Augmentation of eosinophil degranulation and LTC₄ secretion by integrin-mediated endothelial cell adhesion

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Augmentation of eosinophil degranulation and LTC₄ secretion by integrin-mediated endothelial cell adhesion. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L802–L810, 1999.—We examined the effect of eosinophil ligation to cultured human umbilical vein endothelial cells (HUVECs) in augmenting the stimulated secretion of leukotriene (LT) C₄ and eosinophil peroxidase (EPO). The effects of adhesion were compared before and after specific blockade with monoclonal antibodies directed against eosinophil surface integrins or endothelial counterligands. Adhesion to HUVECs augmented EPO release caused by formyl-methionyl-leucyl-phenylalanine plus cytochalasin B from 403 ± 153 (BSA control) to 778 ± 225 ng/10⁶ cells for eosinophils exposed to interleukin-1α-treated HUVECs (P < 0.05) and also caused a twofold increase in stimulated LTC₄ secretion (P < 0.05). To determine whether augmented secretion resulted directly from adhesive ligation, studies were also performed with paraformaldehyde-treated HUVECs; stimulated secretion of both leukotriene C₄ and granular protein in stimulated human eosinophils. In this study, eosinophils purified by negative immunomagnetic separation were allowed to adhere on cultured monolayers of interleukin (IL)-1α-treated HUVECs, which upregulated both ICAM-1 and VCAM-1 on the endothelial surface. Comparisons were made to adhesive interactions between human eosinophils adhering to HUVECs not treated with IL-1α, and the effect of monoclonal antibodies (MAbs) directed against α₅β₃ and β₂-integrins on the eosinophil surface or anti-VCAM-1 and anti-ICAM-1 on the endothelial surface was examined. Additional studies were performed with paraformaldehyde-treated endothelial cells to ensure that augmented secretion related specifically to adhesive augmentation (14) rather than to metabolic or secretory functions of HUVECs.

We found that both α₅- and β₂-integrins caused adhesive upregulation of stimulated eosinophil secretion and that blockade of either eosinophil or endothelial cell surface adhesion molecules prevented the priming effect on eosinophil secretion. Our data are the first demonstration that adhesion to HUVECs through ligation to α₅β₃ or β₂-integrin on the eosinophil surface causes augmentation of stimulated secretion of both EPO and LTC₄ and that blockade of adhesion molecules on either eosinophils or HUVECs prevents the priming effect on eosinophil secretion.

eosinophils; adhesion molecules; leukotriene C₄; eosinophil peroxidase

Eosinophil migration to the conducting airways is associated with increased bronchoactivity in human asthma. This process is regulated by the sequential binding of adhesion molecules on both the capillary endothelium in the conducting airways [e.g., intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)] and the eosinophil cell surface (11, 16, 23, 24). Prior investigations have shown that selective blockade of either endothelial surface adhesion molecules (7, 8) or integrins on the eosinophil cell surface (7) prevents migration of eosinophils into the conducting airways.

Circulating eosinophils, even in atopic asthmatic subjects, are relatively quiescent before transmigration and do not secrete substantial quantities of bronchoactive eicosanoids or granular proteins (27). Because eosinophil transmigration (8, 11, 16, 23, 24) and adhesion begins at the endothelial surface, this study was undertaken to determine whether adhesive ligation of very late antigen-4 (VLA-4), a β₁-integrin, and/or β₂-integrin with the homologous endothelial surface counterligands that occurs in the first stage of inflammatory diapedesis and transmigration caused priming of stimulated eosinophil secretion. We thus hypothesized that eosinophil binding to human umbilical vein endothelial cells (HUVECs) would cause augmented secretion of both leukotriene (LT) C₄ and granular protein in stimulated human eosinophils. In this study, eosinophils purified by negative immunomagnetic separation were allowed to adhere on cultured monolayers of interleukin (IL)-1α-treated HUVECs, which upregulated both ICAM-1 and VCAM-1 on the endothelial surface (3, 4, 6, 7). Comparisons were made to adhesive interactions between human eosinophils adhering to HUVECs not treated with IL-1α, and the effect of monoclonal antibodies (MAbs) directed against α₅β₃ and β₂-integrins on the eosinophil surface or anti-VCAM-1 and anti-ICAM-1 on the endothelial surface was examined. Additional studies were performed with paraformaldehyde-treated endothelial cells to ensure that augmented secretion related specifically to adhesive augmentation (14) rather than to metabolic or secretory functions of HUVECs.

