albino, Memorial Sloan Kettering Institute, New York, USA); 8F2 (anti- α_4 , ascites fluid from C. Morimoto, Dana-Farber Cancer Institute, Boston, MA, USA); mAb 16 (anti- α_5 , 12.5 mg/ml from S. K. Akiyama, Howard University Cancer Center, Washington, DC, USA); GoH3 (anti- α_6 , culture supernatant from A. Sonnenberg, Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands); mAb 13 (anti- β_1 , 11 mg/ml from S. K. Akiyama, see above); MHM24 (anti- β_2 , ascites fluid from A. McMichael, Institute for Molecular Medicine, John Radcliffe Infirmary, Oxford, UK); mAb 15 (anti- β_3 , 5 mg/ml from M. Ginsberg, Scripps Clinic, La Jolla, CA, USA); 439-9B (anti- β_4 , ascites fluid from S. J. Kennel, Oak Ridge National Lab, Oak Ridge, TN, USA); Hermes-1 and Hermes-3 (anti-CD44, culture supernatants from S. Jalkanen, Department of Medical Microbiology, Turku University, Turku, Finland); mAb 29 (anti-CD15, culture supernatant from N. Hogg, Macrophage Lab, Imperial Cancer Research Fund, London, UK).

Other antibodies were obtained from commercial sources: T3 (anti-CD3), T4 (anti-CD4), T8 (anti-CD8) and FITC-conjugated F(ab)₂ fragments of rabbit anti-mouse Ig from DAKO, UK; LeuM3 (anti-CD14) and Leu8 (anti-LECAM-1) from Becton Dickinson, UK; W6-32 (anti-HLA), FITC-anti-human Ig and FITC-F(ab)₂ fragments of rabbit anti-rat IgG from Serotec, UK.

CS1 peptide was used as a diagnostic probe for activity of the type III connecting segment of fibronectin and CS4 peptide was used as an inactive control. GRGDGS was used to indicate RGD-dependent activity and GRDGDG was used as control. Peptides were synthesized and purified as described previously (Humphries et al., 1986). The CS peptide sequences are as follows: DELPQLVTLPHPNLHGPEILDVPST (CS1); FRRTTPPTTATPIRHRPRYPNPGVEE (CS4).

Isolation of human peripheral blood lymphocytes, monocytes and neutrophils

Peripheral blood lymphocytes (PBL) were isolated from heparinized blood by separating mononuclear cells on Histopaque 1077 (Sigma, UK). After washing in Dulbecco's PBS containing 1% foetal calf serum (FCS), the mononuclear cells were resuspended in RPMI 1640 containing 10% heat-inactivated FCS (RPMI 10) at 5×10^6 cells/ml and incubated in 90 mm diameter Petri dishes (5 ml/dish Nunc, Gibco) for 60 min at 37°C to remove plastic-adherent cells.

Monocytes were purified from the mononuclear cell fraction obtained above on a Nycodenz monocyte gradient (Nycomed, UK). Briefly, mononuclear cells were layered on the Nycodenz solution and centrifuged for 15 min at 250 g. Lymphocytes were pelleted while monocytes remained at the interface. Neutrophils were isolated from the red-cell-containing pellet obtained using Histopaque 1077. Briefly, a solution of 2.5% gelatin in 0.9% saline at 4°C was layered on the top of the pellet, mixed by inversion and left to stand. After 30 min at room temperature, red blood cells sedimented and neutrophils remained in the supernatant. Remaining red blood cells in the supernatant were removed by lysis in 0.83% NH₄Cl for 5 min at 37°C. All cells were washed twice and

resuspended in assay medium (HEPES-buffered RPMI 1640 containing 1% FCS; RPMI 1) at 10^7 /ml.

High endothelial cell culture

Primary cultures of high endothelial cells (HEC) were established from cervical lymph nodes of individual AO or (AO \times DA) F_1 rats. Confluent cultures were serially passaged using trypsin/EDTA and plated at 50% of confluent density as described previously (Ager, 1987). Three different strains of HEC were used between 13th and 20th passage in this study. Previous studies have shown that the interactions between lymphocytes and HEC are independent of passage number (Ager and Mistry, 1988). HEC were detached using 0.025% EDTA in PBS and resuspended at 10^7 cells/ml in RPMI 10 for FACS analysis.

FACS analysis of lymphocytes, monocytes, neutrophils and HEC

A 50 μ l sample of a cell suspension (10^7 cells/ml) was stained with 50 μ l of primary monoclonal antibodies diluted in Dulbecco's PBS containing 1% FCS for 60 min at 4°C. After washing three times, cells were stained with 50 μ l of secondary antibody for 45 min at 4°C. After washing again, cells were fixed with 1% formaldehyde in Dulbecco's PBS and analysed on a FACScan (Becton Dickinson, UK) using Consort 30 software. Purified leucocytes were characterised using antibodies against CD3, CD4 and CD8 for T cells, human Ig for B cells, CD14 for monocytes and CD15 for neutrophils. Cells were also stained for a variety of different adhesion molecules that have been implicated in lymphocyte homing (sources of mAbs mentioned above).

Analysis of HEC-adherent lymphocytes

HEC were plated in 30 mm diameter wells of 6-well tissue culture trays (Nunc) at 10^5 per well and grown to confluence over 3 days. HEC were preincubated in assay medium (RPMI 1640 containing 1% FCS) for 30 min at 37°C and then incubated with PBL (10^7 cells/ml; 2 ml/well) for 2 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Non-adherent cells were removed by aspiration and the HEC layer was washed five times with Dulbecco's PBS plus 1% FCS. Wells were analysed by phase-contrast microscopy for human PBL-rat HEC interactions. HEC-adherent lymphocytes were detached by incubating the endothelial layer using 0.025% EDTA in PBS for 5 min at 37°C. PBLs were stained for subsets and homing receptors and analysed by FACS as described above. Incubation of lymphocytes in PBS/EDTA did not affect the expression of phenotypic markers and adhesion molecules as determined by FACS analysis.

