Clin Exp Immunol 1996; **105**:112–119

IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes

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(Accepted for publication 1 March 1996)

SUMMARY

Using a quantitative monolayer adhesion assay, the current report shows that treatment of human umbilical vein endothelial cells (HUVEC) with IL-6 increases their adhesiveness for blood lymphocytes, particularly $CD4^+$ cells, but not for polymorphonuclear cells and monocytes. This effect, which was most pronounced when using low concentrations of the cytokine (0·1–1·0 U/ml) and a short incubation period (4 h), was also apparent with microvascular endothelial cells and a hybrid endothelial cell line. Skin lesions from patients with mycosis fungoides contain high levels of IL-6, and blood lymphocytes from patients with this disorder also exhibited an enhanced adhesion to IL-6-treated HUVEC. The cytokine enhanced intercellular adhesion molecule-1 (ICAM-1) expression and induced the expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on endothelial cells. Antibody blocking studies demonstrated that the vascular adhesion molecules ICAM-1, VCAM-1 and E-selectin and the leucocyte integrin LFA-1 all contributed to lymphocyte binding to endothelium activated by IL-6. It is proposed that IL-6 may be involved in the recruitment of lymphocytes into non-lymphoid tissue.

Keywords IL-6 endothelial cells lymphocytes adherence

INTRODUCTION

A characteristic feature of the cutaneous lymphomas (e.g. mycosis fungoides) and psoriasis is the marked propensity of blood lymphocytes for skin [1,2]. Lymphocyte extravasation into non-lymphoid tissue is dependent upon two closely related events: the first is binding to endothelial cells and the second the migration across blood vessel walls, probably in response to a chemotactic stimulus [3]. Particular emphasis is currently focused on identifying cytokines within areas of lymphocyte infiltrates and determining whether they are inducers of lymphocyte margination or migration. Certain cytokines such as tumour necrosis factor (TNF) and IL-1 act on endothelial cells to enhance their adhesiveness for lymphocytes by up-regulating the adhesion molecule intercellular adhesion molecule-1 (ICAM-1), and inducing the expression of others such as vascular cell adhesion molecule-1 (VCAM-1) [4]. Although these vascular adhesion molecules interact with counter-receptors on lymphocytes, they do not specifically promote lymphocyte–endothelial interaction since they also recognize counter-receptors on neutrophils and monocytes [4]. Moreover, cytokines that augment the entry of blood lymphocytes into tissue also possess chemotactic activity for other populations of leucocytes [5]. Consequently, the pathological accumulation of blood lymphocytes has not been ascribed to the action of a particular cytokine.

IL-6 is one of the most pleiotropic of the interleukins released at sites of injury or infection [6]. Elevated levels of IL-6 are present in the skin lesions of patients with mycosis fungoides [7] or psoriasis [8], and in the blood of joint synovial fluids of patients with rheumatoid arthritis [9,10]. It is released in the cutaneous response to allergen challenge in atopic subjects [11], and may be involved in graft-*versus*-host disease [12]. IL-6 is a systemic mediator of the acute-phase response and many of its activities are shared by IL-1 [13,14]. It induces release of acute-phase proteins from hepatocytes, is efficient in proliferating and differentiating T lymphocytes, and plays an essential role in the terminal differentiation of B lymphocytes into plasma cells. Evidence suggesting that IL-6 is implicated in the recruitment of T lymphocytes into inflammatory exudates is sparse. The cytokine is chemotactic for T lymphocytes [15], and there is no firm consensus of opinion concerning its ability to either induce or enhance the expression of adhesion molecules on endothelial cells [16–21]. However, preliminary studies in our laboratory found that IL-6 treatment of endothelial cells enhanced their binding of lymphocytes. We have confirmed and expanded these observations, and this work forms the basis of the present study, in which we show that IL-6 acts on endothelial cells to increase their adhesiveness for lymphocytes from healthy subjects and from patients with mycosis fungoides.

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MATERIALS AND METHODS

Clinical material

Blood samples were provided by healthy members of staff at St Thomas' Hospital. Twenty-three subjects (mean age 36 years) were included in the study, 11 of whom provided blood samples on two occasions. Five samples of blood were donated by patients with mycosis fungoides whose clinical diagnosis was confirmed by histopathological examination of skin biopsies. All patients had plaque-type disease at stage IA [22]. Two patients had received maintenance photochemotherapy 2 weeks before the study and three were applying bland emollients. Their mean age was 57 years and mean duration of disease 14 years.