We found that both α₅- and β₂-integrins caused adhesive upregulation of stimulated eosinophil secretion and that blockade of either eosinophil or endothelial cell surface adhesion molecules prevented the priming effect on eosinophil secretion. Our data are the first demonstration that the process of eosinophil adhesion at the endothelial surface likely is linked directly to the priming of eosinophil secretion of granular protein and bronchoactive LTs during the cellular transmigration that occurs in human asthma.

METHODS

Isolation of Peripheral Blood Eosinophils

Thirty-six mildly atopic nonsmoking subjects were recruited for eosinophil donation. Atopy was defined by criteria used in the University of Chicago (IL) Asthma Center for the National Heart, Lung, and Blood Institute Human Asthma Genetics Project. Subjects were assessed by a standardized history, and only those demonstrating >1.5% peripheral blood eosinophils were used (19, 20, 22). None of the patients had asthma. There was no difference among individuals in the level of stimulated secretion (see RESULTS) on the basis of atopic history. Phlebotomy was performed, and the eosinophil donor's peripheral blood mononuclear cells were isolated with the use of Ficoll-Hypaque centrifugation.
phils were isolated by the negative immunomagnetic selection technique (12, 19, 20, 22). Briefly, an aliquot of buffy coat was collected from 120 ml of peripheral blood, overlaid onto 1.089 g/ml of Percoll solution, and centrifuged at 1,000 g for 20 min at 4°C. The upper layer, mononuclear cells, and remaining Percoll solution were discarded. The pellet containing granulocytes was separated from contaminating red blood cells by treating the cells with 20 ml of ice-cold double-distilled water, pH 7.40, for 30 s followed by 20 ml of 2× calcium-free Hanks’ balanced salt solution (HBSS). The resuspended cells were mixed gently, and the granulocytes were aspirated, and the cells were washed once with HBSS-BSA, and the pellet was resuspended in 10 ml of HBSS-BSA and loaded onto a magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). The immunomagnetically labeled neutrophils were retained in the magnetic field, and the eosinophils were eluted through the column with 30 ml of HBSS-BSA. The eluate was collected, centrifuged, and resuspended in HBSS containing 5% FCS. Purity of the eosinophils was assessed by differential counts from a Wright-Giemsa air-dried smear, and viability was confirmed by trypan blue exclusion analysis.

Preparation of HUVECs

Purified HUVECs were studied and characterized for factor VIII expression as previously described (13). Immediately after delivery, the umbilical cord was treated with a 1 mg/ml collagenase solution, and the cells were cultured on a 0.2% gel-coated cell culture flask in medium 199 supplemented with 20% FCS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 200 mM l-glutamine, 25 mM HEPES, 50 µg/ml of endothelial cell growth factor, and 100 µg/ml of heparin. The isolated cells were maintained for 3–4 days at 37°C in a 5% CO2 humidified atmosphere until microscopically confluent. After initial seeding with 0.05% trypsin, HUVECs were washed and resuspended in complete medium 199. The cell mixture was centrifuged at 350 g for 10 min, and the supernatant was discarded. The cell pellet was resuspended in complete medium 199, incubated for an additional 3–4 days at 37°C in a CO2 incubator, and grown to confluence. After 2–3 passages, the cells were replated on 96-well microplates and harvested 2–3 days later. HUVECs were treated with either buffer or 10 IU/ml of IL-1α for 4 h, a time previously shown to be optimal for the enhancement of granulocyte adherence (13). Control microplate wells were prepared identically as above except endotoxin-free BSA was used instead of purified HUVECs.