Microscopic assay to distinguish between lymphocytes bound to the upper surface of HEC and lymphocytes underneath the endothelial layer

Previous studies have shown that rat lymphocytes exhibit one of two different morphologies when bound to HEC. Type I cells are phase-light, spherical and stain uniformly with toluidine blue, while type II cells are phase-dark and flattened, in which nuclear and cytoplasmic detail are seen. Electron microscopic analysis has shown that type I lymphocytes are bound to the upper surface of HEC and type II cells are underneath the endothelial layer (Ager and Mistry, 1988). We have analysed HEC-adherent PBL for the distinct morphologies associated with either type I (surface-bound) or type II (migrated) lymphocytes.

HEC were plated at 10^4 per well in 8-well glass tissue culture chamber slides (Lab-Tek slide, ICN, UK) and grown

to confluence over 3 days. PBL were incubated with HEC for up to 2 h at 37°C (10^7 cells/ml; 300 μ l well) after which unbound PBLs were removed by aspiration, the plastic gasket was detached from the slide and the cell layer was washed five times in 50 ml wash solution (D-PBS plus 1% FCS prewarmed to 37°C), fixed for 30 min at 37°C in 3% glutaraldehyde, stained with 0.1% toluidine blue and examined by high-power light microscopy. For each sample, nuclei of adherent PBL (either surface-bound or migrated) and HEC were counted at $\times 1000$ magnification in 25 fields of view. The total number of PBL per HEC was calculated and means \pm s.d. are presented. The effects of monoclonal antibodies to various lymphocyte surface molecules were studied by pre-incubating PBL with the mAbs in assay medium for 30 min at 4°C as described below for the radioisotope assay.

Radioisotope assay to measure total lymphocyte adhesion

HEC were plated at 5×10^3 /well in 96-well tissue culture trays (Nunc, Gibco, UK) and grown to confluence. The adhesion assay was performed essentially as described previously (Ager and Humphries, 1990). PBL were labelled with 10 μ Ci/ml [³H]leucine ([4,5-³H]leucine, 60 Ci/mmol, Amersham International, UK) in leucine-free MEM (containing 5% FCS dialysed against PBS) and resuspended in assay medium. [³H]leucine-labelled PBL were incubated with HEC for 60 min at 37°C (10^7 cells/ml; 100 μ l/well), non-adherent cells were removed by aspiration and the cell layer was washed five times with 100 μ l/well wash solution. Wells were checked by phase-contrast microscopy before HEC and adherent PBL were solubilized in 100 μ l 1 M NH₄OH. Samples were processed for liquid scintillation counting using Optiphase 'Hisafe II' (LKB, UK) and counted on a Beckman LS1801 β -counter. The number of adherent PBL was directly proportional to the radioactivity measured. Bound radioactivity was expressed as a percentage of total plated to give the % lymphocyte adhesion. Results are means \pm s.d. ($n=4$).

The effects of peptides on lymphocyte adhesion were measured using the isotope assay described above. Peptides were dissolved in assay medium at twice the final concentration and 50 μ l was added to quadruplicate wells of HEC. An equal volume of PBL at twice the final concentration was added immediately and the adhesion assay continued as above. The effects of monoclonal antibodies on lymphocyte adhesion were also measured using the radioisotope assay. PBL were resuspended to 10^7 cells/ml in mAb solution and preincubated for 30 min at 4°C. Following warming to 37°C PBL plus mAb were added to the HEC layer and the assay continued as above.

Clustering of PBL

A 50 μ l sample of PBL suspension at 10^7 cells/ml in RPMI 10 was incubated with an equal volume of anti-integrin mAbs or with 50 nM phorbol myristic acetate in 96-well tissue culture trays. PBL were checked for clustering after 2 h and 18 h at 37°C in 5% CO₂ in air.

Cell counts and viability

Cells were counted in suspension by electronic particle counting and viability was monitored by trypan blue exclusion.

Results

Purified leucocyte populations were characterised using

FACS analysis. The differences in size and granularity between lymphocytes, monocytes and neutrophils allowed these 3 cell populations to be separated from one another using 2-dimensional forward versus side scatter analysis on the FACScan (Griffin et al., 1990; data not shown). Analysis of electronically gated populations of the 3 cell types showed that the PBL populations consisted of 65-70% CD3⁺, 50-57% CD4⁺, 18-24% CD8⁺ cells and 3-15% sIg⁺ cells. PBL did not stain with the antibody LeuM3, which recognises CD14. Purified monocytes were 71% positive for CD14 and negative for lymphocyte markers. Neutrophils were 50% positive for CD15 and exhibited neither lymphocyte- nor monocyte-specific markers (data not shown).

Distribution of integrins and other adhesion molecules on lymphocytes, neutrophils and monocytes

The distributions of the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin receptors for fibronectin on lymphocytes, monocytes and neutrophils were determined and compared with the distribution of three other molecules known to play a role in lymphocyte homing, LFA-1, CD44 and LECAM-1. Virtually all PBLs (97%) expressed the common β_1 integrin subunit, 88% of PBL expressed the α_4 subunit and 82% the α_5 subunit (Fig. 1). PBLs did not express the α_1 or α_2 subunits but were 20% positive for α_3 and 67% positive for α_6 (data not shown). Monocytes also expressed α_4 , α_5 and β_1 subunits, although the level of fluorescent staining was consistently lower than that for lymphocytes (Fig. 1). The staining profiles for α_5 and β_1 integrin subunits demonstrated populations of high-

and low-expressing cells in both PBL and monocyte populations. The staining profile for α_4 subunit also showed high- and low-expressing cells in the PBL population; however, monocytes stained uniformly for the α_4 integrin subunit. PBL did not express either the β_3 or the β_4 subunits of other integrin families. Neutrophils did not express either the α_4 or the α_5 integrin subunits. Analysis using an antibody to β_1 subunit showed that neutrophils did not in fact express any members of the β_1 integrin family (Fig. 1).

