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ENDOTHELIAL LEUKOCYTE ADHESION MOLECULE-1 AND INTERCELLULAR ADHESION MOLECULE-1 MEDIATE THE ADHESION OF EOSINOPHILS TO ENDOTHELIAL CELLS IN VITRO AND ARE EXPRESSED BY ENDOTHELIUM IN ALLERGIC CUTANEOUS INFLAMMATION IN VIVO[1]

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We have compared the adhesion of ^{51}Cr-labeled eosinophils and neutrophils to cultured human umbilical vein endothelial cell (EC) monolayers that have been stimulated with IL-1, TNF, or LPS. Each agent stimulated the adhesion to EC of both eosinophils and neutrophils in a similar dose- and time-dependent manner. F(ab')$_2$ fragments of mAb 1.2B6 (anti-endothelial leukocyte adhesion molecule (ELAM)-1) and mAb 6.5B5 (anti-intercellular adhesion molecule (ICAM)-1) each inhibited partially, and to a similar extent, eosinophil and neutrophil adhesion to EC monolayers prestimulated with TNF (10 ng/ml) for 6 h. Greater inhibition of both eosinophil and neutrophil adhesion was achieved by combining the effects of mAb 1.2B6 with either mAb 6.5B5 or mAb TS1/18 (anti-CD18). These observations indicate that both ELAM-1 and ICAM-1 are involved in the adhesion of eosinophils and neutrophils to EC stimulated with TNF. In order to determine whether these molecules are expressed in vivo during allergen-induced late phase allergic responses in the skin, human skin biopsies were examined at 6 h after Ag or saline challenge with the use of an alkaline phosphatase-staining technique. Both ELAM-1 and ICAM-1 were expressed with greater intensities in Ag-challenged biopsies, suggesting that these molecules may be involved in granulocyte recruitment in vivo. The similarities we have established between mechanisms of eosinophil and neutrophil adhesion to cytokine-stimulated EC suggests that factors other than differential leukocyte-EC adhesion may be responsible for the selective accumulation of eosinophils at sites of allergic inflammation.

The presence of tissue eosinophilia is a feature of many allergic disorders and the eosinophil may be a critical effector cell in the mechanisms of allergic inflammation (1, 2). Thus, an increased understanding of basic mechanisms underlying eosinophil recruitment may have important therapeutic consequences. However, there is only limited information about how eosinophils accumulate preferentially at sites of tissue inflammation.

A major factor involved in leukocyte recruitment into inflammatory tissues is thought to be the expression on vascular EC[3] of cytokine-inducible adhesion molecules for leukocytes (3, 4). There is increasing evidence that a number of EC adhesion molecules are involved, with some acting selectively to preferentially bind particular leukocyte types (5–12). The two adhesion molecules that have been characterized most fully are ICAM-1 (5, 6) and ELAM-1 (7, 8, 10). ICAM-1 is a single chain glycoprotein of the Ig supergene family, which is present on unstimulated EC and on a variety of other cell types including activated fibroblasts, EC, macrophages, and lymphocytes (5). The surface expression of ICAM-1 is up-regulated on EC by IL-1, TNF, LPS, or IFN-τ (13). ICAM-1 is a ligand for the leukocyte adhesion molecule LFA-1 (CD11a/CD18) (14) and is likely to be involved in the adhesion of both neutrophils (15, 16) and lymphocytes (6) to cytokine-activated endothelium.

In contrast to ICAM-1, ELAM-1 is found only on activated EC and its expression is induced by IL-1, TNF, or LPS but not by IFN-τ (13, 17). Experiments with mAb and ELAM-1 cDNA-transfected cells indicate that ELAM-1 is a selective adhesion molecule with the ability to bind neutrophils rather than lymphocytes (7, 8, 10). Blocking studies with mAb indicate that the interaction between ELAM-1 with its as yet unidentified ligand on neutrophils, and between CD11/18 and ICAM-1 are likely to be complementary in their overall effect on neutrophil adhesion to EC (18).

After stimulation with IL-1 or TNF, EC become more adhesive for eosinophils as well as for neutrophils and lymphocytes (19). Although Wegner et al. (20) have recently reported a role for ICAM-1 in cytokine-stimulated eosinophil-EC adhesion, the contribution of other adhesion molecules has not been examined in any detail and, in particular, the involvement of ELAM-1 has not been investigated. In this paper we have compared the capacity

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[3] Abbreviations used in this paper: EC, endothelial cells; ICAM, intercellular adhesion molecules; ELAM, endothelial leukocyte adhesion molecules; VCAM, vascular cell adhesion molecule; GM-CSF, granulocyte macrophage CSF; hpf, high power field.

of eosinophils and neutrophils to adhere to cytokine-stimulated EC in the presence of mAb against ICAM-1 and ELAM-1. In addition, we have assessed the expression of these adhesion molecules on the endothelium of the cutaneous vasculature during the evolution of allergic inflammation.

The model of allergic inflammation that we have studied is the late phase cutaneous response to allergen. Introduction of relevant allergen intradermally into sensitized atopic subjects leads to an immediate wheal and flare response within 5 to 10 min (the immediate reaction). In many individuals the immediate response is followed by the development of a tender and indurated inflammatory cutaneous reaction at 6 to 24 h before gradually disappearing. This late phase reaction is characterized by marked eosinophilia and mononuclear cell infiltration, which is maximal at approximately 6 h after allergen introduction (21).