Immunofluorescence Analysis

Passage 3 HUVECs were treated with either buffer or 10 IU/ml of IL-1α for 4 h at 37°C, and the cells were subjected to 0.05% trypsin and 0.02% EDTA in HBSS. After centrifugation, the cells were counted and divided equally into tubes according to the desired protocol. The expression of VCAM-1 and ICAM-1 in the HUVEC-alone and IL-1α-treated HUVEC groups was determined by measuring the fluorescence of 10,000 cells on a FACSscan 400 instrument (Becton Dickinson). Briefly, 5 × 10^5 treated HUVECs were suspended in 100 µl of fluorescence-activated cell sorting (FACS) buffer, and a saturating concentration (1 µg/5 × 10^5 cells) of either ICAM-1, VCAM-1, or IgG1 isotype MAb was added to the sample tubes followed by incubation for 30 min at 4°C. The cells were washed twice in FACS buffer and resuspended in 100 µl of a saturating concentration of fluorescein isothiocyanate goat anti-mouse F(ab')

nate goat anti-mouse F(ab')2 fragments. Twenty minutes later, the cells were washed in FACS buffer and then resuspended in 500 µl of 1% paraformaldehyde until analysis of fluorescence was performed.

Inactivation of HUVECs by Paraformaldehyde

To demonstrate that adhesive ligation rather than activated secretion by HUVECs was the cause of augmented secretion from eosinophils, experiments were performed in HUVECs that were first fixed in a paraformaldehyde solution to cause cell death. Monolayers of HUVECs cultured from four different donors were pretreated with IL-1α (see Preparation of HUVECs) and then fixed with a 2% paraformaldehyde solution containing 0.075% 1-lysine monohydrochloride and 2.1 mg/ml of m-periodate for 10 min. The fixing solution was aspirated, and the cells were blocked with 0.1% BSA and 100 mM glycine in HBSS, pH 7.40. This solution was used to wash and remove any remaining reactive aldehydes before incubation with 20 µg·ml⁻¹ well⁻¹ of an MAb directed against either ICAM-1 (CD54) or VCAM-1 (CD106) on ice for 30 min. The unbound MAb was discarded by gentle washing with HBSS-FCS. This cell preparation was utilized according to the experimental protocol design (see Adhesion Assay and Blockade of Adhesion and Indexes of Eosinophil Activation).

Adhesion Assay and Blockade of Adhesion

Purified human peripheral blood eosinophils from 10 separate eosinophil isolations were resuspended in HBSS containing 5% FCS. For cells treated with an MAb, 10^6 eosinophils were incubated with either buffer control, 20 µg/ml of anti-VLA-4, or 20 µg/ml of anti-CD18 for 20 min before exposure to either BSA control or treated HUVEC-coated microplate wells. Aliquots of eosinophils (5 × 10^4 cells/well) were transferred onto the plate and allowed to adhere for 5, 10, 15, and 30 min at 37°C. Eosinophil adhesion was terminated by removal of nonadherent eosinophils by a plate-inversion technique and washed two times with 200 µl of HBSS-FCS solution. Quantification of adhesion of eosinophils to HUVECs was determined by a function of intracellular eosinophil peroxidase (EPO) content, which was developed specifically for assays required in these studies (19). Adherent eosinophils were lysed with 1% Triton X-100, absorbance at 492 nm was measured every 6 s for 1 min, and maximal uptake (V_max) for each experimental well was calculated by interpolation between successive points (18 s) with Softmax version 2.01 (Molecular Devices, Menlo Park, CA). Standard curves were generated at the same time by adding a known number of eosinophils (1 × 10^3 to 5 × 10^4 cells) to untreated microplate wells. Samples were assayed in triplicate, and the number of adherent cells was calculated from standard curves fitted by linear regression (19). The time at which the adhesion was greatest was used in all subsequent experiments (see results).