In contrast, CD44, LFA-1 and LECAM-1 were widely distributed on all the leucocyte subsets analysed. The majority (>95%) of lymphocytes, monocytes and neutrophils expressed both the CD11a and the CD18 subunits of the β_2 integrin family (Fig. 1 and data not shown). Lymphocytes and monocytes contained populations of high- and low-LFA-1 expressing cells. In contrast, neutrophils stained uniformly for CD11a and CD18. The mean levels of fluorescence were similar to those for lymphocytes and monocytes. CD44 was widely distributed on >95% of all lymphocytes, monocytes and neutrophils. Lymphocytes and monocytes contained high- and low-CD44 expressing cells. Neutrophils stained uniformly for CD44; however, the mean level of fluorescence was considerably lower than that of either lymphocytes or monocytes (Fig. 1). LECAM-1 was also expressed by the majority (>85%) of lymphocytes, monocytes and neutrophils. All three populations were stained equally using the Leu8 antibody.

The antibodies against human integrins, CD44 and LECAM-1, used above did not cross-react on rat

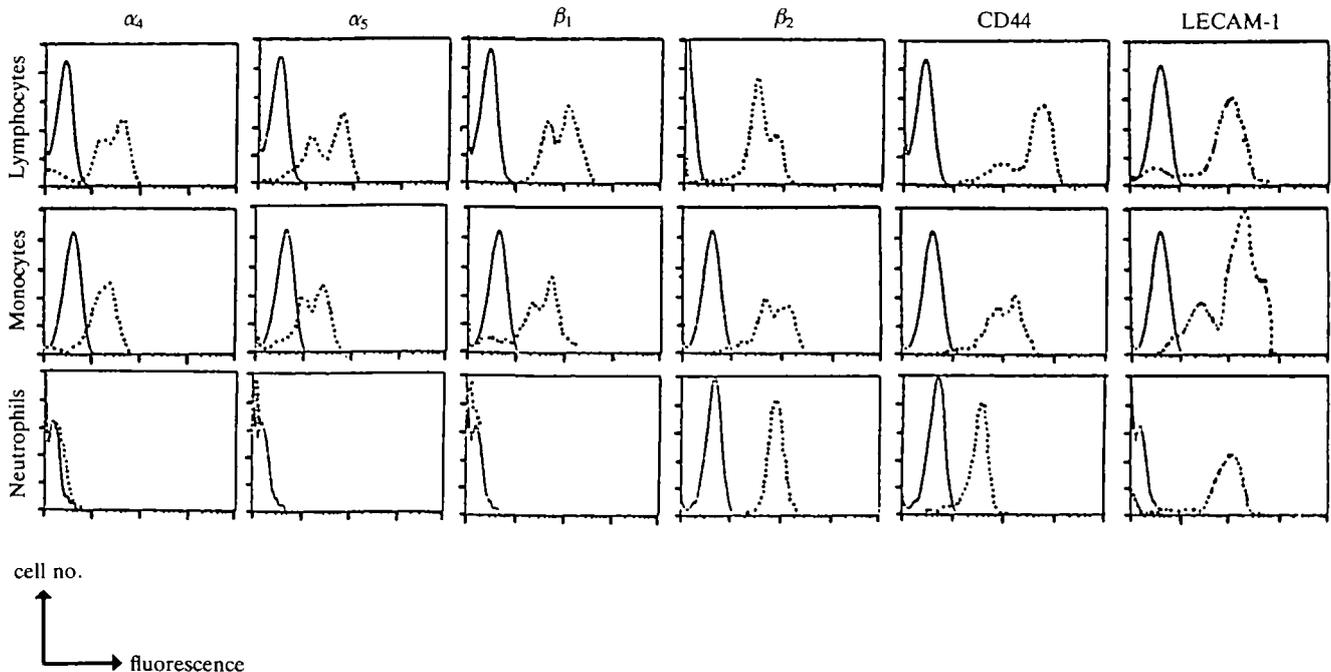


Fig. 1. The distribution of homing-associated adhesion receptors on peripheral blood lymphocytes, monocytes and neutrophils. Purified populations were stained with antibodies to α_4 (8F2), α_5 (mAb 16), β_1 (mAb 13), β_2 (MHM 24) integrin subunits, CD44 (Hermes-1) and LECAM-1 (Leu-8) and compared with unstained cells (continuous lines). Profiles show log fluorescence (0 to 10^4 channels) on the x-axis and cell number (0 to 500) on the y-axis. The staining profiles for CD11a on lymphocytes, monocytes and neutrophils were identical to those given in the figure for β_2 integrin.

tissues, as assessed by indirect immunofluorescence staining and FACS analysis of rat lymph node cells and cultured HEC.