Isolation and culture of human umbilical vein endothelial cells, bovine retinal microvascular endothelial cells and propagation of an immortalized endothelial cell line

Endothelial cells were isolated from human umbilical vein by our standard protocol [23]. Each vein was treated for 10 min at 37°C with 200 U/ml collagenase (Class II; Sigma, St Louis, MO) solution in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Paisley, UK). The cells were collected by washing out the vein with DMEM plus 10% fetal calf serum (FCS), pelleted at 450 ***g*** for 10 min, resuspended in 5 ml medium DMEM (supplemented with 20% FCS, 2 mM glutamine, 200 U/ml penicillin and 100 U/ml streptomycin) and cultured at 37°C in a 7% CO_2-humidified atmosphere in a 25-cm^2 tissue culture flask (Costar, Cambridge, MA) precoated with 1% (w/v) gelatin (Sigma). When confluent monolayers of endothelial cells had formed the cells were disrupted by 1–3 min incubation with 1 ml 0·05% trypsin and 0·025% EDTA in PBS. The enzyme digestion was arrested by addition of 10 ml DMEM plus 10% FCS. Endothelial cells were pelleted at 450 ***g*** for 10 min, resuspended in medium to 1×10^5 cells/ml and 200 μl added to each well of a gelatin-coated 96-well microtitre plate (Costar). Confluent layers of endothelial cells were obtained after 2–4 days culture at 37°C in a humidified atmosphere of 10% CO_2 in air. Identification of human umbilical vein endothelial cells (HUVEC) was confirmed by immunofluorescent staining with an anti-human factor VIII-related antigen antiserum (Nordic Laboratories, Tilburg, The Netherlands) and by their characteristic morphology.

Bovine retinal endothelial cells were isolated by a modification of the method of Wong *et al.* [24]. Removed retinas were washed and disrupted in a glass homogenizer. Microvessels in the homogenate were trapped on a 50-μm nylon mesh and treated with an enzyme content of 500 μg/ml collagenase, 200 μg/ml pronase (BDH, Poole, UK) and 200 μg/ml DNase (Sigma) for 30 min at 37°C on a rotary shaker. The vessel fragments were seeded onto fibronectin-coated floors of culture flasks. On attainment of confluence, endothelial cells were detached by trypsinization. Confirmation of the identity of endothelial cells was by their morphology and staining with an antibody directed against anti-factor VIII-related antigen. Earlier work in our laboratory had shown that treatment of bovine retinal endothelial cells with human IL-1 and TNF up-regulated the expression of ICAM-1 and E-selectin and increased their adhesiveness for human lymphocytes and polymorphonuclear cells.

The immortalized human endothelial cell line, EA.hy926 [23], was grown in DMEM and hypoxanthine, aminopterin and thymidine (HAT) supplemented with 10% FCS, 2 mM glutamine and 200 U/ml penicillin, 100 μg/ml streptomycin at 37°C in 10% CO_2.

Isolation of blood mononuclear cells and enrichment for lymphocytes and monocytes

Fifty millilitres of heparinized (10 U/ml) blood were diluted with an equal volume of DMEM and layered onto Lymphoprep (Nycomed, Oslo, Norway) at a ratio of one part Lymphoprep to two parts diluted blood. After 30 min centrifugation at 1500 ***g*** the mononuclear cell (MNC) layer was removed and washed twice with DMEM. The MNC were further enriched for lymphocytes and monocytes by density gradient centrifugation on Percoll (Pharmacia, Uppsala, Sweden). Approximately $25–50 \times 10^6$ MNC suspended in 2 ml 30% (v/v) Percoll solution were layered onto a gradient comprising 2 ml aliquots of 40%, 50%, 60% and 70% (v/v) Percoll solution and centrifuged at 450 ***g*** for 30 min. Monocytes and lymphocytes were carefully aspirated from the 40%/50% and 50%/60% interfaces, respectively, washed once with DMEM, then twice more with DMEM plus 10% FCS and adjusted to 1×10^6 cells/ml. The purity of the lymphocyte and monocyte preparations was assessed by differential counting of a cytocentrifuge preparation. Only preparations of >95% purity for lymphocytes and >80% purity for monocytes were used in the adherence assay.

Preparation of blood polymorphonuclear cells

Once the MNC had been collected from the Lymphoprep gradient the remaining plasma and Lymphoprep was aspirated, leaving the pellet containing erythrocytes and polymorphonuclear cells (PMN). The erythrocytes were lysed with 0·83% ammonium chloride at 20°C, the PMN pelleted at 450 ***g*** for 10 min, washed twice with 20 ml Hanks' balanced salt solution (HBSS; GIBCO) and resuspended at 1×10^6 cells/ml. Only PMN preparations of >98% purity, assessed by differential counting of a cytocentrifuge preparation, were used in the adherence assays.

Depletion of B cells, $CD4^+$ and $CD8^+$ lymphocytes

Suspensions of lymphocytes (20×10^6/ml) were incubated for 30 min with magnetizable beads coated with anti-pan B cell antibodies (anti-CD19; Dynabeads, Dynal AS, Oslo, Norway) at a cell-to-bead ratio of 1 : 1 with constant gentle mixing at 4°C. The rosetted B cells were removed magnetically and the remaining cell suspension was adjusted to 20×10^6/ml. Repeating the depletion procedure resulted in the removal of 73% of the B lymphocytes (decrease from 11% to 3%). The remaining T lymphocytes were subsequently depleted of either $CD4^+$ or $CD8^+$ cells using Dynabeads coated with anti-CD4 or anti-CD8 antibodies as described above. Aliquots of blood lymphocytes unfractionated and subset-depleted preparations were always stained using directly conjugated anti-CD4 and CD8 antibodies and analysed using flow cytometry. Lymphocytes (25 μl; 5×10^6/ml) suspended in RPMI with 5% FCS were incubated with 10 μl of PE-conjugated antibodies directed against CD4 and CD8 (Becton Dickinson, San Jose, CA) as well as isotype-matched control antibodies (Becton Dickinson) for 30 min at 4°C. The cells were washed three times and suspended in 0·5% paraformaldehyde in PBS for analysis on a FACScan flow cytometer (Becton Dickinson) using the Lysis II program. Results were expressed as the percentage of positive stained cells in comparison with samples incubated with isotype-matched antibodies of irrelevant specificities. The efficiency of depletion of $CD4^+$ cells was 96% (decrease from 54% to 2%) and for $CD8^+$ cells it was 92% (decrease from 25% to 2%).

