

Interactions between interleukin-2-activated lymphocytes and vascular endothelium: binding to and migration across specialized and non-specialized endothelia

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SUMMARY

A prerequisite for the successful immunotherapy of solid tumours with interleukin-2 (IL-2)-activated lymphocytes is their ability to home to the tumour tissue. Lymphocyte homing is a complex process which is known to involve at least two independently regulated events: adhesion to the luminal surface of vascular endothelium and the subsequent transendothelial migration of lymphocytes. In this study we have used an *in vitro* model of lymphocyte homing which employs specialized high endothelium to ask whether IL-2-activated lymphocytes are able to migrate across vascular endothelium in order to leave the blood vessel. Both the adhesion of IL-2-activated cells and their migration across monolayers of cultured high endothelial cells (HEC) were increased in comparison with non-activated lymphocytes. The adhesion of IL-2-activated lymphocytes was mediated by lymphocyte function-associated antigen-1 (LFA-1) and a very late activation antigen-4 (VLA-4)-related pathway. LFA-1-dependent adhesion was mediated by ligands on HEC other than the intercellular adhesion molecule-1 (ICAM-1) and the VLA-4-related pathway was mediated by ligands other than the CS1 domain of fibronectin. HEC-adherent lymphocytes were enriched in natural killer (NK) cells and CD8⁺ T cells which are known to be the tumour-cytotoxic cells in IL-2-activated lymphocytes. However, there was no evidence of cytotoxicity towards the endothelial layer using a syngeneic model. The interaction of IL-2-activated lymphocytes and endothelial cells was not specific for high endothelium since equal numbers of activated lymphocytes bound to and migrated across aortic endothelium. The inability of IL-2-activated lymphocytes to discriminate between high endothelium and non-specialized 'flat' endothelium could be responsible for the widespread dissemination of the cells throughout the body following their adoptive transfer and the unwanted side-effects at non-involved sites.

INTRODUCTION

With the discovery of 'lymphokine-activated killer (LAK) cells', a new type of immunotherapy became available for cancer treatment. Lymphocytes activated either *in vitro* or *in vivo* with interleukin-2 (IL-2) were shown to express cytotoxic activity against allogeneic tumour cell lines and freshly isolated autologous and allogeneic tumour cells.^{1,2} Several years of clinical trials have demonstrated that a small percentage of advanced cancer patients, especially those with melanoma and metastatic renal cell carcinoma, respond to treatment with IL-2 plus IL-2-activated lymphocytes, or even IL-2 alone.^{3,4} However, the

overall results of all clinical trials have been disappointing. The success of this approach will depend not only on the cytotoxic activity of activated lymphocytes but also on their ability to migrate into the tumour mass. The widespread dissemination of IL-2-activated cells throughout the body will dilute out their potential effectiveness and may lead to side-effects at non-involved sites.

Previous experiments using the Boyden chamber assay have demonstrated that human peripheral blood lymphocytes activated with IL-2 have an enhanced motility in comparison to non-stimulated cells.⁵ However, the relevance of these results to their *in vivo* migration across endothelium of tumour vessels is unknown. It is well established that post-capillary venules lined by high endothelial cells (HEC) are specialized vessels for extracting lymphocytes from the blood.⁶ These high endothelial venules (HEV) are found in all lymphoid organs except the spleen. Blood vessels with the characteristic appearance of HEV are observed in a number of chronic inflammatory conditions and autoimmune disorders.^{7,8} The fact that HEV-like vessels

Abbreviations: HEC, high endothelial cell(s); HEV, high endothelial venule(s); ICAM 1(2), intercellular adhesion molecule-1(2); rIL-2, recombinant interleukin-2; LFA-1, lymphocyte function-associated antigen-1; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

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also occur at sites of lymphocytic infiltration in some tumours⁹ suggests that lymphocytes may be recruited to the tumour mass via an HEV-related mechanism. The availability of HEC in culture¹⁰ has now made it possible to investigate the interactions between IL-2-activated lymphocytes and HEC in detail.

In the present study we asked whether circulating tumour-cytotoxic lymphocytes which have already entered the tumour vessel are able to pass the endothelial barrier to accumulate in the tissue or whether they lodge only in the blood compartment. Although the adhesive interactions between IL-2-activated lymphocytes and vascular endothelium have already been reported in humans, the lymphocytes and endothelium were not major histocompatibility complex (MHC) matched and thus the endothelial cells were a target for LAK cells.¹¹ By using a syngeneic model we hoped to avoid the study of cell-cell interactions involved in cytotoxicity and favour those involved in transendothelial passage of IL-2-activated lymphocytes. In the rat, a major source of precursors for LAK cells is the spleen.¹² We have, therefore, activated spleen lymphocytes with IL-2 in order to generate cytotoxic natural killer (NK) and T cells¹³ and investigated their transendothelial passage using the model of the cultured HEC.

MATERIALS AND METHODS

Lymphocyte preparation

Lymphocytes were isolated from spleens and cervical lymph nodes of 8–12-week-old (AO × DA)F₁ hybrid rats bred in the Animal Unit, University of Manchester, U.K.

Mononuclear spleen cells were isolated by centrifugation on Ficoll/Hypaque (density 1.083 g/ml; Sigma Diagnostics, St Louis, MO) for 25 min at 400 *g* and passed over nylon wool.¹⁴ Following this procedure the cell suspension contained <0.5% macrophages and <10% B cells. For studying the mechanism of adhesion, B-cell contamination was reduced to <2% by immunomagnetic beading. Lymphocytes were incubated with a mixture of MRC-OX12 (anti-kappa light chain) and MRC-OX6 (anti-class II) for 30 min on ice to coat B cells and MHC class II⁺ cells, washed and incubated with biomagnetic particles coated with goat-anti-mouse IgG antibody (5 ml particles/10⁸ cells) for 30 min with shaking at 4°. Cells coated with immunoparticles were removed using a magnet. Lymph node cells (LNC) were prepared by teasing apart lymph nodes in medium. Cells released into the supernatant were centrifuged for 10 min at 250 *g*, washed and resuspended in assay medium.

Cell culture

Lymphocytes. Purified spleen lymphocytes were resuspended at 2 × 10⁶ cells/ml and cultured for up to 7 days. Cultures were maintained in RPMI-1640 supplemented with 2 mM glutamine, 0.07 mM 2-mercaptoethanol (2-ME), antibiotics (RPMI) and 10% foetal calf serum (FCS) (RPMI-10) at 37° in a humidified atmosphere of 5% CO₂ in air. Cells were refed every 3 days either with fresh medium or with IL-2 starting on Day 0. Highly purified human rIL-2 from *Escherichia coli*^{15,16} was generously provided by EuroCetus (Amsterdam, The Netherlands). Cells were harvested using a cell scraper to collect both plastic-adherent lymphocytes and lymphocytes in suspension, washed and resuspended in assay medium.