Characterisation of human PBL adhesion to rat HEC

When human PBL were incubated with cultures of rat HEC, lymphocytes adhered preferentially to the endothelial layer within the first 60 min rather than to uncoated areas of tissue culture plastic. After longer incubation (up to 3 h) HEC-adherent lymphocytes adopted one of two distinct morphologies, which were readily identified by light microscopy. Adherent PBL were either 4-5 μm diameter phase-light cells or larger ($\geq 10 \mu\text{m}$ diameter) phase-dark cells. Analysis of fixed and stained preparations of HEC-adherent PBL showed that the small cells stained uniformly with toluidine blue whereas nuclear and cytoplasmic details were visible in the larger, flattened lymphocytes. The identification of two morphologically distinct populations coincides with previous results using rat lymphocytes in which it was shown that the small, uniformly stained, type I lymphocytes were bound to the upper surface of HEC and the larger, flattened, type II lymphocytes were underneath the HEC layer. Quantitation of PBL adhesion to rat HEC using the radioisotope assay showed that there was significant interexperimental variation. In this study the basal level of PBL adhesion after 60 min ranged from 2.5 to 14.6% of the cells plated. About 10% of HEC-adherent PBL demonstrated the morphology of type II, migrated lymphocytes after 120 min of incubation. This was less than half the number found in parallel experiments using rat lymphocytes (data not shown).

In order to study PBL that bound to HEC layers further, all adherent cells (PBL and HEC) were collected in suspension following incubation in 0.025% EDTA in PBS and analysed by FACS. HEC are larger than PBL and were gated out for FACS analysis. HEC-adherent PBL showed identical profiles on forward versus side scatter analysis as the PBL population that was plated (data not shown). However, not all lymphocyte subsets bound equally to the HEC layers. After 120 min of incubation there was an enrichment for B- over T-lymphocytes and the adherent T-cell population was enriched for CD8⁺ over CD4⁺ cells, as found previously using rat lymphocytes (Ager and Mistry, 1988). As shown in Table 1 the number of CD3⁺ cells was reduced from 72% in the plated population to 54% in the adherent fraction and the percentage of sIg⁺ cells was increased fourfold from 4% to 16%. In the adherent T-cell population, the number of CD8⁺ cells at 23% was similar to the population plated (22%), whereas the CD4⁺ population was significantly reduced from 57% to 27%. HEC-adherent PBLs were slightly enriched in CD14⁺ cells from 0.3 to 2%.

Effects of CS1 and GRGDS on PBL adhesion to rat HEC

Inclusion of CS1 peptide in the adhesion assay had a dose-dependent inhibitory effect on PBL adhesion to rat HEC. The control peptide, CS4, had little effect on

Table 1. The adhesion of lymphocyte subsets to cultured HEC

	Plated (%)	HEC-adherent (%)
CD3	72	54
CD4	57	27
CD8	22	23
sIg	4	16
CD14	0.4	2

The distribution of lymphocyte subsets in the plated PBL and HEC-adherent PBL was determined by FACS analysis using phenotype-specific monoclonal antibodies. Results are mean percentage of positive cells.

lymphocyte adhesion (Fig. 2A). Inhibition with CS1 was detectable at 3.7 μM (0.01 mg/ml) and saturated at 37 μM and above. At saturating doses, PBL adhesion was reduced from $9.2 \pm 0.5\%$ in the presence of CS4 to $5.5 \pm 0.1\%$ in the presence of CS1. This represented a maximal inhibition of 43%. CS1 and CS4 peptides tested up to concentrations of 1.0 mM had no effect on the adhesion of HEC to tissue culture plastic or on lymphocyte viability as reported previously (Ager and Humphries, 1990).

Inclusion of GRGDS in the adhesion assay had a dose-dependent inhibitory effect on PBL adhesion to rat HEC at concentrations above 20 μM (Fig. 2B). The blocking activity of GRGDS saturated above 600 μM . The control peptide, GRDGS, had no effect when tested up to 2 mM. The level of adhesion in the presence of GRDGS was not significantly different from that in the complete absence of peptides. In comparison with the control peptide, 60 μM GRGDS inhibited adhesion by 52% and 2 mM GRGDS by 71%. As found previously using rat lymphocytes GRGES also had a dose-dependent inhibitory effect on PBL adhesion to rat HEC. The effects of GRGES were not significantly different from those using GRGDS (Fig. 2B). GRGDS, GRGES and GRDGS at concentrations up to 2 mM had no effect on the adhesion of HEC to tissue culture plastic or on PBL viability as reported previously (Ager and Humphries, 1990). The inhibitory effects of CS1 and GRGDS peptides on the adhesion of human PBL to rat HEC are comparable with those reported previously using rat lymphocytes. Antibodies to the known receptor for CS1 are not available for analysis of rat lymphocytes. The demonstration that human PBL bind to rat HEC via CS1 and RGD peptide-dependent pathways provides a suitable model for determining the role of the CS1 receptor, $\alpha_4\beta_1$ integrin. In addition, the contribution of the RGD-dependent fibronectin receptor, $\alpha_5\beta_1$, could also be determined in order to investigate the role of fibronectin as an adhesive ligand for lymphocytes. Attempts to study the role of fibronectin by testing the ability of polyclonal anti-fibronectin antibodies to block rat lymphocyte adhesion to rat HEC were hampered by their ability to detach HEC from the culture dish (data not shown). Thus, by studying human PBL-rat HEC interactions and using antibodies against human inte-