Adherence assay

Lymphocytes, monocytes or PMN were suspended in 200 μl DMEM and incubated with 3 μCi (0·1 MBq) of Na_2 $^{51}CrO_4$ (Amersham, Aylesbury, UK)/10^6 cells for 1 h at 37°C. The radiolabelled cells were washed three times with 10 ml DMEM plus 10% FCS and finally resuspended at 1×10^6 cells/ml in DMEM plus 10% autologous serum. Only 96-well microtitre plates with intact, confluent monolayers of endothelial cells were used in the adherence assay. The medium was aspirated from each well and replaced with 200 μl radiolabelled cell suspension (1×10^6 cells/ml in DMEM plus 10% autologous serum). Each test was performed in quadruplicate in randomly allotted wells. After 1 h incubation at 37°C, loosely adherent cells were removed by washing the monolayers five times with 200 μl DMEM, and the endothelial cells plus adherent cells lysed by the addition of 200 μl 0·1 M NaOH/well. The lysate was collected and counted in an auto-gamma counter. The percentage of cells adhering to the endothelial monolayer was calculated as follows:

$$\%\ \text{adherence} = \frac{\text{ct/min in } 200 \mu\text{l lysate}}{\text{ct/min in } 200\, l \text{ original cell suspension}} \times 100$$

To examine the binding of leucocytes to cytokine-treated endothelial cells, monolayers were pretreated for 4 h and 24 h with 0·001–10 U/ml IL-6, 0·1–10 U/ml TNF-α or IL-1β, or 2·5–250 U/ml interferon-gamma (IFN-γ). The concentration of all cytokines is expressed in terms of WHO International Units. Individual wells were then washed once with 100 μl DMEM plus 10% FCS before the addition of radiolabelled cells. Recombinant human IL-6 was the generous gift of Dr W. Sebald (Institute of Physiology, Warzburg, Germany). The recombinant human cytokines TNF-α, IL-1β and IFN-γ were kindly provided by Amgen (USA).

Antibody blocking studies

Lymphocytes, at a concentration of 40×10^6 cells/ml in culture media, were incubated with anti-CD11a MoAbs (equivalent to 25 μg/10^6 cells) for 45 min at room temperature. The cells were washed once before addition to the endothelial monolayers. Endothelial monolayers were treated with MoAbs directed against ICAM-1, VCAM-1 and E-selectin for 45 min at concentrations that were 10-fold greater than that needed to demonstrate the optimal expression of these adhesion molecules by ELISA (see below).

Immunocytochemistry

Approximately 1×10^5 endothelial cells were seeded onto multispot microscope slides (Hendley, Loughton, UK) precoated with 0·1% (w/v) gelatin. The endothelial cells were allowed to adhere for 2–3 h at 37°C before the slide was immersed in media, one slide/plastic Petri dish (Bibby Sterilin, Stone, UK) containing 15 ml media. After 4 days culture at 37°C the slides were air-dried and fixed in acetone for 10 min at 20°C. The slides were incubated with 100 μl of 1 : 20 dilutions of pre-immune rabbit serum for 30 min so as to block non-specific binding of the biotinylated second antibody. Then 50 μl of each dilution of primary MoAb (anti-ICAM-1, -VCAM-1 and -E-selectin) and isotype-matched control antibodies of irrelevant specificity (IgG1; Dakopatts, Glostrup, Denmark) were added to designated wells on each slide. To serve as additional controls, other endothelial cells were incubated in the absence of primary antibody or with diamine benzidine (DAB) alone in order to detect non-specific staining due to inappropriate binding of biotinylated antibody or the presence of endogenous peroxidase. Each well had been circled with a wax pen (Miles, West Haven, CT) to prevent mixing of MoAbs from different wells. After 30 min the slides were washed five times by immersion in Tris-buffered saline (TBS), then incubated for a further 30 min with 50 μl/well of a 1 : 400 dilution of biotin-conjugated second antibody (rabbit anti-mouse immunoglobulins; Dako). The slides were washed as before, then treated for 30 min with 1 μg/ml avidin-peroxidase complex (avidin conjugated with type VI peroxidase; Sigma) in TBS. Finally, the slides were incubated with the substrate (0·5 mg/ml DAB, with 0·01% H_2O_2; both Sigma) for 5 min, washed in running tap water, and counterstained with haematoxylin. All incubations were performed at 20°C. Identification of endothelial cells was confirmed by avidin-biotin-peroxidase staining with antisera directed against human factor VIII-related antigen (Nordic Labs; used at 1 : 200 dilution) using 1 : 5000 dilution of appropriate secondary antibody (biotin-conjugated mouse anti-rabbit immunoglobulins; Sigma). Appropriate control isotype irrelevant antibodies were also used for this avidin-biotin-peroxidase staining.