Identical experiments were performed in six separate eosinophil isolations, allowing cells to adhere to paraformaldehyde-fixed HUVECs at 0, 5, 10, 20, 30, and 45 min at 37°C. Adherent eosinophils were lysed and quantified in an analogous manner as above as a function of EPO content. Eosinophil binding to paraformaldehyde-fixed HUVECs was validated under epifluorescent microscopy (Axioskop, Zeiss). Preliminary experiments were conducted to assess the time at which eosinophil adhesion was maximal for both untreated and paraformaldehyde-fixed HUVECs.
Indexes of Eosinophil Activation

EPO release by kinetic assay. EPO content was assessed as previously described (20, 22). Briefly, isolated eosinophils (n = 5 isolations from 5 separate donors) were resuspended in HBSS buffer, pH 7.40, and 10^6 cells were pipetted onto either BSA-, HUVECs alone (no IL-1α), or IL-1α-treated HUVECs-coated microplate wells. The cells were allowed to adhere for 5 min, at which time adhesion was greatest (see RESULTS). For experiments requiring blockade with an MAb directed against VLA-4 and CD18, eosinophils were first treated with an MAb for 20 min before exposure to HUVEC- or BSA-coated plates. Substrate (final concentration 0.006% H_2O_2 and 12 mM o-phenylenediamine) dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 was added to each well according to the experimental protocol or to standard 96-well microplates. The treated cells were activated with 10^−6 M fMLP and CB cause optimal secretion of both LTC4 and granular protein, from which both augmentation and inhibition of adhesive ligation can be demonstrated (17, 20, 29, 30). The reaction mixture was terminated by centrifugation at 350 g for 5 min. In this protocol, the number of eosinophils remained constant for all groups studied (19). Duplicate aliquots of the supernatant (50 µl) were transferred onto another 96-well plate. The EPO content is expressed in picograms per 10^6 eosinophils (pg/10^6 cells) (19, 20, 29, 30) based on standard curves fitted by four-parameter analysis (Softmax version 2.01 software, Molecular Devices), and the concentration of LTC4 is expressed in picograms per 10^6 eosinophils (pg/10^6 cells) (19, 20, 29, 30).

LTC4 secretion by enzyme immunoassay. The secretion of LTC4 caused by activation with buffer or fMLP + CB after eosinophil binding to treated HUVECs or BSA was measured by enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor, MI). Isolated human peripheral blood eosinophils (n = 5 isolations from 5 separate donors) were resuspended in HBSS buffer containing 0.1% gelatin. Eosinophils (10^3 cells·100 µl−1·well−1) were loaded onto ten 96-well microplates coated with either BSA or HUVECs and allowed to adhere for 5 min (see RESULTS). The identical intervention as for blockade of adhesion molecules was used. After exposure, the treated cells were activated with either buffer or fMLP + CB for 30 min, and the reaction was terminated by centrifugation at 500 g for 10 min. Immediately, the supernatant from 10 wells was collected, snap-frozen in liquid nitrogen, and stored at −70°C until assayed. Duplicate samples (50 µl) were pipetted onto the microplate wells coated with a mouse anti-rabbit MAb provided with the EIA kit (Cayman Chemical). Acetylcholinesterase-linked LTC4 tracer (50 µl) and LTC4 antiserum (50 µl) were added, and the samples were incubated for 18 h at room temperature. Each well was aspirated dry and rinsed five times with ready-made wash buffer (EIA kit, Cayman Chemical). Optimum development was obtained after the addition of 500 µl of Ellman’s reagent to each well and 5 µl of tracer to the total activity wells. This assay typically develops in 60–90 min, and microplate reading was performed on a microplate absorbance spectrophotometer (Thermomax, Molecular Devices) at 405 nm. The final concentration of each sample was calculated from standard curves fitted by four-parameter analysis (Softmax version 2.01 software, Molecular Devices), and the concentration of LTC4 is expressed in picograms per 10^6 eosinophils (pg/10^6 cells) (19, 20, 29, 30).

To demonstrate that binding to surface ligands on the HUVECs (rather than stimulated secretory activity from these cells) caused augmented eosinophil secretion, experiments (n = 4 eosinophil isolations from 4 different donors) were conducted as above in nonviable cells. In these studies, monolayers of IL-1α-treated HUVECs were fixed with paraformaldehyde (see Inactivation of HUVECs by Paraformaldehyde) and the superfusate was discarded. After washing, new superfusate was added, and the HUVECs were treated with 20 µg·ml−1·well−1 of either anti-VCAM-1 or anti-ICAM-1 for 30 min. Purified eosinophils (10^6 cells/well) were allowed to adhere to treated HUVECs for 30 min (the time at which maximal cell adhesion occurred) before eosinophil activation with either control or fMLP + CB. Microplate wells then were centrifuged, and the supernatant was collected for assay of LTC4 secretion as above.