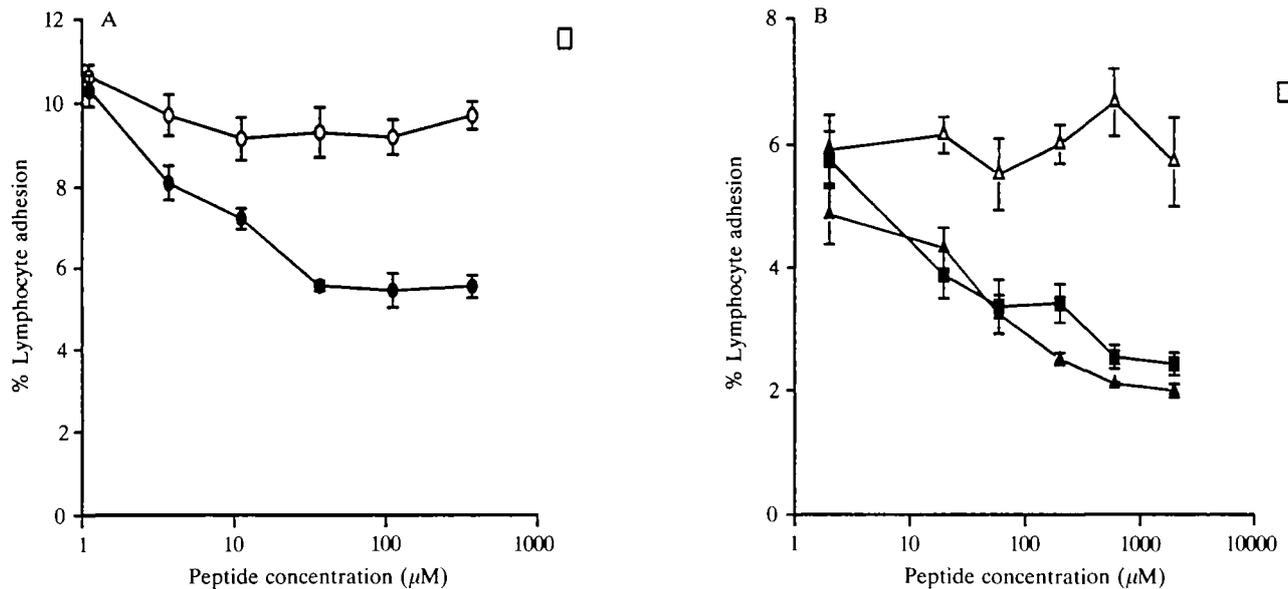


Fig. 2. The effects of fibronectin peptides on human PBL adhesion to rat HEC. The adhesion of [³H]leucine-labelled PBL to cultured HEC was measured in the presence of: (A) CS1 peptide (●) or CS4 (○); (B) GRGDS (▲), GRGES (■) or GRDGS (△). The level of adhesion in the absence of peptides is also shown (□). Results are mean % lymphocyte adhesion ± s.d. (*n*=4).

Table 2. Dose-dependent inhibition of lymphocyte adhesion to HEC by anti-integrin antibodies

	Lymphocyte adhesion (%)	Inhibition (%)
No antibody	9.6 ± 0.6	
mAb13 (β ₁)		
1:10,000	6.6 ± 0.7	31
1:3,000	5.2 ± 0.5	46
1:1,000	4.5 ± 0.4	53
1:300	4.5 ± 0.4	53
mAb16 (α ₅)		
1:10,000	7.8 ± 0.7	19
1:3,000	7.8 ± 0.8	19
1:1,000	7.7 ± 0.8	20
1:300	6.6 ± 0.5	31
8F2 (α ₄)		
1:10,000	8.8 ± 0.4	8
1:3,000	9.3 ± 0.5	4
1:1,000	9.2 ± 0.7	4
1:300	6.7 ± 0.5	30

The adhesion of [³H]leucine-labelled lymphocytes to cultured HEC was measured in the presence of antibodies. Results are mean % lymphocyte adhesion ± s.d. (*n*=4). Results were compared with adhesion in the absence of antibodies to give % inhibition.

grin receptors for fibronectin that do not cross-react on rat tissues, direct effects of the antibodies on the endothelial layer will be avoided.

Effect of anti-integrin antibodies on human PBL adhesion to rat HEC

Monoclonal antibodies directed against the α₄, α₅ and β₁ integrin subunits, which have previously been shown to inhibit cell adhesion to fibronectin, all showed dose-dependent inhibition of PBL-HEC adhesion. Using the radioisotope assay, the inhibitory effects of mAb 13 (anti-β₁) were detectable at 1 in 3000 dilution and those of mAb 16 (anti-α₅) and 8F2 (anti-α₄) at 1 in 300 (Table 2). Using saturating concentrations of each of the

Table 3. Relative effects of anti-integrin antibodies on lymphocyte adhesion to HEC

Expt	α ₄	α ₅	β ₁	β ₂	β ₁ +β ₂
1	65	76	81	—	—
2	53	63	66	—	—
3	65	72	75	51	—
4	69	71	75	66	65
5	43	46	52	25	—
	59 ± 10	66 ± 11	70 ± 10	47 ± 17	

The effect of anti-integrin antibodies on the adhesion of [³H]leucine-labelled lymphocytes was determined in 5 separate experiments. The level of adhesion was compared with that in the absence of antibody. Results are mean levels of inhibition for individual experiments. Pooled data are means ± s.d. Saturating concentrations of the following antibodies were used: 8F2 (α₄), mAb 16 (α₅), mAb 13 (β₁) and MHM24 (β₂).

antibodies there was some variation between experiments in the absolute level of inhibition. However, in 5 separate experiments the inhibitory activities were consistently ranked as follows: anti-β₁ > anti-α₅ > anti-α₄ (Table 3). Mean levels of inhibition were 70% for anti-β₁, 66% for anti-α₅, 59% for anti-α₄ antibodies. Combinations of antibodies to α₄ and α₅ subunits did not inhibit lymphocyte adhesion further than with the β₁ subunit antibody alone (data not shown).