High endothelial cells, aortic endothelial cells and fibroblasts. HEC were isolated and cultured from cervical lymph nodes of

rats syngeneic to lymphocytes as described previously.¹⁰ HEC were maintained in RPMI-1640 supplemented with 20% FCS, 2 mM glutamine and antibiotics. Confluent cultures were serially passaged using 0.1% trypsin/0.025% EDTA. Two different strains of HEC between the twelfth and nineteenth passage were used in this study. Previous studies have shown that the interaction between lymphocytes and HEC is independent of passage number.¹⁷ Aortic endothelial cells and aortic fibroblasts were isolated from (AO × DA)F₁ hybrid rats and cultured as described previously.¹⁰

Microscope assay to distinguish between surface-bound and transmigrated lymphocytes

Previous studies have shown that HEC-adherent lymphocytes adopt one of two distinct morphologies. Type I cells, which stain uniformly with toluidine blue, are bound to the upper surface of HEC and type II cells, which stain differentially, are underneath the HEC layer.¹⁷ Briefly, HEC were plated at subconfluent density (3 × 10⁴ cells/well) in 8-well glass slides (Lab-Tek Tissue Culture Chamber Slides, ICN Biomedicals Ltd, High Wycombe, U.K.) and grown to confluence over 3 days. HEC were preincubated in assay medium (RPMI-1640 supplemented with 1% FCS; RPMI-1) for 30 min and then incubated in triplicate with lymphocytes (2 × 10⁶ cells in 0.3 ml) for 60 min at 37°. Non-adherent lymphocytes were removed by aspiration, the plastic gasket removed and the slide washed five times in wash solution [Dulbecco's phosphate-buffered saline (PBS) plus 1% FCS prewarmed to 37°]. The cell layer was fixed with 3% glutaraldehyde in PBS for 30 min and stained with 0.1% toluidine blue. HEC and lymphocyte nuclei were counted at ×1000 magnification. The numbers of type I and type II lymphocytes bound to 200 HEC were counted/well. The number of lymphocytes bound to HEC was normalized to 10⁵ HEC (estimated as the number of HEC/well at confluence) and expressed as a percentage of cells plated. The means and SD of triplicate observations are presented. Total adherent lymphocytes (% adhesion) include type I and type II cells. Migrated lymphocytes (% migration) are type II cells only.

Differential interference reflection microscopy

Endothelial cells (EC) were plated at 5 × 10⁴–10⁵ cells onto 22 mm² glass coverslips in individual wells (30 mm diameter) of a 6-well cluster tray (Nunc, Roskilde, Denmark) and grown to confluence over 2–3 days. Interleukin-2-activated lymphocytes were incubated with EC as described for microscope assay for up to 120 min at 37°. Following fixation in glutaraldehyde the coverslips were stored in PBS at 4° subsequent to analysis. Coverslips were mounted in a chamber filled with PBS to minimize reflections and examined using a Zeiss Universal microscope in conjunction with a 63 × Plan neofluor phase objective, HBO lamp, reflector (46-62-44), filter (46-78-07) and cross polarizers. Single fields of view were selected by phase contrast microscopy to contain morphologically distinct phase-light, type I and phase-dark, type II lymphocytes¹⁷ and then examined using interference reflection microscopy.¹⁸ Points of contact between lymphocytes and the glass slide were readily seen as interference patterns.

Radioisotope assay to measure total lymphocyte adhesion

Cultured vascular cells (HEC, aortic EC and fibroblasts) were plated at 5 × 10³/well in 96-well tissue culture trays (Nunc) and

grown to confluence over 3 days. As described previously,¹⁹ lymphocytes were labelled for 60 min at 37° with 10 μ Ci/ml [³H]leucine in leucine-free medium containing 5% dialysed FCS, resuspended at 10⁷ cells/ml in assay medium and plated onto quadruplicate wells (0.05 ml/well) of vascular cells which had been preincubated for 30 min at 37°. Following 60 min incubation at 37° in a humidified atmosphere of 5% CO₂ in air unbound cells were removed by washing. Vascular cells and adherent lymphocytes were solubilized in 100 μ l 1 M NH₄OH. Samples were processed for liquid scintillation counting using Optiphase 'Hisafe II' (LKB, Leics, U.K.) and counted on a Beckman LS1801 counter. Bound radioactivity was expressed as a percentage of the total plated to give per cent adhesion and the means and SD of quadruplicate observations are presented. In order to compare lymphocyte adhesion to different vascular cells, the results were normalized to 5 \times 10⁴ vascular cells. In blocking experiments the assay was carried out in two different ways. In the first, peptides and monoclonal antibodies (mAb) were used to preincubate lymphocytes (at 4°) and HEC (at room temperature) for 30 min prior to their inclusion in the assay. In the second, peptides and mAb were added at the start of the adhesion assay without preincubation.

Phenotypic analysis of adherent lymphocytes

HEC-adherent lymphocytes were collected following 60 min incubation on confluent HEC layers in 6-well trays (Nunc) as described above. HEC and lymphocytes were detached following incubation with 0.025% EDTA in PBS for 5 min at 37°. Interleukin-2-activated lymphocytes which were bound to tissue culture plastic (plastic-adherent cells) were harvested using a cell scraper following removal of non-bound cells. For phenotypic analysis, lymphocytes were incubated with primary mAb for 45 min at 4°, washed three times, incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab)₂ fragments of a rabbit anti-mouse immunoglobulin for 30 min at 4°, washed twice and fixed in 1% formaldehyde. 10⁴ cells were analysed on FACScan (Becton Dickinson, Mountain View, CA) using Consort 30 software. The number of positive cells was expressed as percentage of total cells analysed.