Experimental controls. As an additional control, the relative inhibitory effect of 20 µg/ml of IgG1 and anti-CD18 MAbs on augmented eosinophil secretion was assessed. MAbs used to pretreat the eosinophils or HUVECs were dissolved in buffer containing 10 mg/ml of BSA. This diluent blocks the Fc receptors on the cell surface and prevents a nonspecific effect of MAbs on stimulated cells (5). This step was taken as an added control because all MAbs utilized in this study were not available solely as F(ab’)_2 fragments.

Drugs and Suppliers

R15.7 (a CD18 antibody), anti-β2-integrin (IgG1), and RR1/1.1.1 (an ICAM-1 antibody; IgG1) were generous gifts from Dr. Robert Rothlein (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). HP2/1 (a VLA-4 antibody), anti-β1-integrin (CD49d), and anti-VCAM-1 (CD106) were purchased from Immunotech (Westbrook, ME). All supplies used for immunomagnetic separation were obtained from Miltenyi Biotech (Sunnyvale, CA). Chemicals for all buffered and fixing solutions were purchased from either GIBCO BRL (Life Technologies, Grand Island, NY) or Sigma (St. Louis, MO). The LTC4 kit was purchased from Cayman Chemical.

Analysis of Data

Data are expressed as means ± SE for all groups studied. The degree of activation was assessed by comparison of maximal EPO release or LTC4 secretion in the same cell isolation before and after each intervention with Student’s t-test. When multiple comparisons of paired data within a single experimental procedure were made, a Bonferroni correction was applied. In experiments requiring multiple group comparisons, two-way analysis of variance was used. When differences were observed between groups, comparisons were made by Dunnett’s test. Significance was claimed when P < 0.05.

RESULTS

Analysis of Cell Survival

Isolated human peripheral blood eosinophils were 98 ± 5.4% pure, and treated cells utilized in all experimental groups studied remained >98% viable as assessed by trypan blue exclusion analysis. Visually confluent monolayers of cultured HUVECs were readily distinguished with phase-contrast microscopy and remained stably adherent. Microscopic examination with Wright-Giemsa stain revealed vacuolation in 2 of 36 eosinophil isolates, indicating probable activation during or before isolation. These isolated cells were excluded prospectively from all subsequent protocols.
Validation of Upregulated Surface Ligands of HUVECs

Flow cytometric analysis of unstimulated HUVECs confirmed that ICAM-1 is constitutively expressed, with a mean fluorescence intensity (MFI) of $24.9 \pm 6.93$ vs. $5.35 \pm 0.16$ for IgG1 isotype control ($P = 0.03$; Fig. 1A). Coincubation with IL-1$\alpha$ augmented the cell surface expression of ICAM-1 to an MFI of $161.7 \pm 59.4$ ($P = 0.042$ vs. unstimulated HUVECs) that persisted up to 24 h. By contrast, VCAM-1 was not expressed on unstimulated HUVECs (MFI $5.72 \pm 0.42$) but was inducible 4 h after treatment with 10 IU/ml of IL-1$\alpha$ (MFI $31.8 \pm 6.8$; $P = 0.006$ vs. unstimulated HUVECs).

![Fig. 1. A: histogram from flow cytometry of vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression on cultured human umbilical vein endothelial cells (HUVECs). ICAM-1 but not VCAM-1 is constitutively expressed in unstimulated HUVECs (top). Treatment with interleukin (IL)-1$\alpha$ (bottom) upregulated surface ligands on cultured HUVECs. All treated HUVECs in all studies were fixed in paraformaldehyde before fluorescence-activated cell sorting. B: kinetics of eosinophil adhesion to cultured HUVECs. Binding was augmented in cells exposed to IL-1$\alpha$-stimulated HUVECs and was maximal at 5 min. BSA alone, BSA-coated wells, no HUVECs. Data are means $\pm$ SE; $n = 5$ eosinophil isolations from 5 donors.](http://ajplung.physiology.org/)