Antibodies to Class I MHC antigens (which are uniformly expressed by PBL) gave less than 10% inhibition (Fig. 3). The inhibitory effects of α₄, α₅ and β₁ antibodies on PBL adhesion to HEC were not due to the promotion of lymphocyte clustering, which would reduce the number of lymphocytes available for adhesion to HEC. Incubation of PBL with saturating concentrations of these antibodies for up to 18 h did not induce lymphocyte clustering, which was found after

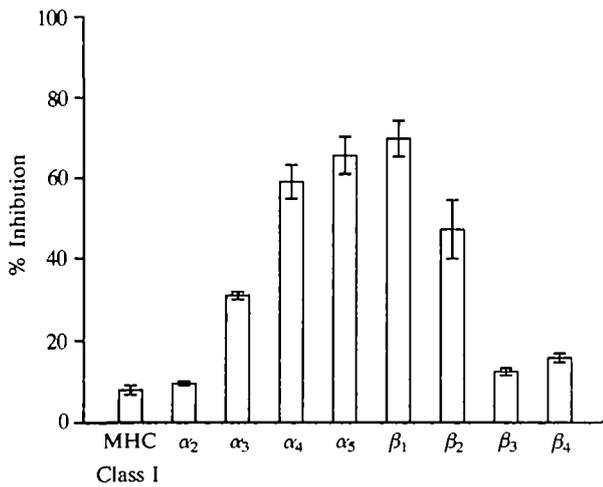


Fig. 3. The effects of monoclonal antibodies to integrin subunits on PBL adhesion to cultured HEC. The adhesion of [³H]leucine-labelled lymphocytes was measured in the presence of antibodies to α_2 (12F1), α_3 (J143), α_4 (8F2), α_5 (mAb 16), β_1 (mAb 13), β_2 (MHM 24); β_3 (mAb 15) and β_4 (439-9B) integrin subunits. Anti-class I MHC (W6/32) was used as control. Lymphocytes were pre-incubated with antibodies for 30 min at 4°C prior to their inclusion in the adhesion assay. Results are compared with adhesion in the absence of antibodies and expressed as % mean inhibition \pm s.e.m. of 3-5 observations.

incubating lymphocytes for 2 h with PMA (data not shown). Pre-incubation of rat HEC with antibodies to α_4 , α_5 and β_1 subunits prior to the adhesion assay had little effect on the subsequent binding of PBL (inhibition <10% for all 3 antibodies, data not shown), which is consistent with the lack of cross-reactivity of these anti-human antibodies on rat integrins as shown above by FACS analysis.

Monoclonal antibodies directed against other integrin subunits were also tested for effects on PBL-HEC adhesion. Antibodies to α_1 , α_2 , β_3 and β_4 subunits all gave less than 15% inhibition of PBL adhesion, which is consistent with their absence from PBL by FACS analysis. Anti- α_3 antibodies inhibited PBL adhesion in a dose-dependent manner, which saturated at a maximum of 30% (Fig. 3). Antibodies to the β_2 subunit also inhibited PBL adhesion to rat HEC; however, the level of inhibition was consistently less than that seen with anti- β_1 antibodies (Table 3 and Fig. 3). Inclusion of anti- β_2 antibodies did not inhibit PBL adhesion over and above that seen with the β_1 subunit antibody alone (Table 3).

The inhibitory effects of α_4 , α_5 and β_1 subunit antibodies were also detectable using a second assay of PBL-rat HEC adhesion, the microscope assay, in which the number of PBL bound per HEC is measured. Using this assay, the levels of inhibition were similar to those found using the radioisotope assay at 73% for anti- β_1 , 72% for anti- α_5 and 63% for anti- α_4 (Tables 3 and 4). Light microscope analysis of HEC-adherent PBL identified two distinct morphologies previously associated with either surface-bound (type I) or migrated (type II) lymphocytes. In order to determine whether

Table 4. The effects of anti-integrin antibodies on the binding and transmigration of lymphocytes

	α_4	α_5	β_1	β_2
Type I	68	72	74	51
Type II	33	73	82	47
Total	64	72	73	49

Results are means levels of inhibition for type I (surface-bound), type II (migrated) and total HEC-adherent lymphocytes in the presence of saturating concentrations of the following antibodies: 8F2 (α_4), mAb 16 (α_5), mAb 13 (β_1) and MHM24 (β_2).

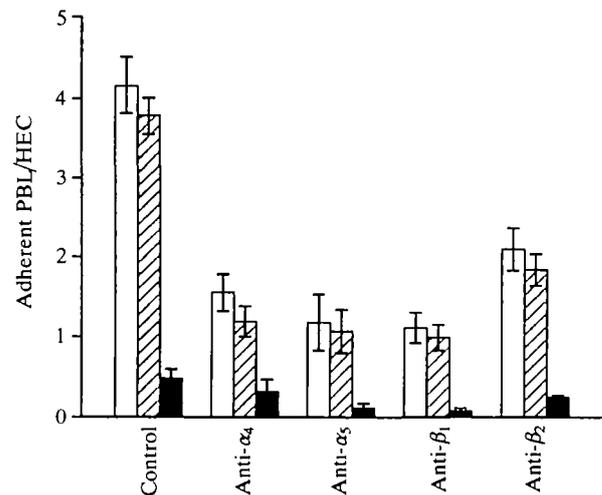


Fig. 4. The effect of anti-integrin antibodies on the binding to and transmigration of PBL across HEC. PBL were incubated with cultured HEC in the presence of antibodies to α_4 (8F2), α_5 (mAb 16), β_1 (mAb 13), β_2 (MHM 24) integrin subunits and the samples processed for microscopic analysis. The numbers of surface-bound (■), migrated (▨) and total (□) HEC-adherent lymphocytes were expressed per HEC. Results are means \pm s.d. ($n=4$).

the integrin antibodies preferentially blocked lymphocyte binding to the upper surface or lymphocyte transmigration, the numbers of type I and type II lymphocytes in the presence and absence of antibodies were determined. As shown in Fig. 4 and Table 4, α_4 , α_5 and β_1 subunit antibodies all blocked binding of lymphocytes to the surface of HEC. Antibodies to α_5 and β_1 also blocked the transmigration of lymphocytes; however, anti- α_4 antibodies had little effect on this population (Table 4).