Antibodies and peptides

Saturating concentrations of the following antibodies against rat antigens were used: MRC-OX6 (anti-MHC class II), MRC-OX8 (anti-CD8), MRC-OX12 (anti- κ light chain detecting 95% B cells), MRC-OX18 (anti-MHC class I), MRC-OX54 (anti-CD2), W3/25 (anti-CD4), R7.3 [anti-T-cell receptor (TcR)- $\alpha\beta$] (all Serotec Ltd, Bicester, U.K.). HP2/1 (mouse anti-human VLA-4) (Immunotech, Marseilles, France), FITC-conjugated F(ab)₂ fragments of rabbit anti-mouse immunoglobulin (Dako Ltd, High Wycombe, U.K.), biomagnetic particles coated with goat-anti-mouse IgG antibody (Metachem Diagnostics Ltd, Piddington, U.K.) were also used. The antibody 3.2.3 (rat anti-NK cell)²⁰ was kindly provided by Dr J. Hiserodt (University of Pittsburgh, PA). The antibodies WT.1 (anti-CD11a), WT.3 (anti-CD18) and IA.29 (anti-ICAM-1) were all kindly provided by Dr Miyasaka (Tokyo Metropolitan Institute of Medical Science, Japan).²¹ CS1 peptide was used as a diagnostic probe for activity of the type III connecting segment of fibronectin and CS1-C as an inactive control.²² Both peptides were kindly provided by Dr M. Humphries (University of Manchester, U.K.).

Cell counts and viability

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

Statistical analysis

The Mann-Whitney *U*-test was used to compare groups of data.

RESULTS

Adhesion of IL-2-activated lymphocytes to and migration across cultured HEC

Spleen lymphocytes were activated with different concentrations of IL-2 for up to 7 days and incubated with cultured HEC for 60 min. Light microscopic analysis of HEC-adherent lymphocytes showed that lymphocytes cultured in the absence of IL-2 adhered exclusively to HEC. During cultivation with IL-2 between 5 and 8% of lymphocytes were found to adhere to tissue culture plastic. However, when plated onto HEC layers

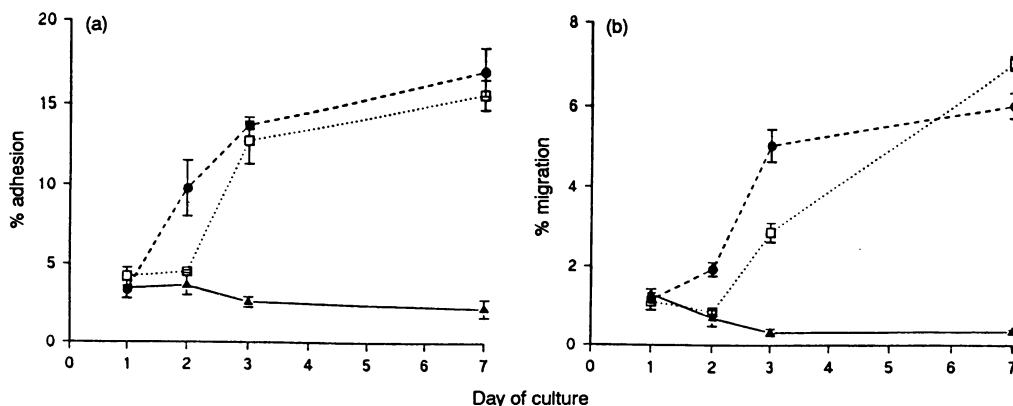


Figure 1. Interaction between IL-2-activated lymphocytes and HEC. Spleen lymphocytes were cultured in the absence [medium (▲)] or presence of IL-2 [100 (□) or 1000 (●) U/ml] for 7 days and incubated with HEC for 60 min. The total number of adherent lymphocytes (a) and the number of migrated cells (b) were expressed as a percentage of the number plated. Values are means of triplicates \pm SD. Results were taken from one experiment representative of five performed.

the majority of adherent cells were bound to HEC and not to exposed areas of tissue culture plastic (data not shown).

The level of lymphocyte adhesion to HEC on Day 1 of culture was $4.5 \pm 1.1\%$ (\pm SD) ($n = 3-5$ experiments). Following culture for 7 days in the absence of IL-2, the level of adhesion was slightly reduced (Fig. 1a). Following activation with IL-2, lymphocyte adhesion increased significantly (Fig. 1a). This effect was dependent on both dose and incubation time in IL-2. The adhesion of lymphocytes incubated with 1000 U/ml IL-2 was increased two-fold by Day 2 of culture to $9.2 \pm 1.6\%$. The effects of 100 U/ml IL-2 were not detectable until day 3 of culture by which time adhesion was increased three-fold to $11.7 \pm 2.7\%$. Between Days 3 and 7 of culture, the effects of 100 U/ml and 1000 U/ml IL-2 were equivalent and the number of adherent lymphocytes had started to plateau. Therefore, in

subsequent experiments lymphocytes activated with 1000 U/ml IL-2 were used between the third and seventh day of culture.

Previous studies using unactivated lymphocytes have shown that following adhesion to the surface of HEC, a subpopulation of lymphocytes migrates across the HEC layer and occupies a position underneath the endothelial layer.¹⁷ Phase contrast microscopic analysis of IL-2-activated lymphocytes also showed two different HEC-adherent populations, one population was phase-light and the other was phase-dark (Fig. 2a). The precise locations of these two morphologically distinct cell populations were investigated further. Using a combination of phase contrast and differential interference reflection microscopy, it was found that phase-light, type I cells were located on top of the HEC layer where they were in contact with HEC only (Fig. 2b). However, the phase-dark, type II cells were located