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Maximal adhesion occurred at 5 min and was sustained for ≥30 min (P < 0.05 for all comparisons vs. HUVECs alone or BSA-treated cells; Fig. 1B). Treatment of HUVECs with 10 IU/ml of IL-1α increased eosinophil adhesion substantially. All wells contained 50,000 eosinophils initially. The maximal number of adherent eosinophils was 2,213 ± 840 eosinophils for wells coated with BSA only and 6,746 ± 1,601 eosinophils for wells coated with HUVECs not pretreated with IL-1α (P < 0.01). By contrast, cells exposed to HUVECs plus IL-1α had 15,572 ± 2,032 adherent eosinophils (P < 0.002 vs. BSA control or HUVECs alone; Fig. 1B). Because adhesion to upregulated HUVECs (nonparaformaldehyde fixed) was maximal at 5 min, all subsequent experimental procedures in living HUVECs were therefore performed with a 5-min exposure time.

**Blockade of Adhesion With MAb Directed Against Eosinophil Surface Ligands**

Blockade of the α4-chain of VLA-4 with a specific MAb (HP2/1) decreased adhesion of eosinophils to IL-1α-treated HUVECs by 50.1 ± 4.6% (P < 0.02; Fig. 2). Similarly, pretreatment with an anti-CD18 MAb substantially blocked the adhesion of eosinophils from 15,572 ± 2,032 to 7,216 ± 996 eosinophils/well (P < 0.01 vs. IL-1α-treated HUVECs; Fig. 2). Maximal blockade of adhesion was approximated by each MAb used alone; combined treatment with both MAbs did not inhibit further the augmented eosinophil binding to IL-1α-treated HUVECs (see Discussion).

**Effect of Adhesive Ligation to HUVECs on Eosinophil Secretion of Granular Protein**

fMLP + CB caused comparable EPO release at 30 min for cells exposed to either BSA (403 ± 15.3 ng/10⁶ cells) or HUVECs alone (401 ± 77 ng/10⁶ cells; P = not significant (NS)). Ligation of eosinophils to IL-1α-treated HUVECs caused augmented release of EPO after fMLP + CB activation (Fig. 3A). EPO secretion increased from 401 ± 77 ng/10⁶ cells for HUVECs alone (no IL-1α) to 778 ± 225 ng/10⁶ cells for eosinophils exposed to IL-1α-treated HUVECs. This augmented secretion caused by adhesion to IL-1α-treated HUVECs was significantly blocked to control levels by preincubation of eosinophils with either anti-VLA-4 MAb (262 ± 62 ng/10⁶ cells) or anti-CD18 MAb (434 ± 102 ng/10⁶ cells; P < 0.01 vs. no MAb; Fig. 3A). Inhibition was maximal after blockade with either ligand; hence the combined effects of the two MAbs caused no further inhibition of EPO secretion. Pretreatment with IgG1 isotype control antibody had no effect on EPO release for stimulated eosinophils exposed to IL-1α-treated HUVECs (P = NS vs. IL-1α-treated HUVECs, no MAb; Fig. 3B).

**Effect of Ligation to HUVECs on LTC₄ Secretion**

Baseline secretion (before activation) of LTC₄ was insignificant in all treatment protocols (Fig. 4). For BSA-exposed eosinophils, LTC₄ concentration in the supernatant after 30 min was 1.81 ± 1.13 pg/10⁶ cells, 2.71 ± 0.22 pg/10⁶ cells for eosinophils exposed to HUVECs without IL-1α, and 3.40 ± 1.34 pg/10⁶ cells for eosinophils exposed to IL-1α-treated HUVECs (P = NS vs. baseline for all comparisons). Activation with fMLP + CB caused comparable LTC₄ secretion for eosinophils exposed to BSA (372 ± 50.4 pg/10⁶ cells) and for cells exposed to HUVECs alone (323 ± 38.2 pg/10⁶ cells; P = NS). However, LTC₄ secretion was substantially augmented for stimulated eosinophils exposed to IL-1α-treated HUVECs (624.3 ± 150.9 pg/10⁶ cells; Fig. 4; P < 0.05 vs. HUVECs without IL-1α or BSA).