Discussion

The tissue-specific migration or homing of lymphocytes involves an undetermined number of cell-cell and cell-matrix interactions, which together are referred to as lymphocyte-EC recognition. Thus far, several different lymphocyte surface molecules have been implicated in homing to lymphoid organs; the selectin, LECAM-1, the integrins, LFA-1 and VLA-4, and CD44. The

precise roles of each of these different adhesion receptors are not completely elucidated, although, by analogy, with neutrophil-EC interactions (Lawrence and Springer, 1991) the selectins are thought to mediate the initial, reversible binding of lymphocytes to the luminal surface of EC and the integrins to mediate the stabilisation of adhesive contacts and the subsequent transendothelial migration of lymphocytes.

Using a new *in vitro* model of lymphocyte homing we have previously identified an adhesive ligand on HEC using anti-adhesive synthetic peptides. CS1 peptide, which encodes the major cell-adhesion domain in the type III CS of fibronectin, supported the adhesion of rat lymphocytes and blocked adhesion to cultured HEC by at least 50%. The receptor for CS1 is the integrin $\alpha_4\beta_1$. In this study a heterologous *in vitro* adhesion assay was developed to study the functional aspects of $\alpha_4\beta_1$ integrin as well as other β_1 receptors in lymphocyte-endothelial cell interactions. The essential features of lymphocyte-HEC interactions that we have described previously were retained using human PBL. HEC-adherent lymphocytes adopted one of two differing morphologies, which are associated either with lymphocytes bound to the upper surface of HEC or lymphocytes that have migrated to a position underneath the endothelial layer. The relative binding of CD4⁺, CD8⁺ and B-lymphocytes to cultured HEC was similar to that found previously using rat lymphocytes (Ager and Mistry, 1988). The differential binding of lymphocyte subsets to high endothelium has been reported using other models of lymphocyte homing (Kimpton et al., 1989; Stevens et al., 1982); however, the molecular basis of this remains to be determined. The effects of anti-adhesive synthetic peptides on PBL adhesion to cultured HEC (see below) were similar to those previously reported using rat lymphocytes. Thus the adhesion receptors that lymphocytes use to bind to cultured HEC cross-react functionally between rat and human cells. Inter-species cross-reactivity of lymphocyte homing receptors has also been reported using the frozen section assay (Wu et al., 1988) and *in vivo* assays of homing (Binns and Licence, 1990). After the establishment and characterisation of the heterologous human PBL-rat HEC adhesion assay, functional analysis of β_1 integrin receptor-ligand interactions was carried out employing the purified peptide ligands of these receptors and integrin subunit-specific monoclonal antibodies.

CS1 and GRGDS both showed dose-dependent inhibition of PBL adhesion to HEC reaching maxima of 40% and 60%, respectively. A modified form of the latter ligand (GRGES) also exhibited dose-dependent inhibition. The effects of GRGES were equivalent to those of GRGDS as found previously using rat lymphocytes (Ager and Humphries, 1990). The activities of CS1 and GRGES suggest a role for $\alpha_4\beta_1$ integrin, since both peptides are diagnostic inhibitors of fibronectin III CS adhesive activity (Mould et al., 1991). However, the activity of GRGDS cannot be readily attributed to a specific integrin receptor.

In order to examine further the role of $\alpha_4\beta_1$ and to explore the site of action of RGD-containing peptides, we have used antibodies to the α_4 subunit and to other fibronectin binding β_1 integrins. The use of monoclonal antibodies against human integrins that did not cross-react on rat integrins allowed effects on human lymphocytes to be investigated independently of any effects on rat endothelium. FACS analysis of PBL confirmed that the majority (80-90%) of cells expressed both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins. The integrin $\alpha_3\beta_1$, which has been reported to recognise the RGD-dependent central cell-binding domain of fibronectin (Elices et al., 1991), was expressed by a subpopulation of lymphocytes (30%). The prototype member of the VLA family, $\alpha_1\beta_1$, was not expressed on fresh PBL, neither was $\alpha_2\beta_1$ integrin, as reported previously (Hemler, 1990). Monoclonal antibodies to β_1 , α_4 , α_5 and α_3 subunits, which have all been shown to inhibit cell adhesion to fibronectin, gave dose-dependent inhibition of lymphocyte adhesion to rat HEC; the effects were ranked $\beta_1 > \alpha_5 \geq \alpha_4 \gg \alpha_3$. Antibodies to the other integrin subunits, not expressed by PBL, and to MHC Class I, which is uniformly expressed by PBL, were without effect. Antibodies to α_4 and α_5 subunits gave similar levels of inhibition at 55-65%, while β_1 antibodies consistently gave the highest levels of inhibition at 65-75%. Combinations of antibodies to α_4 and α_5 subunits did not inhibit adhesion further, indicating that maximal levels of inhibition were achieved with the β_1 subunit antibody alone. In addition, these results suggest that the α subunit activities were associated with β_1 integrin subunits rather than with alternative β chains such as β_p , which is known to hybridise with the α_4 subunit (Holzmann and Weissman, 1989). The dominant roles of both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ in lymphocyte-HEC adhesion strongly suggest that fibronectin is a ligand on HEC. The additional activity of $\alpha_3\beta_1$ integrin also supports this suggestion, although $\alpha_3\beta_1$ is known to recognise other extracellular matrix molecules in addition to fibronectin (Elices et al., 1991). Although $\alpha_4\beta_1$ integrin is known to recognise an alternative cell surface ligand, V-CAM-1, the activity of CS1 peptide has been mapped to a different site on $\alpha_4\beta_1$ and thus is an indicator of fibronectin, rather than V-CAM-1, binding (Pulido et al., 1991).