MATERIALS AND METHODS

Isolation and Culture of EC

EC were obtained by collagenase (type II, Sigma, Poole, U. K.) digestion of human umbilical veins with the use of a modification of a method of Jaffe et al. (22, 23). Cells from different cords were cultured separately at 37°C in 5% CO_2 in EC growth medium consisting of basic medium (RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 15% heat-inactivated FCS (Seralab, Crawley Down, U. K.), 10% heat-inactivated human serum from normal blood donors, 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO)), 5 U/ml sodium heparin (Leo, Princes Risborough, U. K.) and 15 μg/ml EC growth factor (Sigma) in tissue culture flasks (GIBCO) pretreated with 1% tissue culture grade gelatin (Sigma). At confluence, cells were detached and subcultured by using 0.125% trypsin-EDTA (GIBCO) in Puck's saline A (GIBCO) with 10 mM HEPES. At fourth passage confluent EC were removed from culture flasks with 0.125% trypsin-EDTA in Puck's saline A, centrifuged, and resuspended in 15% FCS, 10% heat-inactivated human serum, and antibiotics in RPMI 1640 (basic medium) at a concentration of 2×10^5 cells/ml. Aliquots of 0.2 ml were then cultured overnight in flat bottomed, gelatin-coated 96-well microtiter plates (Costar, Cambridge, MA) resulting in confluent EC monolayers. Cytokine stimulation of the EC was performed by adding 22 μl of cytokine at 10 times the desired final concentration in basic medium to the appropriate wells.

After stimulation for the required length of time the wells were washed twice with warm HBSS and immediately used in the adhesion assays. Cultures were judged to consist of >99% EC by morphologic criteria and by positive staining with the anti-EC mAb EN4 (24), as determined by cytofluorographic analysis. Monocyte contamination was <1%, as demonstrated by negative staining with the monocyte-specific mAb anti-Leu-M3.

Preparation of Leukocytes

Neutrophils. Neutrophils were isolated from heparinized (25 U/ml) peripheral blood. E were sedimented for 45 min by mixing with 0.2 volumes of 6% dextran (Dextran 110, Fisons, Loughborough, U. K.). The white cell rich supernatant was then layered over Ficoll/Hypaque (Lymphoprep; Nycomed, Oslo, Norway) and the neutrophils were separated by density-gradient centrifugation at 350 × *g* for 30 min as described by Boyum (25). E contaminating the neutrophil pellet were removed by incubating for 10 min on ice with lysing solution (10 mM potassium bicarbonate containing 0.15 M ammonium chloride and 0.1 mM EDTA) and washing with HBSS without calcium and magnesium ($HBSS^-$) (GIBCO). The cells were then washed again before radiolabeling. The purity of cells was over 95% as determined by Kimura stain (26) and viability was greater than 95% by trypan blue exclusion.

Eosinophils. Eosinophils were separated from venous blood of mildly hypereosinophilic (>5%) patients by the method of Vadas et al. (27). A total of 100 ml of blood was anticoagulated with 5 ml 0.2 M EDTA and E were sedimented with 6% dextran. The white cell-rich plasma was washed twice by centrifugation at 200 × *g* for 10 min at 20°C in Eagle's MEM with Earle's salts, supplemented with 25 mM HEPES, 2% heat-inactivated FCS (GIBCO), 2 mM L-glutamine (GIBCO), and 3 mg/ml deoxyribonuclease-1 (D5025, Sigma) (washing buffer). The leukocytes were then resuspended in 8 ml washing buffer.

A stock solution of 30% metrizamide (Sigma) was prepared in Tyrode's solution containing 1 mg/ml gelatin (BDH Ltd., Poole, U. K.). Two-milliliter aliquots of 24, 23, 22, 20, and 18% metrizimide were carefully overlayed sequentially in 15-ml conical Falcon tubes, and 2 ml leukocyte suspension applied to the top. Eosinophils were then obtained from 22 to 23% and 23 to 24% interfaces after centrifugation for 45 min at 1200 × *g* at 20°C. The purity of cells from each band was determined by Kimura stain and cells of >90% purity were used in the study.

T cells. T cells were prepared from nonadherent mononuclear cells by rosetting with SRBC as described (28).

Radiolabeling of Leukocytes

Leukocytes were suspended in 150 to 300 μl buffer solution in which they have been prepared and 75 to 150 μCi 51-sodium chromate (Amersham International, Amersham, U. K.) were added. The cell suspension was incubated at 37°C in 5% CO_2 for 1 h for eosinophils and neutrophils or for 1.5 h for T cells, with intermittent shaking. The cells were then washed four times in $HBSS^-$ and resuspended in HBSS at 1×10^6/ml.

Cytokines and Other Reagents

Human rIFN-γ was kindly provided by Biogen (Cambridge, MA). Human rIL-1β was a kind gift from Dr. R. Lewis, (Syntex, Palo Alto, CA). Human rTNF-α (sp. act. 32 U/ng) was a kind gift from Chiron Corporation (Emeryville, CA). GM-CSF and IL-3 were obtained from the National Institute for Biological Standards and Control, Potters Bar, U. K.). Human rIL-4 was purchased from Genzyme, Boston, MA. Human rIL-5 was from Amgen Biologicals, Thousand Oaks, CA. Human rIL-6 was generously provided by Prof. W. Fiers (Rijksuniversiteit, Gent, Belgium). LPS (*Escherichia coli*, serotype 0127:B8) was purchased from Sigma.

mAb

mAb 1.2B6 (anti-ELAM-1, IgG1), mAb 6.5B5 (anti-ICAM-1 (CD54), IgG1) and mAb 1.4C3 (IgG1) were prepared as described (23). mAb 1.4C3 is now known to react with an epitope on VCAM-1 which is not required for T lymphocyte adhesion, as determined by mAb inhibition studies (29). VCAM-1 has previously been shown to be expressed in a similar density on the EC surface as ELAM-1 after IL-1, TNF, or LPS stimulation for 6 h (23). mAb EN4 was purchased from Sanbio, Uden, Holland and was used as a positive control to identify vascular endothelium in tissue sections (24). Tissues were also stained with isotype-matched mAb of irrelevant specificity as negative controls. mAb TS1/18 (anti-CD18, IgG1) and BB7.5 (anti-HLA class I framework, IgG1) were obtained from the American Type Culture Collection (Rockville, MD) and were used at saturating concentrations of protein A-purified protein and ascites, respectively.