In separate experiments intact endothelial cell monolayers

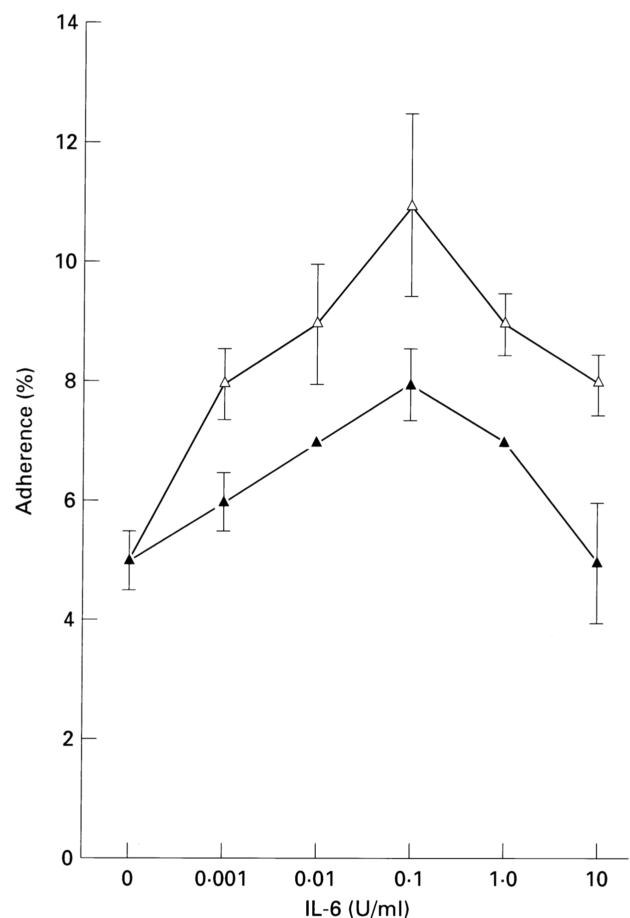

Fig. 1. Adherence of lymphocytes to endothelial cells treated with IL-6. In this representative experiment lymphocytes from a healthy subject were added to endothelial monolayers pretreated with IL-6 for either 4 h (△) or 24 h (▲). The ordinate describes the percentage of adherent lymphocytes and the abscissa the concentration of IL-6 used to treat the endothelial cells. Vertical bars denote the s.d. of the mean adherence from quadruplicate wells. Optimum increase in adherence occurred with 0·1 U/ml IL-6 at 4 h (120% increase; $P<0·001$) and 24 h (59% increase; $P<0·01$).

cultured in 96-well microtitre plates were stained with the anti-ICAM-1, anti-VCAM-1 and anti-E-selectin MoAbs or with isotype-matched antibody of irrelevant specificity as negative control. The plates were washed twice with 100 μl/well PBS and the endothelial cell monolayers fixed for 20 min at 20°C with 0·4% formaldehyde in PBS. After four washings with PBS the cells were incubated for 30 min at 20°C with a range of dilutions of the MoAbs, and the endothelial cells further stained using the avidin-biotin-peroxidase method described above. At the end of the incubation with the avidin-peroxidase label, the plates were washed four times with PBS and 200 μl of substrate (tetramethyl benzidine; Sigma; 100 μg/ml in 0·1 M sodium acetate buffer pH 6·0 plus 0·01% H_2O_2) added/well for 10 min, the colour reaction was stopped with 12·5% H_2SO_4 and photospectrometry (450 nm) performed using a micro ELISA autoreader (Dynatech MR5000). Results were expressed as the absorbance of the test wells at 450 nm minus the absorbance of the negative control wells where the primary antibody had been omitted. All tests were performed in quadruplicate.

The mouse MoAbs directed against ICAM-1 (RR1) and CD11a (R15) were provided by Dr R. Rothlein (Boehringer, Ingelheim, Germany). The $F(ab)_2$ fragments of the mouse MoAb ENA2 which recognizes the ENA1 epitope of E-selectin was the gift of Dr J. Leewenburg (Rijksuniversiteit Limberg, The Netherlands). The anti-VCAM-1 mouse MoAb was purchased from Immunotech (Marseille, France). All antibodies were diluted with 0·05 M TBS pH 7·6 with 0·1% bovine serum albumin (BSA).

Statistical analysis

Results of adherence experiments were expressed as the mean ± s.d. Differences in the binding of lymphocytes to untreated and cytokine-treated endothelium were analysed by the Mann–Whitney *U*-test, whilst the blocking effects of antibody to adhesion molecules on lymphocyte adherence were evaluated by the Wilcoxon signed ranked test.

Table 1. IL-6 enhances lymphocyte adherence to microvascular endothelial cells and a hybrid endothelial cell line

	Percent increase in lymphocyte adhesion			
	Microvascular cells			Hybrid cells
Experiment no.	IL-6 (1 U/ml)	TNF (1 U/ml)	IFN-γ (250 U/ml)	TNF (1 U/ml)
1	35	24	25	43
2	28	35	27	37
3	51	39	35	28
4	43	33	24	
Mean	***39 ± 10	**33 ± 6	*28 ± 5	**36 ± 7

Prior to the assay, microvascular endothelial cells from bovine retinas were pretreated with IL-6 or TNF for 4 h and with IFN-γ for 24 h and the hybrid human umbilical vein endothelial cell (HUVEC) line EA.hy926 was pretreated with IL-4 for 4 h. Results are expressed as the percentage increase in lymphocyte adhesion.