Addition of the anti-VLA-4 MAb blocked the augmented LTC₄ secretion caused by activated eosinophils exposed to IL-1α-treated HUVECs to 208 ± 51 pg/10⁶ cells (P < 0.05 vs. cells receiving no MAb; see above; Fig. 4). As for EPO release, augmented LTC₄ secretion caused by stimulated adhesive eosinophil binding to IL-1α-treated HUVECs was also blocked to baseline after pretreatment with the anti-CD18 MAb (327 ± 72
for fixed HUVECs treated with IL-1α occurred at 30–45 min (Fig. 5A). Treatment with the anti-ICAM-1 MAb caused a decrease in LTC4 secretion after fMLP + CB from 2,055 ± 503 pg/10^6 cells (basal) for eosinophils adhered to fixed HUVECs pretreated with IL-1α to 1,473 ± 319 pg/10^6 cells (P < 0.05 vs. basal LTC4 secretion). Blockade with anti-VCAM-1 had a similar inhibitory effect on LTC4 secretion (1,484 ± 689 pg/10^6 cells) caused by fMLP + CB activation (P < 0.05; Fig. 5B).

DISCUSSION

This study was undertaken to examine the hypothesis that adhesion of eosinophils at the endothelial surface also is a process by which stimulated secretion of bronchoactive substances is primed. Prior investigations (2, 15, 19, 22) have demonstrated that eosinophils adhere through the β1-surface ligand VLA-4 to the extracellular matrix protein fibronectin (FN). Adhesion of eosinophil VLA-4 to FN required ~60 min and was decreased at 120 min. This was associated with augmented stimulated secretion of eosinophils caused by fMLP at both 60 and 120 min but not at earlier times. A prior investigation has also demonstrated augmented superoxide anion generation from stimulated eosinophils after exposure to exogenous soluble VCAM-1 (21).

In this study, we examined the effect of adhesion of VLA-4 and β2-integrin on eosinophils to VCAM-1 and ICAM-1 on living HUVECs. Studies were conducted to determine whether the degree of adhesion was related to priming of stimulated eosinophil degranulation and/or secretion of LTC4. Additional experiments were performed with paraformaldehyde-fixed HUVECs to assess whether the augmented stimulated eosinophil secretion was specifically mediated by adhesive ligation (14) rather than to stimulation of secretory augmentative substances from viable HUVECs. Although some adhesion of eosinophils to HUVECs was observed in the "basal" (i.e., untreated) state, the greatest adhesion occurred for HUVECs that were first pretreated with IL-1α, which is known to upregulate VCAM-1, the specific endothelial surface ligand for VLA-4 (7, 26).

Adhesion to IL-1α-treated HUVECs (living and paraformaldehyde fixed) was augmented nearly twofold at 5 and 30 min, respectively, and diminished slightly thereafter (Figs. 1 and 5). Blockade of adhesion below baseline levels (Fig. 2) after the addition of the MAb against the surface ligand suggests that isolates of peripheral cells even from mildly atopic donors have some intrinsic adhesive capacity.

We also found that adhesion to IL-1α-treated HUVECs caused augmented secretion of granular protein (EPO) and LTC4, after stimulation with fMLP + CB. Secretion was comparably less for untreated eosinophils incubated with either BSA control or HUVECs with no IL-1α (Figs. 3 and 4). This augmented secretion caused by adhesive ligation of human eosinophil VLA-4 and β2-integrin to IL-1α-treated HUVECs occurred at 5 min (Fig. 1B); by contrast, Muñoz et al. (19) and Neeley et al. (22) have shown that eosinophil binding to FN through VLA-4 requires 60–120 min. Accordingly, both binding and priming of eosinophil secretion caused by
VLA-4 have substantially different kinetics for different counterligands. Priming of eosinophil secretion occurs almost instantaneously even in the presence of blockade of all $\beta_2$-integrins, whereas adhesion alone requires 60 min for VLA-4 and the matrix protein FN.

