Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7430–7433, August 1991
Medical Sciences

Human eosinophil adherence to vascular endothelium mediated by binding to vascular cell adhesion molecule 1 and endothelial leukocyte adhesion molecule 1

(leukocytes/adherence proteins)

PETER F. WELLER*†, THOMAS H. RAND*, SUSAN E. GOELZ‡, GLORIA CHI-ROSSO‡, AND ROY R. LOBB‡

*Department of Medicine and the Harvard Thorndike Laboratories, Charles A. Dana Research Institute, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215; and ‡Biogen, Inc., Cambridge, MA 02142

Communicated by Paul B. Beeson, May 28, 1991 (received for review March 13, 1991)

ABSTRACT **Adherence of human eosinophils to cytokine-stimulated endothelial cells, which was only partially due to CD18-dependent pathways, was also mediated by binding to endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Eosinophils bound specifically to both recombinant soluble ELAM-1 and recombinant soluble VCAM-1. Eosinophil binding to recombinant soluble VCAM-1 and to transfected CHO cells expressing VCAM-1 was inhibited with anti-VCAM-1 (4B9) and anti-very late activation antigen 4 (anti-VLA-4; HP1/2 or HP2/1) monoclonal antibodies. Eosinophils, but not neutrophils, expressed VLA-4 detected by cytofluorography. Eosinophil adherence to tumor necrosis factor α-stimulated human umbilical vein endothelial cells was partially blocked by monoclonal antibodies against ELAM-1 (BB11) and VCAM-1 (4B9) and against VLA-4 (HP2/1). Thus, while both eosinophils and neutrophils can bind to activated endothelial cells by adherence to ICAM-1 and ELAM-1, only eosinophils expressed VLA-4 and adhered to VCAM-1 on activated endothelial cells. Eosinophil adherence to VCAM-1 might provide a mechanism contributing to the selective recruitment of eosinophils into tissue sites of inflammation.**

The adherence of leukocytes to vascular endothelium is of importance in immune responses and in the mobilization of leukocytes into tissue sites of inflammation. Binding of lymphocyte function-associated antigen 1 (LFA-1; CD11a–CD18 complex) to intercellular adhesion molecule 1 (ICAM-1; CD54) constitutes a major mechanism of adhesion for lymphocytes (1), monocytes (2), neutrophils (3), and eosinophils (4). In the leukocyte adhesion-deficiency syndrome, genetic deficiencies in CD18 integrins are associated with pyogenic infections because neutrophil adherence is impaired and neutrophils cannot migrate into lesions (3). While neutrophils are absent from inflammatory lesions in this syndrome, both lymphocytes and eosinophils can be found in affected tissue sites (3), indicating that these cells possess mechanisms for endothelial adherence independent of CD18/ICAM-1. In the present study, we have evaluated mechanisms by which human eosinophils adhere to cytokine-stimulated vascular endothelial cells. While both eosinophils and neutrophils bound to ICAM-1 and endothelial leukocyte adhesion molecule 1 (ELAM-1), only eosinophils expressed very late activation antigen 4 (VLA-4; CD49d–CD29) and bound to vascular cell adhesion molecule 1 (VCAM-1). Eosinophil expression of VLA-4 and adherence to VCAM-1 would enable eosinophils, in contrast to neutrophils, to be preferentially localized in tissue sites of specific immune reactions.

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MATERIALS AND METHODS

Eosinophil Isolation. Eosinophils were isolated by Ficoll/Hypaque sedimentation from the blood of two donors with the hypereosinophilic syndrome and by Percoll density gradient centrifugation from the blood of two mildly eosinophilic donors with helminthic infections and four normal donors as described (5). For adherence studies eosinophils were enriched to purities between 45% and 90% and were resuspended in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum (RPMI 1640/10% FCS) at 2–12 × 10^5 per ml. For flow cytometric comparisons with neutrophils, eosinophils were enriched to 26–94% purities.

Monoclonal Antibodies (mAbs). The following murine mAbs were used: 4B9, an IgG1 mAb against VCAM-1 (6); 60.3, an IgG2a that recognizes the common β subunit of the CD11–CD18 complex (7); BB11, an IgG2b that blocks adherence to ELAM-1 (8); C_2E_5, an IgM against ligands for ELAM-1 (9); and HP2/1, HP1/2, HP1/3, and B-5G10, IgG1 mAbs against the α subunit of VLA-4 (10–12). Control myeloma proteins included MOPC 195 or MOPC 141 (IgG2b), MOPC 21 (IgG1), TEPC 183 (IgM), UPC 10 (IgG2a) (Cappel Laboratories).