***$P < 0·01$ compared with untreated monolayers;
**$P < 0·02$ compared with untreated monolayers;
*$P < 0·05$ compared with untreated monolayers.

RESULTS

IL-6 treatment of endothelial cells enhances lymphocyte adhesion

Figure 1 shows the results of an experiment in which incubation of HUVEC with IL-6 for 4 h and 24 h produced a dose-dependent increase in the adhesion of lymphocytes from a healthy volunteer. For both incubations, the maximum increase in the adhesion of this subject's lymphocytes occurred when endothelial cells were pretreated with 0·1 U/ml IL-6. However, the increase at 4 h (mean 120% increase; $P<0·001$) was greater than that at 24 h (mean 59% increase; $P<0·01$). When lymphocytes were analysed from an additional six healthy subjects, the largest increase in binding to endothelium treated with IL-6 for 4 h occurred with 0·1 U/ml (mean 77 ± 28%; $P<0·005$) and 1·0 U/ml of the cytokine (mean 64 ± 31% increase; $P<0·02$). Overall, there was no significant increase in the adhesion of lymphocytes from these subjects to endothelial cells pretreated for 24 h with IL-6 (range 0·01–10 U/ml).

To determine if IL-6 also increased the adhesiveness of HUVEC for other leucocytes, enriched preparations of monocytes and PMN, together with lymphocytes, were incubated on endothelial monolayers that had been treated with IL-6 for 4 h and 24 h. For each experiment, samples of PMN, monocytes and lymphocytes

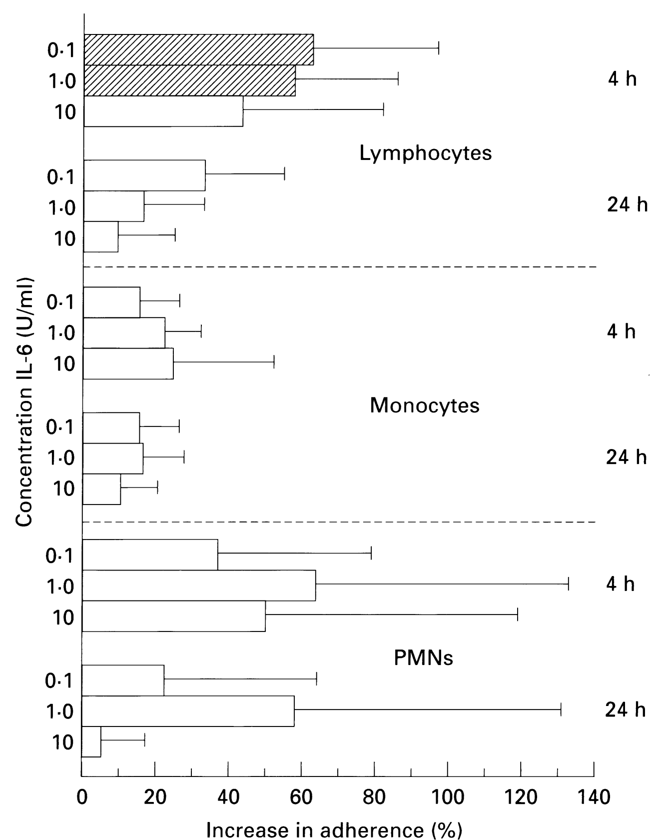

Fig. 2. Comparative binding of lymphocytes, monocytes and polymorphonuclear cells (PMN) to IL-6-treated endothelial monolayers. Results are expressed as the mean percentage increase in adherence of lymphocytes, monocytes and PMN prepared from five normal subjects. All leucocytes were incubated on endothelial monolayers that had been pretreated with IL-6 for 4 h and 24 h. A significant increase in leucocyte attachment ($P<0·01$) occurred only when lymphocytes were added to monolayers treated with 0·1 and 1·0 U/ml of IL-6 for 4 h (▨). Horizontal bars represent s.d. of the mean.

Table 2. Binding of lymphocytes from patients with mycosis fungoides to endothelial cells pretreated with IL-1 or IL-6

	Percent increase in lymphocyte adhesion			
	IL-1 (U/ml)		IL-6 (U/ml)	
Patient	0·1	1·0	0·1	1·0
1	19	41	38	62
2	0	8	8	39
3	14	14	17	42
4	16	50	40	81
5	25	19	6	44
Mean	15 ± 9	26 ± 17	22 ± 16	*†54 ± 18

Endothelial monolayers were pretreated with either IL-1 or IL-6 for 4 h, washed and overlaid with enriched preparations of lymphocytes.

*$P < 0{\cdot}001$ compared with untreated endothelium; †$P < 0{\cdot}001$ compared with endothelium treated with 1·0 U/ml IL-1.