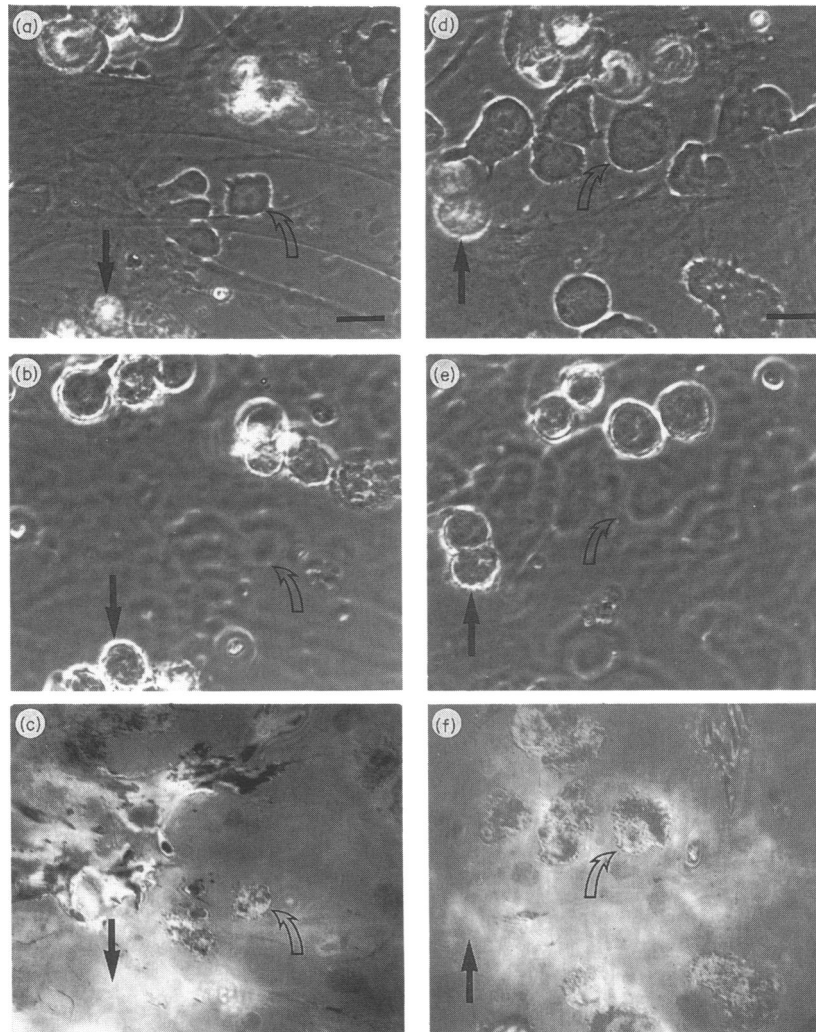


Figure 2. Interactions between IL-2 activated lymphocytes and cultured endothelial cells. Micrographs of lymphocytes activated with 1000 U/ml IL-2 for 3 days bound to either cultured HEC (d-c) or aortic endothelial cells (d-f). Two populations of adherent lymphocytes were seen by phase contrast microscopy in HEC (a) and aortic EC (d) cultures; types I, phase-light lymphocytes (closed arrow) and type II, phase-dark lymphocytes (open arrow). The phase contrast images in (b) and (e) are focused $4 \mu\text{m}$ above those in (a) and (d) showing that the phase-light lymphocytes are located on the surface of the endothelial layers (closed arrow) and the phase-dark cells are out of focus. Differential interference reflection microscopy revealed that only the phase-dark lymphocytes were in direct contact with the glass surface (c, f) as demonstrated by the interference patterns (open arrow). Contact points between the endothelial cells and the glass surface are also seen. Type I, surface-bound lymphocytes did not show interference patterns demonstrating that they were not in direct contact with the glass slide. Bars, $10 \mu\text{m}$.

Table 1. Phenotypic analysis of spleen lymphocytes following IL-2 activation. Cells were cultured in the absence (medium) or presence of 1000 U/ml IL-2 (IL-2) for 7 days and analysed by FACS on Days 1, 3 and 7 of culture. The following markers were used: W3/25 (CD4 cell), MRC-OX8 (CD8 cell), 3.2.3. (NK cell) and MRC-OX12 (B cell). Results are the number of positive cells as a percentage of cells counted. Values represent means \pm SD of $n=3-6$ observations

	Medium			IL-2		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
CD4	48 \pm 4	51 \pm 6	66 \pm 8	46 \pm 2	26 \pm 3	7 \pm 3
CD8	33 \pm 1	34 \pm 3	23 \pm 6	32 \pm 1	44 \pm 4	45 \pm 4
NK	10 \pm 1	7 \pm 3	3 \pm 2	10 \pm 1	26 \pm 6	42 \pm 7
B	10 \pm 3	7 \pm 3	8 \pm 1	10 \pm 4	8 \pm 4	6 \pm 2

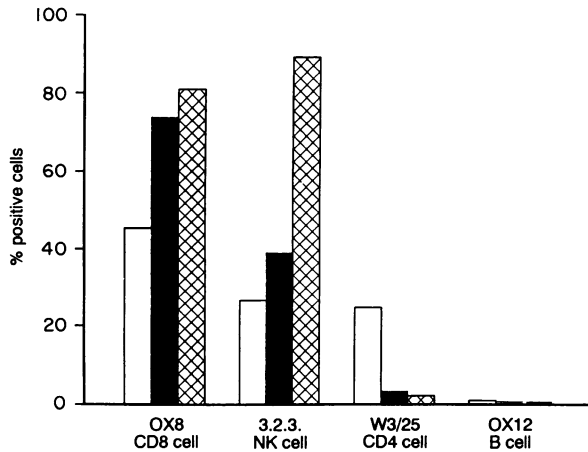


Figure 3. Phenotypic analysis of adherent IL-2-activated lymphocytes. Cells were cultured in the presence of 1000 U/ml IL-2 for 4 days. Plastic-adherent (▨) and HEC-adherent (■) cells were separated from the whole population of IL-2-activated lymphocytes and analysed by FACS; culture (□). The following markers were used: MRC-OX8 (CD8 cell), W3/25 (CD4 cell), 3.2.3. (NK cell) and MRC-OX12 (B cell). Results are the number of positive cells as a percentage of cells counted. Results are taken from one experiment which is representative for four performed.

underneath the HEC where they were adjacent to the glass slide, as shown by the interference patterns of these lymphocytes in Fig. 2c. There were no interference patterns associated with phase-light, type I cells demonstrating that these cells were not in direct contact with the glass slide. These observations suggest that a subpopulation of HEC-adherent IL-2-activated lymphocytes migrated across the HEC layer, in a manner analogous to unactivated lymphocytes.

Following IL-2 activation, the number of lymphocytes that migrated across the HEC layer increased significantly (Fig. 1b). Lymphocyte migration was dependent on dose and incubation time in IL-2 as described above for total lymphocyte adhesion. In the presence of 1000 U/ml IL-2 the percentage of migrated lymphocytes increased two-fold after 2 days and four-fold after 3 days of culture. The effects of 100 U/ml IL-2 appeared later. However, the effects of both IL-2 doses were similar after 7 days

up to a maximum increase of six- to eightfold. The proportion of total adherent lymphocytes that had migrated following 60 min incubation was \sim 50% at all incubation times and doses of IL-2.

Phenotypic analysis of HEC-adherent IL-2-activated lymphocytes

Interleukin-2-activated lymphocytes contained a mixture of small and large cells. Following 7 days of culture with 1000 U/ml IL-2, CD4⁺ cells decreased from 50% on Day 1 to 7% on Day 7. CD8⁺ cells increased from 33% to 46% and NK cells from 9% to 39% (Table 1). FACS analysis using two-dimensional forward versus side angle light scatter showed that the arising population of lymphoblasts generated in IL-2 was exclusively composed of CD8⁺ and NK cells and did not contain any CD4⁺ cells (data not shown).