Preparation of $F(ab')_2$ Fragments

The protein content of monoclonal ascites fluid was checked at 280 nm and diluted with HPLC buffer (0.2 M sodium dihydrogen phosphate in 0.2% sodium chloride solution, pH 7.4) to 10 mg protein/ml; 0.5-ml aliquots were then passed through a TSK 3000 size exclusion HPLC column at 1 ml/min and 1-ml fractions were collected. The presence of specific Ig in the fractions was identified by ELISA by using IL-1- or TNF-stimulated EC as the solid phase (23). Positive fractions were then pooled, concentrated to 5 mg/ml, and dialyzed against pepsin digestion buffer (20 mM sodium acetate, pH 3.5). A 0.25-ml slurry of immobilized pepsin (Pierce, Chester, U. K.) containing 50% glycerol in 0.1 M sodium acetate, pH 4.5, was washed twice and resuspended in 0.5 ml digestion buffer. One milliliter of Ig solution was then added and incubated for 4 h at 37°C with continuous shaking at 200 rpm. The reaction was then stopped by addition of 100 μl 1 M Tris HCl, pH 8.6, after which the supernatant was dialyzed against 0.5 M glycine, 3 M NaCl, pH 8.9, and passed over a protein A Sepharose CL-4B column (Sigma) to remove undigested Ig and Fc fragments. The $F(ab')_2$ preparations were then dialyzed against PBS twice and finally against HBSS. The purity of the resulting $F(ab')_2$ preparation was checked by ELISA (23) and by double immunogel-diffusion (Ouchterlony) with the use of anti-whole IgG (Serotec, Kiddlington, U. K.) and anti-Fc (Binding Site, Birmingham, U. K.) as second antibodies.

Leukocyte-EC Adhesion Assays

EC at confluence were removed from culture flasks with 0.125% trypsin-EDTA and resuspended in 15% FCS, 10% human serum in

RPMI 1640 (basic medium) at a concentration of 2×10^5 cells/ml. Aliquots (0.2 ml) were then cultured overnight or longer in flat bottomed gelatin-coated 96-well microtiter plates (Corning, Corning, NY), resulting in confluent monolayers, as determined by phase contrast microscopy. Cytokine stimulation of EC monolayers was conducted by addition of 22 μl cytokine solution in basic medium at 10 times final concentration.

After washing EC with HBSS, 200 μl leukocyte suspension containing 2×10^5 cells were added to each assay well and incubated for 30 min at 37°C in 5% CO_2 to allow cells to settle. Nonadherent cells were then washed twice with HBSS by gentle aspiration and replacement of the medium. The cultures were then checked by phase-contrast microscopy to confirm leukocyte-EC adhesion and the integrity of the EC monolayers. After aspiration of the medium, the cells were lysed by addition of 200 μl 1% Nonidet P-40 (BDH Ltd.) for >10 min. One hundred-μl aliquots were taken from each well and radioactivity was counted in a gamma counter (LKB 1260 Multigamma). The percent adhesion of leukocytes was calculated as (cpm in 100 μl of lysate ÷ cpm in 100 μl of original leukocyte suspension) × 100.

mAb were titrated in preliminary experiments by ELISA (1.2B6, 1.4C3, and 6.5B5) or flow cytometry (BB7.5, TS1/18) and subsequently used in adhesion assays at 10 times saturating concentrations. In order to test the effects of mAb directed against EC (1.2B6, 6.5B5, and 1.4C3), EC monolayers were preincubated with 100 μl of mAb for 15 min before addition of 100 μl ^{51}Cr-labeled leukocyte suspension. In experiments designed to test the effect of anti-CD18 (mAb TS 1/18), leukocytes were preincubated with mAb for 15 min and transferred in the continuous presence of mAb to the EC monolayers. The percentage inhibition by mAb of the enhanced adhesion of leukocytes attributable to cytokine stimulation of EC was calculated as follows: % inhibition = 1 − (δ in presence of mAb ÷ δ in control without mAb) × 100, where δ = % leukocyte-EC adhesion in cytokine-stimulated cultures − % leukocyte-EC adhesion in control cultures without stimulation. Test and control samples were performed in triplicate unless otherwise stated.

Atopic Subjects

We studied atopic human subjects aged between 18 and 50 years, who were known to have late phase responses to timothy pollen or house dust mite allergens when injected intradermally. Subjects with any medical disorder other than allergic asthma and rhinitis were excluded, as were individuals who had ever received injection immunotherapy or who were currently taking oral medication. The studies were approved by the Ethical Committee of Guy's Hospital and the study was performed with each volunteer's informed consent.

Skin Testing

Lyophilized extracts of *Dermatophagoides pteronyssinus* or *Phleum pratense* were kindly supplied by ALK Laboratories, Copenhagen, Denmark (courtesy of Dr. Henning Lowenstein). The lyophilized extracts (1 million sq. U) were reconstituted in 1 ml of ALK diluent (isotonic solution with 0.3 mg/ml human serum albumin and 0.5% phenol) and then diluted to 12,000 sq. U/ml, 1,200 sq. U/ml, or 400 sq. U/ml with the same diluent. One batch of allergen was used throughout the study. On the subject's arrival in the clinic, skin prick tests were carried out with concentrated grass pollen and house dust mite Ag and diluent control. The mean diameter of the wheal and flare were recorded at 15 and 20 min after each test. Ag was then injected intradermally into the nondominant supinated forearm. The concentration of the Ag employed for intradermal injection was determined by the size of the reaction to the prick test. If the prick test was less than 8 mm in diameter, 0.2 ml of the 12,000 sq. U/ml solution was administered; if the prick test wheal was between 8 and 10 mm, 0.02 ml of the 12,000 sq. U/ml solution was given, and if the prick test wheal was greater than 10 mm, 0.02 ml of the 400 sq. U/ml solution was injected. The control solution was injected at a site approximately 7 cm below the Ag injection. Perpendicular diameters of the wheal and flare responses were measured at 10, 15, 20, and 30 min after the skin test and the size of late response was measured at 4 and 6 h after injection. The diameters for each response were summed and then averaged to give an indication of size of response. After 6 h the allergen and control sites were both biopsied.