Taken together, these data provide evidence that lymphocytes use at least two different fibronectin receptors for adhesion to cultured HEC. If this is the case, it is curious that blocking either of the receptors gives almost maximal inhibition of lymphocyte adhesion compared to that obtained by blocking all β_1 integrins. The formation of stable, adhesive contacts between lymphocytes and cultured HEC is an active process, which is blocked by pre-treatment of the lymphocyte with the protein kinase inhibitor, staurosporine (Hourihan and Ager, unpublished). Previous reports using the frozen section assay have demonstrated the requirement for an intact lymphocyte cytoskeleton in order to bind to HEC (Woodruff et al., 1977). It is therefore possible that simultaneous engage-

ment of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ by immobilised fibronectin in the HEC layer is, in fact, required by the lymphocyte in order to bind to HEC in this model. Attempts to identify the potentially overlapping roles of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ showed that combinations of antibodies to the α_4 and α_5 subunits or CS1 and RGD-containing peptides did not inhibit adhesion further than with either antibody or peptide alone, suggesting that a single antibody or peptide can give almost maximal inhibition. There is a growing body of evidence to suggest that activation of T-lymphocytes via cross-linking of surface CD3 can upregulate the affinities of integrins and other adhesion receptors for their respective ligands (Dustin and Springer, 1989; Shimizu et al., 1990; Spertini et al., 1991). As found using other models of lymphocyte homing, lymphocyte adhesion to cultured HEC is independent of antigen-receptor engagement (May and Ager, 1992). The proposal that cross-linking of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ on the lymphocyte surface by immobilised fibronectin results in the formation of stable, adhesive contacts either *via* fibronectin itself or *via* altered affinities of other adhesion receptors, e.g. LFA-1, is a possibility in this model. The observation that antibodies to β_2 integrins also inhibited adhesion and that the effects were overlapping rather than additional to those of β_1 integrins supports this proposal. In addition, these results suggest that β_1 integrins may play a more important role than β_2 integrins in lymphocyte-HEV interactions.

The fact that antibodies to α_4 and α_5 subunits blocked binding to the upper surface of HEC layers suggests that fibronectin is localised there. The additional observation that antibodies to α_5 , but not α_4 , blocked the transmigration of lymphocytes in this model suggests different roles for these two adhesion domains in fibronectin: $\alpha_4\beta_1$ -CS1 recognition mediates binding to the upper surface of HEC and $\alpha_5\beta_1$ -fibronectin recognition mediates the subsequent transendothelial migration of bound lymphocytes. In support of this proposal, we have already reported that CS1 peptide preferentially blocked binding of lymphocytes to the upper surface of HEC layers rather than transendothelial migration in this model (Ager and Humphries, 1990). In addition, lymphocytes bound to the upper surfaces of cultured HEC are exclusively distributed to the peripheral edges of HEC, not over the cell body (Ager and Mistry, 1988). Together, these results suggest preferential localisation of CS1 domain-containing fibronectin at the peripheral edges of HEC. The successful transmigration of bound lymphocytes may then depend on $\alpha_5\beta_1$ -mediated recognition of fibronectin in the intercellular or subendothelial matrix of HEC. The results presented here raise important questions about the precise distribution of fibronectin in high endothelial venules *in vivo* and it will be important to determine whether HEC-associated fibronectin, in particular the CS1 domain, is accessible to circulating leucocytes.

The molecular basis of the selective homing of lymphocytes, but not other leucocytes, to lymph nodes

is not completely worked out. The selection of lymphocytes for entry into lymph nodes could occur either during the initial adhesion step or during the subsequent transendothelial migration phase. As shown here, LECAM-1, which is the prototype lymph node "homing receptor", is widely distributed on the majority of circulating leucocytes and it is known to function on these other types of leucocyte. A soluble form of LECAM-1 has been shown to inhibit the migration of neutrophils into sites of inflammation (Watson et al., 1991) and antibodies to LECAM-1 block the initial, reversible adhesion or "rolling" of neutrophils on the luminal surface of inflamed EC (von Andrian et al., 1991). Although there is evidence that lymphocyte LECAM-1 and neutrophil LECAM-1 may recognise different ligands on endothelial cells (Picker et al., 1991) and thus regulate their distinct migration pathways via differential adhesion to HEC, it is possible that lymphocyte LECAM-1 on its own may not function as the homing receptor for lymph nodes and that additional signals will be required. Studies of lymphocyte interactions with HEV *in vivo* have shown that lymphocytes initially attach to HEC via their surface microvilli (Anderson and Anderson, 1976) and that this is a reversible, adhesive interaction (Bjerknes et al., 1986). Lymphocytes that remain attached to HEC enter apical clefts between individual HEC, adopt a motile morphology and subsequently penetrate the endothelial lining to enter the lymph node. In this context, fibronectin may function to stabilise the initial reversible, adhesive contacts between lymphocytes and HEC that precede transendothelial migration and thus fibronectin may be crucial for the successful homing (i.e. entry) of lymphocytes into lymph nodes. There are several observations that support a role for fibronectin in the selective migration pathways of lymphocytes. The CS1 sequence of fibronectin is subject to alternative splicing (Hynes, 1985) and thus is a possible source of tissue specificity in lymphocyte migration, e.g. fibronectin expressed by HEC contains the CS1 sequence whereas fibronectin expressed by other types of endothelia does not. As we have shown here, the selective expression of β_1 integrins by lymphocytes and not neutrophils contrasts with the widespread distribution of other homing-associated adhesion molecules on these two populations. Thus, $\alpha_4\beta_1$ integrin on peripheral blood lymphocytes and the CS1-containing splice variant of fibronectin on HEC may contribute to constitutive migration of lymphocytes, but not neutrophils, into organised lymphoid tissues. The use of cultured high endothelium may allow the precise roles of β_1 integrins and the other homing-associated adhesion molecules, LECAM-1, CD44 and LFA-1, in the initial binding and subsequent transendothelial migration of lymphocytes to be delineated.

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