Analysis of IL-2-activated lymphocytes that bound to HEC indicated that the lymphoblasts bound preferentially. HEC-adherent cells were enriched in CD8⁺ cells and NK cells and depleted in CD4⁺ cells (Fig. 3). As previously reported, a subpopulation of IL-2-activated lymphocytes adhered to the culture vessel during their cultivation. FACS analysis of these plastic-adherent cells showed that they were exclusively lymphoblasts (data not shown). Plastic-adherent lymphocytes were also enriched in CD8⁺ and NK cells (Fig. 3). The percentage of CD8⁺ cells was similar in HEC-adherent and plastic-adherent cells at 75% and 80% respectively. The number of cells expressing the NK marker was significantly higher in plastic-adherent cells than in HEC-adherent cells (89% and 39%). The total sum of CD8⁺ and 3.2.3⁺ cells was higher than 100% in both populations which suggests that some IL-2-activated lymphocytes expressed both markers. Further analysis of HEC-adherent and plastic-adherent cells showed that both populations contained between 40 and 50% CD3⁺ T cells (data not shown).

Mechanism of the interaction between IL-2-activated lymphocytes and HEC

In the following experiments a radioisotope assay was used to measure total lymphocyte adhesion to HEC. Previous studies have shown that the microscope assay and the radioisotope assay are comparable methods to measure the adhesion of lymphocytes to HEC.²³ Previous reports have characterized the mechanism of adhesion of thoracic duct lymphocytes and lymph node cells to HEC.^{17,19} In this study freshly isolated LNC were used as a reference population of unactivated lymphocytes with which to compare the behaviour of IL-2-activated lymphocytes. The behaviour of unactivated lymphocytes in this model was independent of their source; lymphocytes harvested from lymph nodes, spleen and peripheral blood of the same animal bound equally well to cultured HEC (data not shown).

Role of adhesion structures in lymphocyte adhesion to HEC

Saturating doses (which were determined in separate assays to be 50 μ g/ml) of the following mouse anti-rat mAb were used: anti-CD11a, anti-CD18, anti-ICAM-1, anti-TcR- $\alpha\beta$, anti-CD2, anti-CD8 and anti-MHC class I. The anti-human VLA-4 antibody HP2/1 was also used. CS1 peptide and the inactive control peptide, CS1-C, were used at saturating doses which have been previously determined.¹⁹

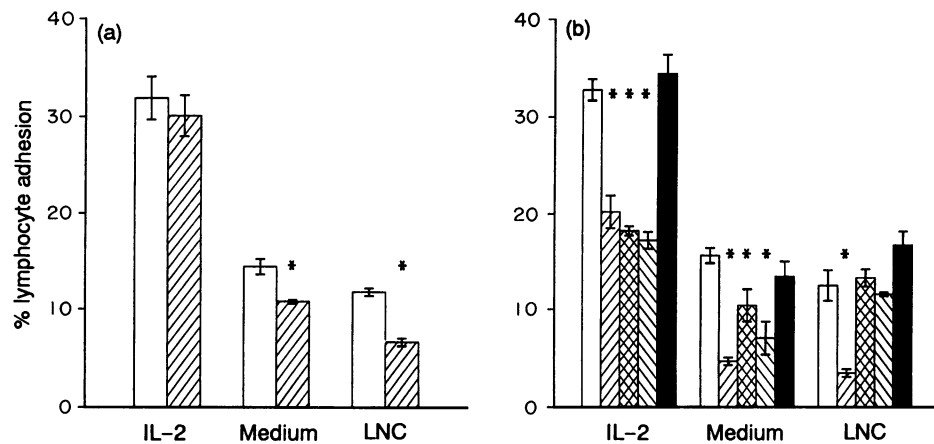


Figure 4. Adhesion blocking assay using CS1 peptide and anti-integrin antibodies. Lymph node cells (LNC) and spleen cells cultured in the absence (medium) or presence of 1000 U/ml IL-2 for 4 days (IL-2) were labelled with [3 H]leucine. (a) Effect of CS1 peptide on lymphocyte adhesion to HEC. Lymphocytes were incubated with HEC for 60 min in the presence of either CS1 (■) or the inactive control peptide CS1-C (□). (b) Effects of anti-integrin antibodies on lymphocyte adhesion to HEC. Lymphocytes and HEC were preincubated with the following mAb: WT.1 [anti-CD11a (■)], WT.3 [anti-CD18 (■)], HP2/1 [anti- α_4 -integrin subunit (■)], MRC-OX8 [anti-CD8 (■)] for 30 min on ice; no mAb (□). Lymphocytes were plated onto HEC in the continued presence of these antibodies for 60 min at 37°. The total number of adherent lymphocytes was expressed as a percentage of the number plated. Values are means \pm SD of quadruplicates of one experiment which is representative for four performed. * $P < 0.05$ with respect to control peptide CS1-C in (a). * $P < 0.05$ with respect to control without antibody (b).

Lymphocytes were resuspended in assay medium containing mAb or peptides and incubated with HEC for 60 min. In the presence of CS1 peptide, the adhesion of both LNC and medium-cultured lymphocytes were decreased significantly compared to the control peptide, CS1-C (Fig. 4a). The inhibition of freshly isolated LNC was consistently higher than that of lymphocytes cultured in the absence of IL-2, at 40% and 25% respectively. In contrast, the binding of IL-2-activated lymphocytes was not inhibited by CS1 peptide. None of the mAb had any inhibitory effect on lymphocyte adhesion when they were included in the assay without prior preincubation (data not shown). Since the known receptor for CS1 peptide is VLA-4, the lack of effect of HP2/1 was likely due to suboptimal effects. To test for maximal effects, lymphocytes and HEC were also preincubated with peptides prior to their inclusion in the adhesion assay. Preincubation with CS1 peptide did not inhibit the adhesion of LNC and medium-cultured lymphocytes further. The adhesion of IL-2-activated lymphocytes was still unaffected by CS1 peptide following preincubation. However, following preincubation of lymphocytes with mAb HP2/1, the adhesion of LNC and medium-cultured lymphocytes were significantly inhibited (Fig. 4b). The inhibitory effects of HP2/1 on LNC and medium-cultured cell adhesion were similar at 70% and 75% respectively. In contrast to the lack of effect of CS1 peptide, HP2/1 also inhibited the adhesion of IL-2-activated cells, however inhibition was lower at 40%. Antibodies to CD11a and CD18 inhibited the adhesion of medium-cultured and IL-2-activated lymphocytes but had no effect on the binding of freshly isolated lymphocytes (Fig. 4b). The effect of anti-CD11a was greater on IL-2-activated lymphocytes than on medium-cultured cells at 45% and 34% inhibition, respectively. Maximal inhibition with anti-CD18 and anti-CD11a antibodies was variable between individual experiments ranging from 25% to 55% inhibition. However, in the same experiment the effects of both mAb were not significantly different, suggesting that the β_2 -integrin-mediated lymphocyte adhesion is an LFA-1-depen-

dent mechanism. The blocking effects of the mAb were not due to non-specific effects since anti-CD8 antibodies, which bind to the majority of HEC-adherent IL-2-activated cells, did not inhibit adhesion (Fig. 4b). In addition, antibodies to MHC class I, which bind to all cells, did not block adhesion of any of the populations tested (data not shown). LFA-1-dependent adhesion was not mediated by ICAM-1 on the HEC, since the anti-functional ICAM-1 antibody, IA29, did not block adhesion either with or without prior incubation of HEC with the antibody (data not shown). Inclusion of antibodies to CD2 and TcR- $\alpha\beta$ also had no effect on the binding of any of the populations tested (data not shown).