Skin Biopsies

The biopsy site was first anesthetized with 1% lignocaine without epinephrine. The biopsies were performed by using a 4-mm punch in all subjects at 6 h after the intradermal injection of either Ag or control. After biopsy the wound was closed with one suture to facilitate healing. The biopsies were divided into two: one specimen was immediately placed into liquid nitrogen and stored −70°C before processing. The other biopsy was placed in 10% formal saline for 24 h and embedded in paraffin.

Histology

Paraffin-embedded biopsies were sectioned and stained with hematoxylin and eosin. Sections were coded and read blind by investigaters who had no involvement with the clinical portion of the study. Cell counts were performed of extravascular eosinophils, neutrophils, and mononuclear cells, based upon standard histologic appearances. The number of cells in each biopsy was counted per 10 hpf (using an Olympus BH-2 microscope, Olympus WHK 10x/20L eyepiece, 1.25 × 10 and an Olympus SPlan 40.0.70 160/0.17 objective) of papillary and mid-dermis covering an area of 1.0 sq. mm. Cryostat sections (5 μ) were cut and allowed to dry before fixing in acetone for 10 min. Sections were then stored in sealed containers at −70°C until used. Sections were stained by sequential incubation with primary mAb from culture supernatant, biotin-conjugated rabbit anti-mouse Ig, and streptavidin-biotin-alkaline phosphatase complex (Vector, Burlingame, CA). They were then developed with Vector Red with 125 mM levamisole included in the substrate to inhibit nonspecific tissue alkaline phosphatase activity. Sections were counterstained with Mayer's hematoxylin, dehydrated with alcohol, and mounted. A 5-point scale (0 to 4) was used to document the changes in global extent and intensity of Ag expression in the cryostat sections, as previously described for ELAM-1 by Messadi et al. (30) and ICAM-1 by Leung et al. (31).

Statistics

Differences between values in the leukocyte-EC adhesion assays were analyzed by using Student's *t*-test. Differences in Ag expression between Ag and paired control biopsies were compared by using the Wilcoxon test for paired differences. Correlations between the different Ag and leukocyte types in Ag and control biopsies were performed by using Spearman's rank correlation.

RESULTS

Comparison of cytokine-induced adhesiveness of EC for eosinophils and neutrophils. After stimulation for 6 h with either IL-1, TNF, or LPS, EC monolayers showed a dose-related enhancement of adhesiveness for eosinophils (Fig. 1). The dose-response relationship for eosinophils was found to be similar to that for enhanced neutrophil adhesion, with optimal enhancement of adhesion occurring with 10 U/ml IL-1, 10 ng/ml TNF, and 1 to 10 μg/ml LPS (Fig. 1). When the kinetics of enhanced EC adhesiveness for eosinophils were compared with those for neutrophils, a similar time-course was also observed. Although for IL-1 and LPS stimulation there was a tendency for EC adhesiveness for eosinophils to be maximal at 8 h rather than at 4 h and for the effect with all cytokines to decay more slowly for eosinophils than for neutrophils (Fig. 2), these differences were not statistically significant.

Preincubation of EC with IL-4 (200 U/ml) or IFN-γ (250 U/ml) did not lead to any increase in adhesion of eosinophils or neutrophils, whereas both cytokines enhanced the adhesion of T cells. In three experiments, 200 U/ml IL-4 increased the adhesion of T cells to EC by an average of 43% ($p < 0.01$), 56% ($p < 0.001$), and 26% ($p < 0.02$) at 4, 8, and 24 h, respectively, and 250 U/ml IFN-γ increased T cell adhesion by a mean of 71% ($p < 0.01$) and 116% ($p < 0.01$) at 4 and 24 h, respectively. Other cytokines that had no detectable effect on the adhesiveness of EC for eosinophils, neutrophils, or lymphocytes after stimulation of EC for 4 to 72 h included IL-3 (0.25 to 250 U/ml), IL-5 (0.01 to 10 U/ml), IL-6 (0.50 to 500 U/ml), and GM-CSF (0.5 to 500 U/ml) (data not shown).