It is interesting to note that adhesion (Figs. 1B and 5A) and stimulated secretion of EPO (Fig. 3A) and LTC$_4$ (Figs. 4 and 5B) caused by exposure to IL-1$\alpha$-treated HUVECs all were blocked to basal levels (i.e., comparable to control or non-IL-1$\alpha$-treated HUVECs) after pretreatment with an MAb directed against surface adhesion ligands on eosinophils ($\alpha_4$ or $\beta_2$-integrin) or on HUVECs (ICAM-1 or VCAM-1). Combined administration of an MAb directed against both ligands together thus had no incremental inhibitory effect. The mechanism by which blockade of one ligand on the eosinophil surface causes blockade of the augmenting effects of the other was not defined in this study, but steric interference among these macromolecules is a possibility. Hence blockade with either MAb could cause maximal inhibition of adhesion and corresponding adhesive priming.

It is important also to specify some other limitations of our findings. All studies were performed in vitro, and it is not possible to extrapolate these data to events of cellular migration in human asthma. Molecular adhesion of eosinophils to endothelium in vivo occurs under conditions of flow and shear stress (1, 10, 18) not replicated in these studies. Nonetheless, these dynamic events may have relatively little effect on the experimental conditions observed in these studies because the ligands in these investigations are active only after firm ligation to the endothelial surface occurs (25, 28).

Blockade of VLA-4 was effected through an $\alpha_4$-chain MAb. Recently, an $\alpha_4\beta_7$ ligand has been identified (9). However, its specific role in eosinophil function remains to be established. A study in vitro (9) has shown that mucosal addressin adhesion molecule-1 (MadCam-1), expressed specifically by gut endothelial cells, is a preferential ligand for $\alpha_4\beta_7$. Although $\alpha_4\beta_7$ can also bind to VCAM-1, this cell-cell ligation requires greater integrin activation than binding instantaneously to Mad-Cam-1 (25). In these studies, we elicited eosinophil secretion with the formylated tripeptide FMLP, a chemo-
proteins (20, 29), and metabolic burst activity (21). Muñoz et al. (19) have previously shown that activation with platelet-activating factor results in eosinophil secretion of bioactive metabolites that contracts expanded human airways, largely through the activation of eosinophil 5-lipoxygenase and secretion of LTC₄, and the secretory process is blocked by relatively selective inhibitors of phospholipase A₂ (30). However, the actual trigger causing eosinophil activation in human asthma remains unknown. Thus the priming process caused by eosinophil ligation to HUVECs cannot yet be related to a specific stimulus in vivo that provokes eosinophil secretion that is presumed to occur in the human asthmatic state.

Finally, as for other studies, the precise mechanism by which ligation of eosinophil integrins to HUVECs augments LTC₄ secretion was not established. We did establish, however, that priming was caused directly as the consequence of adhesive ligation rather than by secretory products from activated HUVECs. Parafomaldehyde-fixed HUVECs caused similar upregulation of eosinophil secretion to living cells, and this was attenuated by ligand-specific blockade (Figs. 2 and 5B). Our data demonstrate that eosinophil adhesion to IL-1α-treated HUVECs in vitro is associated temporally with increased augmented secretion of stimulated secretion of both granular protein and LTC₄ in concentrations substantially greater than those required to cause contraction of human bronchial airway explants (19). Because adhesion and augmented secretion for cells killed with parafomaldehyde and washed after upregulation with IL-1α was comparable to that for living cells before treatment with parafomaldehyde for FACScan, it thus is unlikely that substances secreted from HUVECs have a role in augmented eosinophil secretion of LTC₄. Stimulated LTC₄ secretion from eosinophils adhering on monolayers of parafomaldehyde-fixed IL-1α-treated HUVECs did not differ from eosinophils exposed to buffer-treated IL-1α-treated HUVECs. These data further confirm that augmentation of stimulated eosinophil secretion is related solely to the process of eosinophil binding at the endothelial surface. Augmentation occurs much more rapidly than that previously reported to be caused by VLA-4-FN ligation (19, 20, 22) and appears to be mediated mutually by both β₁- and β₂-integrins on the eosinophil surface.

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