Expression of LFA-1 and VLA-4 by IL-2-activated lymphocytes

The previous results provide some evidence for down-regulation of VLA-4/CS1-dependent adhesion and up-regulation of LFA-1-dependent adhesion in IL-2-activated lymphocytes. We have therefore studied the expression of VLA-4 and LFA-1 by IL-2-activated lymphocytes. It was only possible to study expression of the α -subunit of VLA-4 since antibodies against rat β_1 integrin subunit are not available. However, antibodies against both subunits of LFA-1 were used in this analysis.

As shown in Fig. 5, the majority (80–90%) of freshly isolated spleen lymphocytes stained uniformly with the anti-CD11a antibody, WT.1. Neither the number of positive cells nor the fluorescence intensity were changed following culture in the absence of IL-2. Following IL-2 activation, the majority of lymphocytes were also LFA-1 positive, however two populations were readily identified. One showed a similar low level of staining as unactivated lymphocytes (LFA-1^{lo}) and the other expressed higher levels of LFA-1 (LFA-1^{hi}) (Fig. 5). Identical results were obtained using the anti-CD18 antibody, WT.3 (data not shown). Electronic gating by forward versus side angle light scatter indicated that LFA-1^{hi} lymphocytes were exclusively located in the lymphoblast population (data not shown). This

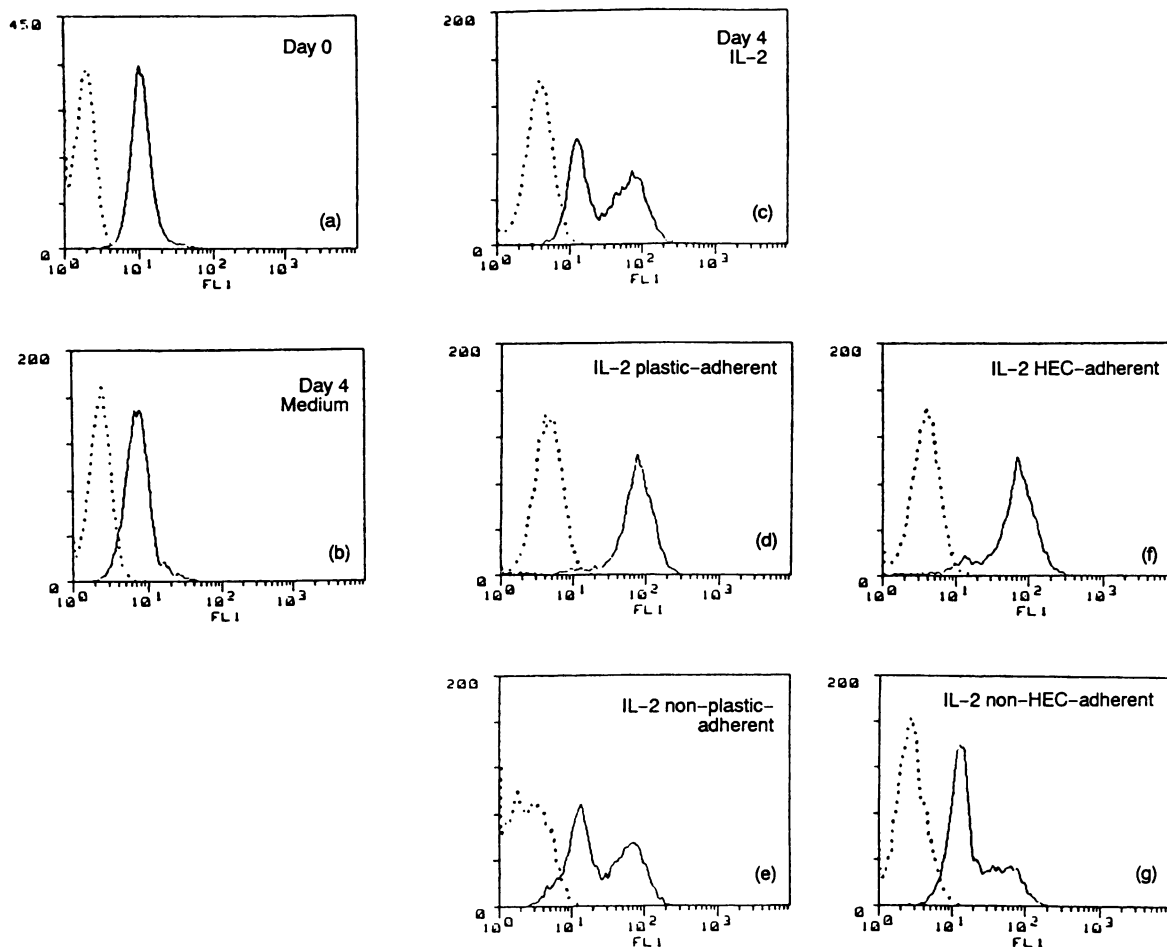


Figure 5. LFA-1 expression by IL-2-activated lymphocytes. Freshly isolated cells (a) and cells cultured in the absence (b) and presence of 1000 U/ml IL-2 for 4 days (c–g) were stained with the anti-CD11a antibody WT.1 (—) or with the non-reactive control antibody, OX21 (· · · ·) and analysed by FACS. The whole population of IL-2-activated lymphocytes (c) was separated into plastic-adherent (d) and non-adherent cells (e). The whole population of IL-2-activated lymphocytes was plated onto HEC for 60 min and HEC-adherent (f) and non-HEC-adherent lymphocytes (g) were analysed. Profiles show log fluorescence (0 to 10^4 channels) on the x-axis and cell number (0–250) on the y-axis.