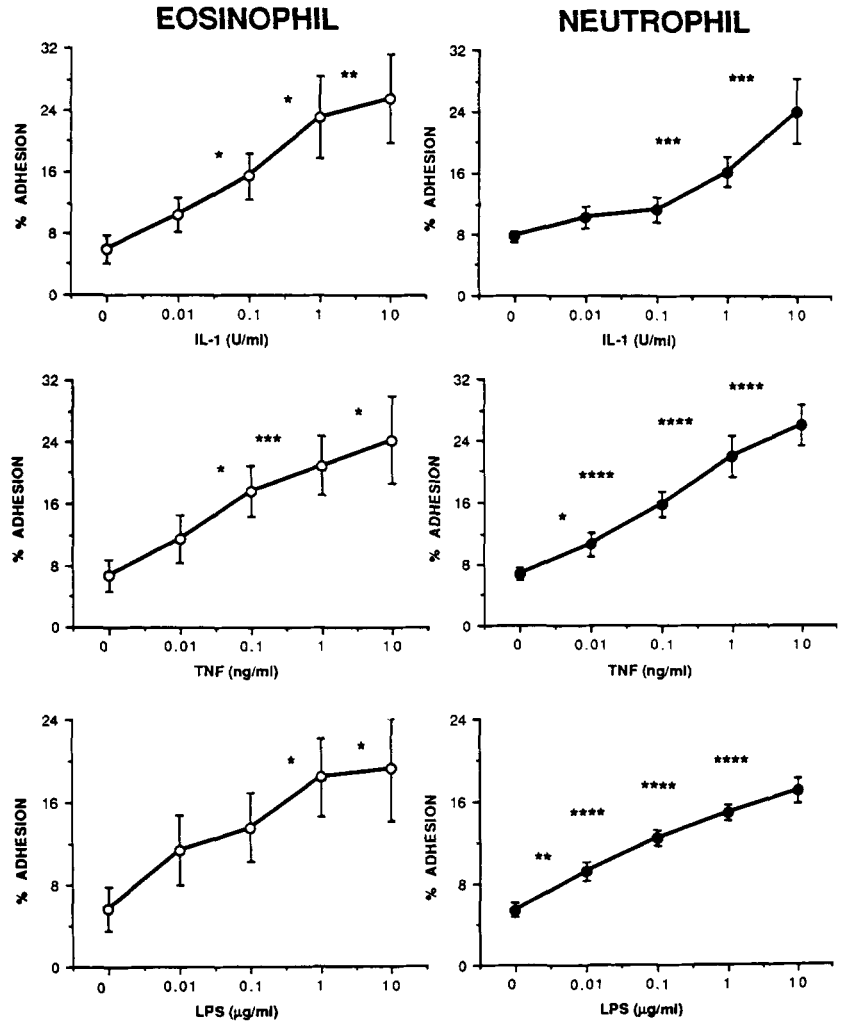

Figure 1. Dose responses of IL-1-, TNF-, or LPS-enhanced EC adhesiveness for eosinophils or neutrophils. EC cultures were preincubated with varying concentrations of IL-1, TNF, or LPS for 6 h and washed before addition of ^{51}Cr-labeled leukocytes. Probability values compared with unstimulated control EC cultures were * < 0.05, ** < 0.02, *** < 0.01, and **** < 0.0001.

The effects of anti-ELAM-1 and anti-ICAM-1 mAb on eosinophil adhesion to EC monolayers. In six experiments, F(ab')$_2$ fragments of anti-ELAM-1 (mAb 1.2B6) and anti-ICAM-1 (mAb 6.5B5) inhibited the adhesion of eosinophils to EC prestimulated with TNF (10 ng/ml) for 6 h, whereas mAb 1.4C3 had no inhibitory effect. The overall inhibition with mAb 1.2B6 and mAb 6.5B5 of enhanced eosinophil adhesion caused by TNF stimulation of EC was 42.3 ± 11.6% (mean ± SEM) and 35.1 ± 9.5% (mean ± SEM), respectively, each being significant ($p < 0.001$) compared with mAb 1.4C3.

Having established that F(ab')$_2$ preparations of mAb 1.2B6 (anti-ELAM-1) or mAb 6.5B5 (anti-ICAM-1) were able to each partially inhibit the adhesion of eosinophils to TNF-stimulated EC, we examined the effects of applying the mAb in combination. As shown in a representative experiment (Fig. 3), stimulation of EC with TNF enhanced eosinophil adhesion from a basal level of approximately 7% to 43%. Preincubation of EC with either mAb 1.2B6 or mAb 6.5B5 partially inhibited eosinophil adhesion to 27% and 28%, respectively, whereas preincubation of EC with mAb 1.4C3 did not alter eosinophil adhesion to EC. Preincubation of EC with both mAb 1.2B6 and mAb 6.5B5 led to greater inhibition of eosinophil adhesion to TNF-stimulated EC than when either mAb was used in combination with the control mAb 1.4C3 ($p < 0.02$, $p < 0.001$, respectively).

Despite the greater inhibition seen when anti-ELAM-1 (mAb 1.2B6) and anti-ICAM-1 (mAb 6.5B5) were used in combination rather than alone, eosinophil adhesion to TNF-stimulated EC was still not abolished to basal levels. Although ICAM-1 is known to act as a ligand for LFA-1 (CD11a/CD18) and possibly also Mac-1 (CD11b/CD18), functional components of the CD11/CD18 complex on eosinophils might react with other molecules on TNF-activated EC apart from ICAM-1. To investigate this possibility we tested the effect of mAb TS1/18, a mAb directed against CD18, the common β-chain shared by CD11a, CD11b, and CD11c, on eosinophil adhesion. In a preliminary experiment, the adhesion of eosinophils or neutrophils to TNF-activated EC was not influenced by incubation with a control IgG1 mAb BB7.5 directed against the HLA class I framework (data not shown). In subsequent experiments, therefore, the adhesion in the presence of anti-CD18 mAb (TS 1/18) was compared with control cultures incubated without mAb. As can be seen in Figure 3, inclusion of mAb TS1/18 in the adhesion assays leads to significant ($p < 0.001$), but only partial, inhibition of eosinophil adhesion to TNF-stimulated EC. Thus, in three experiments mAb TS 1/18 inhibited eosinophil adhesion to TNF-stimulated EC by 53.2 ± 4.0% (mean ± SEM). In these experiments the overall degree of inhibition caused by anti-CD18 was not significantly greater than that seen with anti-ELAM-1 or anti-ICAM-1