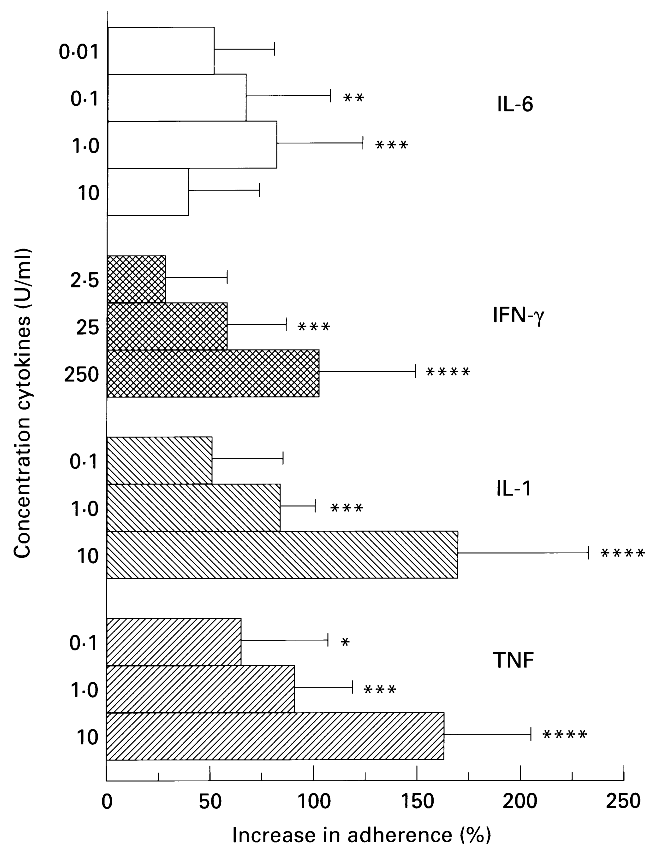

Fig. 3. Binding of lymphocytes to endothelial cells treated with IL-6, IFN-γ, IL-1 or tumour necrosis factor (TNF). Lymphocytes from five healthy subjects were incubated with endothelial monolayers that had been pretreated with IL-6, IL-1 or TNF for 4 h and IFN-γ for 24 h. Results are expressed as the mean percentage increase in adherence. Vertical bars represent s.d. of the mean. *P<0·05; **P<0·02; ***P<0·01; ****P<0·001 compared with lymphocyte attachment to untreated monolayers.

isolated from a normal subject were added to IL-6-treated endothelial cells derived from one umbilical cord vein. In 2/5 experiments, IL-6 enhanced the attachment of PMN, but as shown in Fig. 2 there was considerable variation in the binding properties of these cells. Overall, there was no statistical increase in the adherence of PMN and monocytes to IL-6-treated monolayers. Only the adhesion of lymphocytes was significantly increased to HUVEC pretreated with 0·1 U/ml IL-6 (mean 63% increase; P<0·01) and 1·0 U/ml IL-6 (mean 58% increase; P<0·01) for 4 h.

Since most lymphocytes that infiltrate skin are $CD4^+$, we investigated the binding properties of $CD4^+$ and $CD8^+$ lymphocytes to HUVEC stimulated with IL-6. In three experiments, CD4-enriched lymphocytes exhibited a mean 91% increase (P<0·01) in attachment to IL-6-treated monolayers and CD8-enriched lymphocytes a 54% enhanced adherence (P<0·05).

To investigate whether IL-6 also increased the adherence of lymphocytes to endothelial cells from other sources, monolayers of bovine retinal microvascular cells and the EA.hy926 immortalized HUVEC cell line [23] were included in the study. In four experiments 1 U/ml IL-6 induced a mean 39% increase in the adhesion of human lymphocytes (P<0·01) to microvascular endothelial cells which was comparable with the action of 1 U/ml TNF (Table 1). This table also shows that treatment of EA.hy926 cell lines with IL-6 resulted in a mean 36% increase in lymphocyte adhesion. Pretreatment of the recombinant IL-6 with an anti-IL-6 neutralizing antibody (R&D Systems, Abingdon, UK) abrogated its effect on endothelial cells, and 1 U of our IL-6 preparation was found to contain only 7·5 femptograms of endotoxin. Thus, the adherence-enhancing activity of recombinant IL-6 was unlikely to be due to the effect of a contaminant in the preparation.

Comparison of lymphocyte binding to HUVEC pretreated with IL-6, IFN-γ, IL-1 or TNF

The next stage of the study compared the adherence-enhancing activity of IL-6 with that of other cytokines known to increase the adhesiveness of endothelium for lymphocytes. Each experiment consisted of adding lymphocytes from a healthy subject to endothelial cells, isolated from one umbilical cord vein, pretreated with IL-6, IL-1 or TNF for 4 h and with IFN-γ for 24 h. The concentration and incubation times for IL-1, TNF and IFN-γ were selected on the basis of previous experiments undertaken in our laboratory [23]. The results from five experiments are shown in Fig. 3. The largest increase in adhesion was produced by 10 U/ml IL-1 (mean 172% increase in adhesion; P<0·001) and 10 U/ml TNF (mean 163% increase; P<0·001), both of which were significantly greater (P<0·05) than the optimal adhesion produced by IL-6 (mean 84% increase; P<0·01). Over the concentration of cytokines used, the increase in adhesiveness induced by IL-6 was comparable with that generated by IFN-γ.