LFA-1^{hi} lymphoblast population was enriched in both plastic-adherent and HEC-adherent cells and depleted in the non-adherent fractions. LFA-1^{lo} cells were only found among non-adherent lymphocytes (Fig. 5). The majority (80–90%) of fresh isolated spleen cells stained uniformly using HP2/1 which recognizes the α_4 -integrin subunit. In contrast to LFA-1 expression, the expression of a α -subunit of VLA-4 did not alter significantly following IL-2 activation. The staining profiles of medium-cultivated and IL-2-activated lymphocytes using HP2/1 mAb were similar (Fig. 6). Separate analysis of the lymphoblast population showed that staining with HP2/1 was slightly reduced in comparison with unactivated lymphocytes in these cultures (data not shown).

Adhesion of IL-2-activated lymphocytes to different vascular cells

The adhesion of IL-2-activated lymphocytes to plastic suggested that the adhesion mechanisms operating may not be restricted to HEC recognition. The adhesion of IL-2-activated lymphocytes to other types of vascular cell was therefore investigated. IL-2-activated lymphocytes bound to aortic endothelial cells

almost as well as to cultured HEC. However, adhesion to aortic fibroblasts was up to 10 times lower than to endothelial cells (Table 2). Microscopic analysis of IL-2-activated lymphocytes bound to aortic EC revealed both phase-bright and phase-dark cells. Interference reflection microscopy showed that these represented surface bound and migrated lymphocytes respectively (Fig. 2).

DISCUSSION

The accumulation of adoptively transferred IL-2-activated lymphocytes into tumour tissue *in vivo* is dependent not only on their delivery into the tumour blood vessels but also their subsequent transendothelial passage. Previous studies have shown that IL-2-activated lymphocytes preferentially accumulate in the lung, liver and spleen whereas accumulation in tumour tissue is low and non-selective.^{24–26} As yet, it is not clear whether these cells actually enter the tumour itself or only lodge in the vasculature. In the present study we have investigated whether IL-2-activated lymphocytes are able to migrate across vascular endothelium and thus enter target tissue using the model of cultured high endothelium.

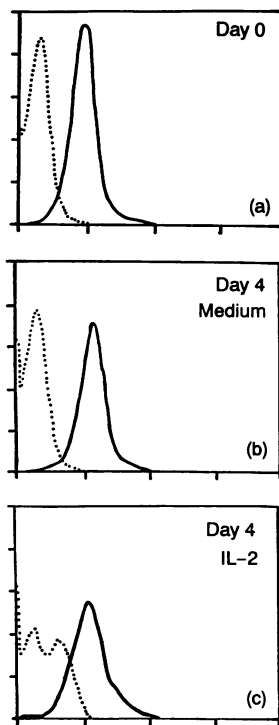


Figure 6. The expression of α_4 -integrin subunit by IL-2 activated lymphocytes. Freshly isolated cells (a) and cells cultured in the absence (b) and presence (c) of 1000 U/ml IL-2 for 4 days were stained with the anti- α_4 -integrin subunit antibody, HP2/1 (—) or with the non-reactive antibody OX21 (·····) and analysed by FACS. Profiles as given in legend to Fig. 5.

Table 2. Adhesion of IL-2-activated lymphocytes to different vascular cells. Spleen lymphocytes cultured in the presence of 1000 U/ml IL-2 for 4 days were labelled with [3 H]leucine and plated on either high endothelial cells, aortic endothelial cells or aortic fibroblasts for 60 min. The total number of adherent lymphocytes was expressed as a percentage of the number plated. Values represent means \pm SD of $n = 10$ observations

	% adhesion/ 50,000 cells	Relative adhesion
High endothelial cells	32 \pm 5	1
Aortic endothelial cells	27 \pm 4	0.84
Aortic fibroblasts	5 \pm 2	0.16

Cultivation of B-cell-depleted lymphocytes from rat spleens in IL-2 induced an active cell proliferation and generated plastic-adherent lymphoblasts and clusters of lymphoblasts in suspension which expressed CD8 and NK cell markers. CD4⁺ cells, which were not detectable in the lymphoblast population, decreased in number during the culture. These results indicate that compared with the human system²⁷ the same lymphocyte subpopulations were preferentially stimulated by IL-2. Previous experiments have shown that the IL-2-activated lymphocytes used in this study expressed high levels of cytotoxicity against tumour cells *in vitro* (G. Pankonin, unpublished observations) indicating that we have actually generated cytotoxic cells. It has

been shown that IL-2-activated lymphocytes can kill allogeneic endothelial cells.^{11,28} Using a syngeneic model we did not observe morphological changes in HEC following short-term (1 hr) interaction with IL-2-activated lymphocytes. In addition, using a 4-hr chromium release assay allogeneic HEC were killed by IL-2-activated lymphocytes whereas syngeneic HEC were relatively poor targets (G. Pankonin and J. T. Vaage, unpublished observations). Thus we have been able to study the cellular interactions involved in transendothelial migration rather than endothelial killing by IL-2-activated lymphocytes.

The increased adhesion of IL-2-activated lymphocytes to syngeneic rat HEC was directly related to dose and incubation time in IL-2. HEC-adherent lymphocytes were a mixture of CD8⁺ cells and NK cells which comprised the blast population. NK cells, which express the CD8 marker, and a small subset of CD8⁺ T cells are known to comprise the plastic-adherent population (also known as adherent lymphokine-activated killer cells or A-LAK cells) in IL-2-activated lymphocytes.¹³ NK cells are the main precursor for tumour-cytotoxic lymphocytes which kill without MHC restriction following IL-2 activation.^{3,27,29} Therefore, our results indicate that both cytotoxic subpopulations of IL-2-activated lymphocytes are able to interact with HEC and preferential adhesion of activated NK cells was not seen. IL-2-activated lymphocytes were also shown to transmigrate the HEC layer. This effect was dependent on dose and incubation time in IL-2 as found for total lymphocyte adhesion. The fraction of adherent cells that transmigrated was similar in all cases suggesting that a high level of lymphocyte adhesion *in vivo* might be a prerequisite for a successful accumulation of lymphocytes in the tumour tissue. Preliminary studies using A-LAK cells in this model have shown that they bind and transmigrate HEC layers *in vitro* to a slightly greater extent than the whole population (G. Pankonin, unpublished observation). Previous studies of the effects of cytokines on cultured HEC have shown that the binding and transmigration of unactivated lymphocytes are independently regulated.²³ The precise microenvironment of the tumour vessel may therefore influence not only whether IL-2-activated lymphocytes bind, but also whether they migrate across the endothelial layer in order to enter the tumour tissue *in vivo*.