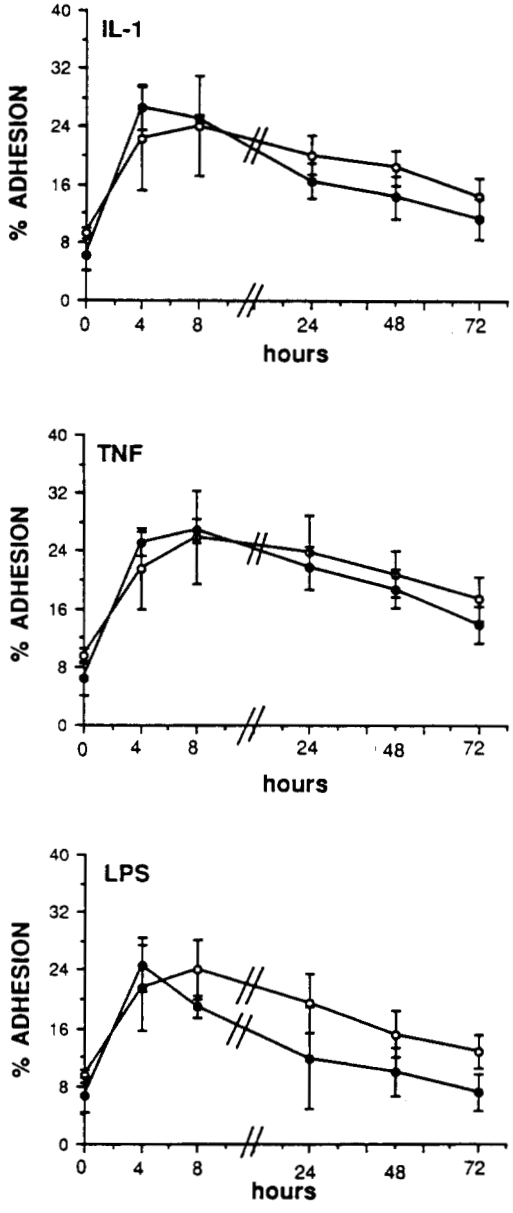

Figure 2. Kinetics of IL-1-, TNF-, or LPS-enhanced EC adhesiveness for neutrophils (●——●) or eosinophils (○——○). EC were preincubated with IL-1 (10 U/ml), TNF (10 ng/ml), or LPS (1 μg/ml) for varying times and washed before addition of ^{51}Cr-labeled leukocytes.

mAb.

When mAb TS1/18 (anti-CD18) was combined with the F(ab')$_2$ preparation of mAb 1.2B6 (anti-ELAM-1), additive effects were observed. In three experiments this combination of mAb resulted in significantly more inhibition of adhesion to EC than anti-ELAM-1 alone ($p < 0.01$) or anti-CD18 alone ($p < 0.01$), and the adhesion of eosinophils to TNF-stimulated EC was reduced to that of adhesion to unstimulated EC (Fig. 3). In contrast, the addition of either anti-ICAM-1 (not shown) or control mAb 1.4C3 (Fig. 3) conferred no greater inhibitory activity than anti-CD18 alone.

Effects of anti-ELAM-1 and anti-ICAM-1 mAb on neutrophil adhesion to EC monolayers. F(ab')$_2$ fragments of either anti-ELAM-1 (mAb 1.2B6) or anti-ICAM-1 (mAb 6.5B5) partially inhibited the adhesion of neutrophils to EC prestimulated with IL-1 (10 U/ml) or TNF (10 ng/ml) for 6 h. In 16 experiments, mAb 1.2B6 and mAb 6.5B5 inhibited neutrophil-EC adhesion enhanced by TNF by 50.2 ± 5.3% (mean ± SEM) and 45.9 ± 4.1% (mean ± SEM), respectively. In contrast a F(ab')$_2$ preparation of mAb 1.4C3 had no inhibitory activity (−5.1 ± 8.7%, mean ± SEM). When compared with the effects of control mAb 1.4C3, the overall inhibition of neutrophil-EC adhesion by mAb 1.2B6 and 6.5B5 were highly significant ($p < 0.001$ for each mAb).

We next examined the effect on neutrophil adhesion to EC of F(ab')$_2$ preparations of mAb 1.2B6 or mAb 6.5B5 in combination. As shown in a representative experiment (Fig. 3), stimulation of EC with TNF enhanced neutrophil adhesion from a basal level of approximately 2% to 49%. Preincubation of EC with either mAb 1.2B6 (anti-ELAM-1) or mAb 6.5B5 (anti-ICAM-1) partially inhibited neutrophil adhesion to 32% and 37%, respectively, with preincubation of EC with the control mAb 1.4C3 having no inhibitory effect. Preincubation of EC with both mAb 1.2B6 and mAb 6.5B5 led to greater inhibition of neutrophil adhesion to TNF-stimulated EC than when either mAb was used in combination with control mAb 1.4C3 ($p < 0.02$, $p < 0.001$, respectively). As can be seen in Figure 3*B*, inclusion of mAb TS 1/18 in the adhesion assays lead to significant ($p < 0.001$), but only partial, inhibition of neutrophil adhesion to TNF-stimulated EC, with neutrophil adhesion being inhibited by 47.9 ± 5.6% (mean ± SEM, five experiments). As with eosinophil adhesion, the overall degree of inhibition of neutrophil-EC adhesion caused by anti-CD18 was not significantly greater than that seen with anti-ELAM-1 or anti-ICAM-1 mAb.

In three experiments, anti-CD18 (mAb TS1/18) was combined with the F(ab')$_2$ preparation of mAb 1.2B6 (anti-ELAM-1). This combination of mAb resulted in significantly more inhibition of neutrophil adhesion to TNF-stimulated EC than anti-ELAM-1 alone ($p < 0.01$) or with anti-CD18 alone ($p < 0.01$), with the level of neutrophil-EC adhesion being reduced by the combination of mAb to the level of adhesion to unstimulated EC (Fig. 3). In contrast, the addition of either anti-ICAM-1 (not shown) or control mAb 1.4C3 (Fig. 3) was not more inhibitory than TS1/18 alone.

Enhanced expression of ELAM-1 and ICAM-1 on endothelium in dermal blood vessels in allergic inflammation in vivo. The stimulation by IL-1 or TNF of both eosinophil and neutrophil adhesion to EC, together with the inhibitory effects of anti-ELAM-1 and anti-ICAM-1 mAb, suggested that induction or up-regulation of ELAM-1 and ICAM-1 on endothelium in dermal venules might be an important mechanism for recruitment of eosinophils and neutrophils into the tissues during the late phase response. We therefore looked for expression of these molecules in human skin at 6 h after the injection of allergen intradermally.

The size of the wheal and flare responses in the early reaction after intradermal introduction of Ag was 14.3 ± 1.1 mm and 48.5 ± 4.8 mm (mean ± SEM, $n = 12$), respectively. The respective sizes after intradermal introduction of saline were 1.9 ± 6.7 mm and 4.9 ± 2.7 mm. The size of the late reaction after Ag challenge was 41.6 ± 8.6 mm, whereas there was no late response after saline challenge.