IL-6 is present in the skin of patients with mycosis fungoides [12], as are large numbers of infiltrating lymphocytes [2]. Experiments were undertaken to determine whether blood lymphocytes from these patients also responded to IL-6-treated HUVEC. Table 2 shows that incubation of HUVEC with 1·0 U/ml IL-6 significantly increased the binding of lymphocytes from patients with mycosis fungoides and that the enhancing effect was greater than that induced by 1·0 U/ml IL-1 (P<0·001).

Adhesion molecules and the binding of lymphocytes to IL-6-treated endothelial cells

Immunocytochemical studies were performed to investigate

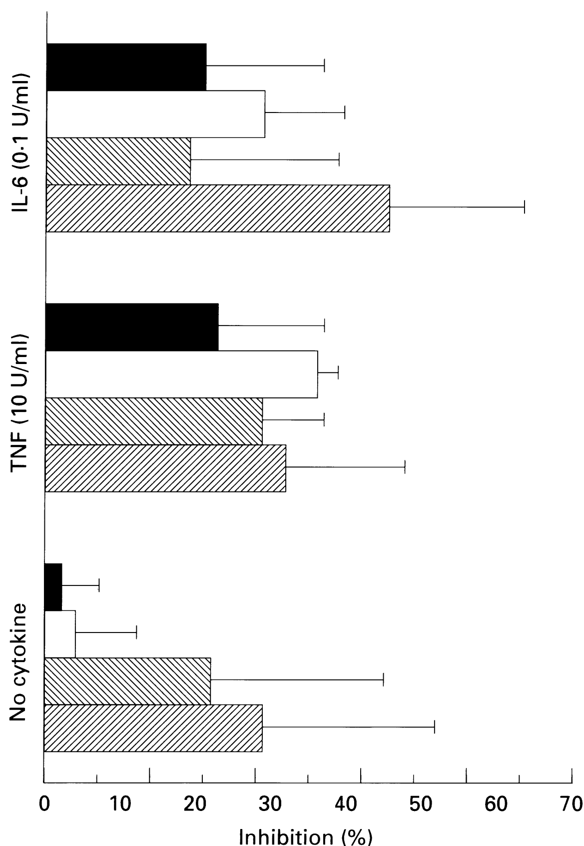

Fig. 4. Effect of anti-adhesion molecule antibodies on the binding of lymphocytes to IL-6-treated endothelial cells. Endothelial monolayers incubated with either 0·1 U/ml IL-6, 10 U/ml tumour necrosis factor (TNF) or diluent culture medium for 4 h were washed and further treated for 45 min with antibodies against E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) before being overlaid with lymphocytes from normal subjects. Also, other preparations of lymphocytes were incubated with anti-LFA-1 antibodies prior to their introduction onto untreated and cytokine-treated endothelial cells. Antibodies used against the four adhesion molecules: ■, E-selectin; □, VCAM-1; ▧, ICAM-1; ▨, LFA-1. Results are from 10 experiments and are expressed as percentage inhibition of adhesion. Horizontal lines represent the s.d. of the mean. Antibodies against E-selectin, VCAM-1, ICAM-1 and E-selectin inhibited adhesion to IL-6- and TNF-treated endothelium ($P<0·01$) and anti-ICAM-1 impeded lymphocyte binding to untreated endothelium ($P<0·05$). Lymphocyte adhesion to all monolayers was inhibited by anti-LFA-1 ($P<0·01$).

whether IL-6 up-regulated the surface expression of ICAM-1, E-selectin and VCAM-1 on endothelial cells. In the first series of experiments the expression of all three adhesion molecules was found to be enhanced on cells cultured on multispot microscope slides. To obtain a quantitative assessment of the up-regulation of these molecules, endothelial monolayers were analysed by ELISA and stimulated for 4 h with 0·1 U/ml IL-6. Resting endothelial cells did not express either E-selectin or VCAM-1. IL-6 increased the optical density (OD) of endothelial cells stained for ICAM-1 (mean OD 0·21–0·47), VCAM-1 (0·06–0·18), and E-selectin (0·02–0·37). The kinetics of this response was very similar to that induced by TNF (10 U/ml).

The contribution of the endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin and the β_2-integrin LFA-1 (CD11a) to the binding of lymphocytes to IL-6-treated endothelial cells (0·1 U/ml for 4 h) was assessed by antibody blocking studies. These experiments were also extended to the study of untreated endothelium and endothelial cells pretreated with 10 U/ml TNF. Figure 4 illustrates the results from 10 experiments, and shows that lymphocyte binding to IL-6-treated endothelium was impeded by antibodies directed against E-selectin (mean 21% inhibition; $P<0·01$), VCAM-1 (mean 29% inhibition; $P<0·01$), and ICAM-1 (mean 19% inhibition; $P<0·01$). Similar results were obtained when lymphocytes were added to TNF-treated endothelium in the presence of anti-E-selectin (mean 23% inhibition; $P<0·01$), anti-VCAM-1 (mean 36% inhibition; $P<0·01$), and anti-ICAM-1 antibodies (mean 29% inhibition; $P<0·01$). With untreated endothelium only the anti-ICAM-1 antibody inhibited adhesion (mean 22% inhibition; $P<0·05$) due to the absence of E-selectin and VCAM-1 from the surface of these cells. Pretreatment of lymphocytes with anti-LFA-1 antibodies inhibited lymphocyte binding to untreated endothelium (mean 29% inhibition; $P<0·01$) and endothelial cells pretreated with IL-6 (mean 46% inhibition; $P<0·01$) and TNF (mean 32% inhibition; $P<0·01$). These findings demonstrate that lymphocyte attachment to IL-6-treated endothelium is dependent upon the expression of E-selectin, ICAM-1 and VCAM-1 on endothelial cells and CD11a on lymphocytes.