Previous reports have suggested that the adhesion of resting and activated lymphocytes to endothelium are mediated via different pathways.³⁰ Most of these studies have used purified CD4⁺ T cells. Since the migration of CD8⁺ cells is regulated independently of CD4⁺ cells,³¹ their adhesive interactions with endothelium should be studied separately. One mechanism by which lymphocytes bind to HEC was recently demonstrated using anti-adhesive synthetic peptides. CS1 peptide, a 25-mer sequence representing the major cell recognition site within the type III connecting segment of fibronectin, was shown to inhibit the adhesion of unactivated lymphocytes to HEC suggesting that lymphocytes can bind to HEC via a fibronectin-related mechanism.¹⁹ The receptor for CS1 peptide is the integrin $\alpha_4\beta_1$,²² also known as VLA-4. Using cultured HEC, CS1 peptide inhibited the adhesion of both freshly isolated lymphocytes and medium-cultured lymphocytes. The adhesion of IL-2-activated lymphocytes to HEC was not inhibited by CS1 peptide. This suggests that the CS1-dependent adhesion pathway is down-regulated in IL-2-activated lymphocytes. The anti-human VLA-4 antibody, HP2/1, stained the majority of freshly isolated rat lymphocytes which suggests that it cross-reacts phenotypically

with rat α_4 .³² HP2/1 also blocked the adhesion of rat lymphocytes to immobilized CS1 peptide and VCAM-1 (M. May and A. Ager, unpublished observations) demonstrating that it cross-reacts functionally with rat VLA-4. Consistent with the inhibitory effects of CS1 peptide, HP2/1 inhibited the adhesion of both freshly isolated and medium-cultured lymphocytes to HEC. The adhesion of IL-2-activated lymphocytes was also inhibited by HP2/1, although the level of inhibition was lower than that seen with medium-cultured cells. The level of HP2/1 staining did not change following IL-2 activation. Together these results suggest that the specificity of lymphocyte VLA-4 is altered following IL-2 activation. VLA-4 on activated lymphocytes could recognize alternative ligands on HEC such as VCAM-1³³ or Hep II domain of fibronectin.³⁴ In fact, Allavena *et al.*³⁵ observed the induction of VLA-4-dependent adhesion between IL-2-activated NK cells and human umbilical vein endothelium following treatment of the endothelial cells with the cytokine IL-1, which is known to induce VCAM-1 expression.³³ Alternatively the α_4 -integrin subunit on IL-2-activated lymphocytes could hybridize with another β -chain, e.g. β_p ,³⁶ to alter its ligand specificity. The absence of antibodies to β_1 and β_p in the rat means that we cannot distinguish between these two possibilities at this stage. It will therefore be important to identify the ligands on HEC for VLA-4 on resting and IL-2-activated lymphocytes. Although we used lymph node cells as a reference population of unactivated lymphocytes our results did not suggest that the behaviour of unactivated lymphocytes from different organs of the rat were significantly different in this model. Previous studies have shown that the *in vivo* migratory behaviour of unactivated lymphocytes harvested from spleen and lymph nodes were similar.³⁷

Another important adhesion molecule on lymphocytes is the β_2 -integrin LFA-1, known to interact with two ligands on endothelial cells, ICAM-1 and ICAM-2.^{38,39} Our results with IL-2-activated lymphocytes demonstrate that LFA-1-dependent adhesion is up-regulated following lymphocyte activation. Antibodies against CD11a and CD18 inhibited the adhesion of IL-2-activated lymphocytes but did not block adhesion of freshly isolated lymphocytes. The majority of lymphocytes expressed LFA-1 and the percentage of positive cells did not change following IL-2 activation. However, the lymphoblast population of IL-2-activated lymphocytes which bound preferentially to both HEC and tissue culture plastic was enriched in LFA-1^{hi} cells and contained few LFA-1^{lo} cells. The high expression of LFA-1 might be one reason for the high adhesiveness of IL-2-activated lymphoblasts. The functional activity of LFA-1 might also be up-regulated by an increased avidity of the LFA-1 molecule as shown previously following lymphocyte stimulation.^{21,38,39} The functional relationship between LFA-1^{hi} expression and plastic adherence of lymphoblasts is unclear. A comparison of the percentages of cells adhering to tissue culture plastic (5–8%) and HEC (15–20%) and the compositions of both populations, suggests that plastic adherence may be a special property of a subpopulation of IL-2-activated lymphocytes that bind to HEC. The observation that the adhesive mechanisms of lymphocytes are changed to a LFA-1-dependent pathway after IL-2 activation are supported by other reports that the increased adhesion of activated lymphocytes to vascular endothelium was mediated via LFA-1.^{21,40,41}

One ligand for LFA-1 is the adhesion molecule ICAM-1. FACS staining of cultured HEC with the ICAM-1 mAb, IA.29,

has shown that the majority of the cells are ICAM-1⁺.²³ Previous studies by Tamatani *et al.*²¹ have shown that the ICAM-1 antibody, IA.29, inhibited the adhesion of mitogen-activated lymphocytes to HEC. The lack of any effect of this mAb in our system suggests that the LFA-1-dependent adhesion occurs via an alternative ligand of LFA-1. This alternative ligand could be ICAM-2 which was shown to be constitutively expressed by vascular endothelium.³⁹ In summary, the VLA-4/CS1-dependent and LFA-1-dependent adhesion pathways are inversely regulated in lymphocytes following IL-2 activation. The results provide some evidence for a biological cell sorting of IL-2-activated lymphocytes on HEC via a functional up-regulation of LFA-1 which is normally expressed on the cell surface.

Our hypothesis that IL-2-activated lymphocytes might pass a barrier of high endothelium to enter the tumour tissue *in vivo* is based on previous observations of HEV-like vessels in some lymphocyte infiltrated tumours. However, we found that IL-2-activated lymphocytes bound to and migrated across non-specialized 'flat' endothelium and therefore had lost the property of unactivated lymphocytes to interact preferentially with high endothelium.¹⁷

In conclusion, the lymphocyte subpopulations known to be the main candidates for tumour-cytotoxic cells in IL-2-activated lymphocytes are able to bind to and migrate across HEC. However, the increased interaction of IL-2-activated cells and endothelial cells was non-specific and was not restricted to HEC. These observations suggest that IL-2-activated lymphocytes *in vivo* may adhere to and migrate across endothelium in different tissues. This type of increased non-specific adhesion of IL-2-activated lymphocytes in connection with their functional properties might account for the dramatic vascular leakage syndrome which is one of the main side-effects in adoptive immunotherapy of tumour patients.

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