When compared with the control response to saline, the development of the late phase reaction at 6 h was

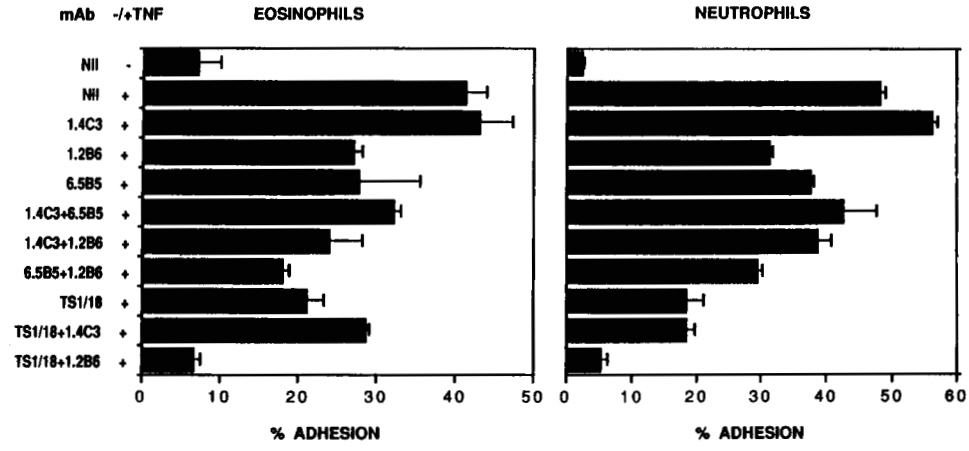

Figure 3. Effects of mAb on the adhesion of eosinophils (*left*) and neutrophils (*right*) to EC. EC monolayers were preincubated with TNF (10 ng/ml) or medium control for 6 h before assessment of the inhibitory effects of mAb, either alone or in combination (see *Materials and Methods*). The final concentrations of mAb used in the experiments were 1/200.

accompanied by substantial infiltration with eosinophils, as detected by the staining of paraffin-embedded sections (mean increase in eosinophils 199-fold ($p < 0.001$) (Table I). There was a smaller (two- to fourfold) increase in both neutrophil ($p < 0.02$) and mononuclear cell ($p < 0.05$) numbers in the same hematoxylin and eosin-stained sections.

When frozen sections of the biopsies were examined for the expression of ELAM-1 and ICAM-1 by vascular endothelium, there was a significant increase in the staining of vascular endothelium by mAb 1.2B6 (ELAM-1) ($p < 0.01$) and by mAb 6.5B5 (ICAM-1) ($p < 0.02$) but not by mAb 1.4C3 (VCAM-1) after intradermal injection of Ag (Table I), with expression of ELAM-1 showing a positive correlation with the expression of ICAM-1 ($r = 0.8$, $p = 0.003$).

DISCUSSION

Alterations in the EC in response to cytokines may be of critical importance in the interactions of circulating leukocytes with the vessel wall, because changes in the expression of adhesion receptors may regulate the number and type of leukocytes emigrating from the blood into inflammatory tissues (3, 4). In this study we have compared the adhesion of neutrophils to cytokine-stimulated EC with that of other leukocyte types in order to explore the possibility that mechanisms determined to be involved in the adherence of neutrophils or lymphocytes to cytokine-stimulated EC might also be responsible for the binding of eosinophils. Such mechanisms might be responsible for the recruitment of eosinophils into the tissues during the late phase response to allergen.

We have demonstrated that the enhancement of adhesion of eosinophils to IL-1-, TNF-, or LPS-stimulated EC is similar in terms of dose-response and kinetics to that of neutrophils, although there was a tendency for enhanced eosinophil adhesion to peak later and to persist for longer than enhanced neutrophil adhesion. Previous studies comparing the adhesion of neutrophils and T cells to cytokine-stimulated EC have indicated that, although IL-1 and TNF enhance EC adhesiveness for both cell types, the lymphokines IL-4 and IFN-τ have a selective action on EC adhesiveness for T cells without stimulating neutrophil adhesion (28, 32). In this study we found that IL-4 and IFN-τ did not enhance EC adhesiveness for eosinophils, suggesting that the factors controlling EC adhesiveness for eosinophils have more in common with those for neutrophils than those for lymphocytes.

Previous reports have shown that the enhanced adhesion of neutrophils to cytokine-stimulated EC is largely attributable to adhesive interactions between ELAM-1 and its receptor on polymorphonuclear leukocytes and

TABLE I

Cell count of eosinophils, neutrophils, and mononuclear cells (cells/10 hpf) compared with EC expression of ELAM-1, ICAM-1 and VCAM-1[a] in skin biopsies

Patient No.	Eosinophils		Neutrophils		Mononuclear Cells		ELAM-1		ICAM-1		VCAM-1	
	C[b]	A	C	A	C	A	C	A	C	A	C	A
1	0	88	3	23	32	65	0.50	2.75	2.00	3.00	0	0.75
2	0	49	8	13	45	136	0	2.50	1.00	2.75	1	0
3	0	21	0	7	5	160	1.50	2.75			1	0
4	0	29	52	50	7	90	1.00	1.25	1.25	0.75	0	2.00
5	0	31	0	79	65	53	1.00	2.50	0.25	2.00	0.5	1.25
6	1	207	8	161	19	44	0.75	1.75	1.50	1.00	1.00	2.00
7	0	222	25	31	51	65	1.00	2.50	0	2.75	0	3.00
8	2	0	0	0	66	0	1.00	0.75	0	0.75	0.75	1.75
9	0	34	0	2	44	124	0.75	1.25	0.25	1.00	0	0.75
10	1	22	3	10	4	13	0	0	0	0	0	0
11	0	83	0	63	14	38	0.5	3.00	1.00	3.50	0.5	2.25
12	0	0	0	3	4	6	0	1.00	0	1.50	0.25	0.25
Mean	0.33	65.7	8.25	36.8	29.7	66.2	0.66	1.83	0.66	1.73	0.41	1.17
SEM	0.19	21.5	4.47	13.5	6.9	15.0	0.14	0.28	0.22	0.34	0.12	0.30
	$p < 0.001$		< 0.02		< 0.05		$p < 0.01$		$p < 0.02$		NS	

[a] A 5-point scale (0 to 4) was used as previously described by Messadi et al. (30) and Leung et al. (31) for estimating intensity of staining of ELAM-1 and ICAM-1.