DISCUSSION

This study shows that treatment of endothelial cells with IL-6 increases their adhesiveness for lymphocytes, but not for monocytes or PMN. The finding that adhesiveness of endothelial cells for lymphocytes was also increased by the action of IL-1, TNF-α and IFN-γ agrees with previous reports [25]. In contrast to the activity of the other cytokines studied, the enhancing effect of IL-6 was most apparent when using low concentrations and a short incubation period (4 h) with endothelium. IL-6 is known to increase the permeability and proliferation of endothelial cells [26,27], and our findings further implicate the endothelial cell as a target for this potent inflammatory factor [5,6]. The demonstration that IL-6 increased the adhesiveness of HUVEC, a hybrid endothelial cell line, and bovine retinal microvascular endothelial cells shows that its activity extends to other forms of vascular endothelial cells. Dense infiltrates of lymphocytes are present within skin lesions of patients with mycosis fungoides [1,2], and lesional samples contain high levels of IL-6 [7]. When blood lymphocytes from patients with mycosis fungoides were added to endothelial cells treated with IL-6 they also exhibited an increased adhesion to the monolayers which was even greater than that recorded with endothelium treated with a similar concentration of IL-1. However, in an ongoing study we find that blood lymphocytes from patients with mycosis fungoides do not differ from control cells in their adherence to IL-6-treated endothelium or in the distribution of their CD4 : CD8 subpopulations (unpublished observations). Most of the lymphocytes that infiltrate the skin of patients with mycosis fungoides are of helper T cell origin [1,2,28], which may be related to the present finding that $CD4^+$ lymphocytes were highly adherent to IL-6-treated endothelium.

In the current investigation both IL-6 and TNF had a similar kinetic effect on the up-regulation of ICAM-1, VCAM-1 and E-selectin. Antibody blocking studies demonstrated that both E-selectin and VCAM-1 were partly involved in enhancing

lymphocyte binding to IL-6-treated endothelial cells, although the blocking activity of anti-ICAM-1 and anti-CD11a to such monolayers was comparable to that seen with untreated endothelium. Since one or more of these vascular adhesion molecules are recognized to some degree by all leucocyte populations [4], it is surprising that IL-6-treated endothelial cells did not support the binding of all preparations of PMN and monocytes, particularly as TNF activation of endothelium is known to increase the adherence of phagocytic cells [25]. The promotion of lymphocyte adherence by IL-6 could arise from a selective modification in the conformation or phosphorylation status of certain vascular adhesion molecules [29], or from the induction of novel adhesion determinants that are recognized preferentially by lymphocytes. Experiments are planned to investigate these possibilities.

There is conflicting information in the literature regarding the effect of IL-6 upon the expression of endothelial adhesion molecules and the support of leucocyte binding. The cytokine is reported to up-regulate [16–18] or to have no effect [19,20] upon ICAM-1 expression on endothelial cells, and not to induce E-selectin synthesis [21]. IL-6 activation of endothelial cultures derived from bovine brain capillaries increases their adhesiveness for lymphocytes [30], whereas incubation of HUVEC with this cytokine did not promote PMN binding [31]. When used as a chemotherapeutic agent, its injection into the skin of a cancer patient produced a cutaneous eruption associated with an infiltrate of mononuclear inflammatory cells [32]. However, IL-6 did not induce leucocyte infiltration of the skin in transgenic mice [33].

IL-6 is found in skin lesions from patients with mycosis fungoides [7], psoriasis [8,34] and atrophic skin disease [35], and there is uncertainty as to whether IL-6 is present in normal skin [8,33]. Its promotion of lymphocyte adherence in the present study may also have a bearing on the observation that blood lymphocytes from normal subjects bind to dermal endothelia exposed in tissue sections of psoriatic plaques, but not to those of uninvolved psoriatic skin or skin from normal subjects [36]. IL-6 is released from epidermal cells [8], keratinocytes [37] and fibroblasts [38], and the demonstration that IL-6 production by endothelial cells is stimulated by IL-1 [30] or IFN-γ [39] introduces the consideration that IL-6 could be having an autocrine feedback effect on endothelial cell function. An overproduction of IL-6 by fibroblasts from patients with progressive systemic sclerosis is thought to have a profound effect upon the course of this disease [40]. Elevated levels of IL-6 are present in the blood and knee joint synovial fluids of patients with rheumatoid arthritis [9,10] and in the cerebrospinal fluids of patients with inflammatory neurological disease [41]. Common to these clinical disorders is an excessive tissue infiltration by circulating lymphocytes. The ability of IL-6 to promote lymphocyte–endothelial cell interaction combined with its enhancement of lymphocyte migration [15] suggests that this cytokine may have a distinct role in the recruitment of blood lymphocytes into inflammatory lesions.

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