[b] C, control biopsy site; A, Ag challenge biopsy site; NS, not significant.

between the neutrophil CD11/CD18 complex and ligands on EC, including ICAM-1 (7, 8, 10, 15, 16, 18). In view of the similarity between TNF, IL-1, and LPS in inducing or up-regulating EC expression of ELAM-1 and ICAM-1 (6, 13, 17), it is likely that each of these agents stimulates EC adhesiveness for neutrophils by similar mechanisms. As our experiments indicated that TNF, IL-1, and LPS were also similar in their effects on EC adhesiveness for eosinophils, we chose to concentrate on the effects of TNF for our comparison of the roles of these molecules in the adhesion of eosinophils and neutrophils.

Consistent with the similarities between the mechanisms of eosinophil and neutrophil adhesion to cytokine-stimulated EC at the cellular level, blocking studies with mAb strongly suggest for the first time that the neutrophil-selective endothelial adhesion molecule ELAM-1 is also able to bind eosinophils. In contrast to the combination of anti-ICAM-1 with anti-CD18 mAb, the combination of anti-ELAM-1 mAb with anti-CD18 mAb inhibited eosinophil adhesion to TNF-stimulated EC additively, suggesting that ELAM-1 does not act as a ligand for CD11/CD18 as previously demonstrated for neutrophils (18).

The observations that molecular interactions other than those between CD11/CD18 and ICAM-1 are involved in eosinophil adhesion to activated EC is in contrast to the results of Wegner et al, who found that anti-ICAM-1 mAb eliminated baboon eosinophil adhesion to 1% glutaraldehyde-fixed, LPS-stimulated umbilical vein EC in vitro (20). However, it is possible that ELAM-1 might be more sensitive to glutaraldehyde fixation than ICAM-1. Moreover, the existence of a CD11/18-independent pathway for adhesion of eosinophils to EC was indicated by the data of Lamas et al., who found that anti-CD18 mAb only partially inhibited eosinophil adhesion to IL-1, TNF, or LPS-stimulated EC (19).

Two further points of discussion derive from the experiments using combinations of mAb to inhibit adhesion. First, the combination of anti-ELAM-1 and anti-CD18 virtually eliminated adhesion of both neutrophils and eosinophils to TNF-stimulated EC. Although we cannot exclude the possibility that other adhesion molecules may be participating, the data indicate that the separate interactions involving ELAM-1 and CD18 are likely to be major components determining both neutrophil and eosinophil adhesion to TNF-activated EC. Second, when used in combination with anti-ELAM-1, anti-CD18 mAb were more inhibitory for both eosinophil and neutrophil adhesion to TNF-activated EC than the combination of anti-ELAM-1 with anti-ICAM-1. This could either be caused by a greater functional effect of mAb directed at leukocyte rather than endothelial determinants, or by the presence of other ligands on EC for CD11/18 molecules. One such molecule may be ICAM-2 (33).

We used mAb 1.4C3 as a control mAb in our inhibition experiments, because previous experiments had shown the 1.4C3 Ag was present on TNF-stimulated EC at 6 h in approximately equal density to ELAM-1 (23). While these experiments were in progress we discovered that the Ag recognized by mAb 1.4C3 is the mononuclear cell-selective EC ligand VCAM-1 (9, 11, 12). 1.4C3 does not inhibit T cell adhesion (29) and is presumably directed at a nonfunctional epitope. Thus, the lack of inhibition of neutrophil or eosinophil adhesion with the use of mAb 1.4C3 does not exclude a role for VCAM-1 in the adhesion of these cells to 6-h TNF-stimulated EC. However, such a role seems unlikely in view of the nearly total inhibition of the adhesion of each of these cell types with the combination of anti-ELAM-1 and anti-CD18. Furthermore, neither eosinophils nor neutrophils showed enhanced adhesion to EC stimulated by IL-4, which is now known to stimulate EC adhesiveness by a mechanism dependent upon the induction of VCAM-1 (29). The determination of any possible role for VCAM-1 in this model will, however, await inhibition experiments with mAb directed at appropriate functional epitopes.

Cultured human umbilical vein EC monolayers have been used in this study and many other studies as a model for leukocyte endothelial cell interactions at inflammatory sites. The appropriateness of this model has been supported by the demonstration of ELAM-1 and ICAM-1 induction or up-regulation on the cutaneous microvasculature during delayed hypersensitivity reactions (34, 35), IL-2 immunotherapy (36), vasculitis (31), and after injection of recombinant cytokines (37). Moreover, Messadi et al. have demonstrated the induction of ELAM-1 on dermal venules during short term organ culture of human skin in cytokine-containing media (30). Our demonstration of increased expression of both ICAM-1 and ELAM-1 in cutaneous allergic inflammation in vivo adds further weight to the argument that these molecules are relevant to inflammatory responses. At 6 h, the maximal time for eosinophil recruitment (21), both ELAM-1 and ICAM-1, but not VCAM-1, showed significantly greater expression on vascular endothelium in cutaneous late phase lesions than in control skin injected with saline. Judging from in vitro studies, the mechanism whereby ELAM-1 and ICAM-1 are induced on vascular endothelium during the late phase response may well involve the local release of cytokines such as IL-1 or TNF, perhaps through the mediation of mast cell degranulation (38).

Although tissue eosinophilia is a characteristic feature of allergic inflammation, the mechanisms for this selective eosinophil migration are still poorly understood. The present study does not elucidate how eosinophils accumulate selectively in the tissues during allergic responses. Our data suggests that the mechanism for this selectivity is not caused by differential eosinophil adhesion to ELAM-1 or ICAM-1 on EC. It remains possible that agents other than IL-1 or TNF may stimulate an eosinophil-selective adhesion mechanism, or that cytokine action on the eosinophil may lead to a selective interaction with adhesion molecules on the EC. It is equally possible that eosinophils may accumulate at allergic lesions as a consequence of a greater capacity to survive in the tissues as compared with neutrophils, perhaps in response to cytokines such as GM-CSF (39, 40).

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