Eosinophil Adherence Assays. Human umbilical vein endothelial cells (HUVEC) were harvested and grown to confluence in 48-well tissue culture plates as described (13). Recombinant human tumor necrosis factor α (TNF-α; Biogen) at 10 ng/ml was added to half of the wells on each plate 3–5 hr prior to adherence assays. CHO cell lines that stably express VCAM-1 were generated and grown as confluent monolayers as described (6). TNF-α-stimulated and unstimulated HUVECs and VCAM-1-expressing and control untransfected CHO cells were pretreated for 30 min with mAbs 4B9 (10 μg/ml) or BB11 (1 μg/ml) or subclass control myeloma proteins prior to addition of eosinophil-enriched granulocytes. Recombinant soluble VCAM-1 and ELAM-1 (rsVCAM-1 and rsELAM-1) were produced as described (ref. 14 and R.R.L., unpublished data). Briefly, rsELAM-1 and rsVCAM-1, lacking putative transmembrane and cytoplasmic domains, were stably expressed in CHO cells and purified by immunoaffinity chromatography. Both molecules, when immobilized on plastic, are functional adherence proteins. Microtiter well plates were coated at 4°C overnight either with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) or with 0.5 μg of recombinant protein

Abbreviations: VCAM-1, vascular cell adhesion molecule 1; ELAM-1, endothelial leukocyte adhesion molecule 1; rsVCAM-1 and rsELAM-1, recombinant soluble VCAM-1 and ELAM-1; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; VLA, very late activation antigen (integrin); TNF-α, tumor necrosis factor α.

†To whom reprint requests should be addressed at: Beth Israel Hospital, DA-617, 330 Brookline Avenue, Boston, MA 02215.

(in PBS) that subsequently was blocked with 1% BSA. Eosinophils were pretreated at room temperature with mAb 60.3 (10 μg/ml), mAb HP2/1 or HP1/2 (1:50 dilution of ascites), or subclass control myeloma proteins (10 μg/ml) for 20 min prior to adherence assays.

Eosinophils in 250 μl of RPMI 1640/10% FCS were added to each well and incubated for 20 min at room temperature. Plates were decanted with agitation to remove nonadherent cells and washed three times with RPMI 1640/10% FCS, and then adherent cells were fixed by addition of 1% glutaraldehyde (Polysciences) in PBS. Eosinophils were stained and enumerated by methods adapted from those previously applied for assays of eosinophil chemotaxis (15). Eosinophils were stained for 20 min with 2% aniline blue in PBS (pH 7.2) and washed three times with 0.02 M sodium borate/0.02 M KCl/0.02 M NaOH, pH 9. With aniline blue staining, eosinophils, but not neutrophils, were brightly fluorescent when viewed under fluorescein excitation. The numbers of adherent eosinophils in three to six low-power (10× objective) fields per well were enumerated under fluorescence microscopy (Leitz Laborlux S with 50-W mercury arc lamp and wide-band blue H3 excitation/emission filters) using an automated image analyzer (Optomax V, Analytical Instruments, Shaffron Walden, Essex, U.K.) as described (15). Adherent eosinophils in triplicate wells were counted for each experimental condition and for each control myeloma protein; the net adherence was calculated by subtracting from the total adherence the control adherence of eosinophils (in the presence of immunoglobulin subclass control proteins) to either unstimulated endothelial cells, untransfected CHO cells, or BSA-coated plates. Control adherence ranged from about 10–50 eosinophils per field. The numbers of adherent eosinophils, which varied with the input numbers of eosinophils, increased in different experiments to means of about 125–600 eosinophils per field on TNF-α-treated HUVECs and were comparable in magnitude to the numbers of adherent neutrophils enumerated under the same conditions.

Flow Cytometry. Isolated eosinophil-enriched granulocytes at 10^7 per ml in calcium/magnesium-free Hanks' balanced salt solution containing 0.1% BSA and 0.01% sodium azide were stained at 4°C for 30 min with saturating quantities of mAbs B-5G10, HP2/1, HP2/1, HP1/3, or C_2E_5 or with subclass control myeloma proteins, washed in the same medium, stained with phycoerythrin-conjugated goat anti-mouse Ig (Tago) with or without subsequent staining with fluorescein isothiocyanate-conjugated anti-CD16 mAb (Leu 11a, Becton Dickinson), and fixed in 0.5% paraformaldehyde. Flow cytometry was performed on a FACStarPlus and analyzed by the program CONSORT 30 (Becton Dickinson). Granulocytes were gated by forward and orthogonal light scatter, and 20,000-gated cells were analyzed per sample.

RESULTS

Eosinophils Adhere to Both Recombinant ELAM-1 and VCAM-1. To evaluate the capacity of eosinophils to bind to ELAM-1 and VCAM-1, eosinophil adherence to plates coated with rsELAM-1 or rsVCAM-1 was studied (Fig. 1). Eosinophils bound to rsELAM-1-coated plates, and binding was inhibited with the blocking anti-ELAM-1 mAb, BB11. Eosinophils bound to rsVCAM-1-coated plates, and binding was inhibited with an anti-VCAM-1 mAb (4B9) as well as with a mAb (HP1/2) against VLA-4, the recognized VCAM-1 ligand (11, 16). Eosinophils also bound to transfected CHO cells expressing VCAM-1, and this adherence was inhibited by mAbs against VCAM-1 (4B9) and against VLA-4 (HP2/1) (Fig. 1). Eosinophil binding to each recombinant protein was specifically inhibited only by the mAb specific for that protein (not shown), confirming the ability of eosinophils to adhere to either ELAM or VCAM.

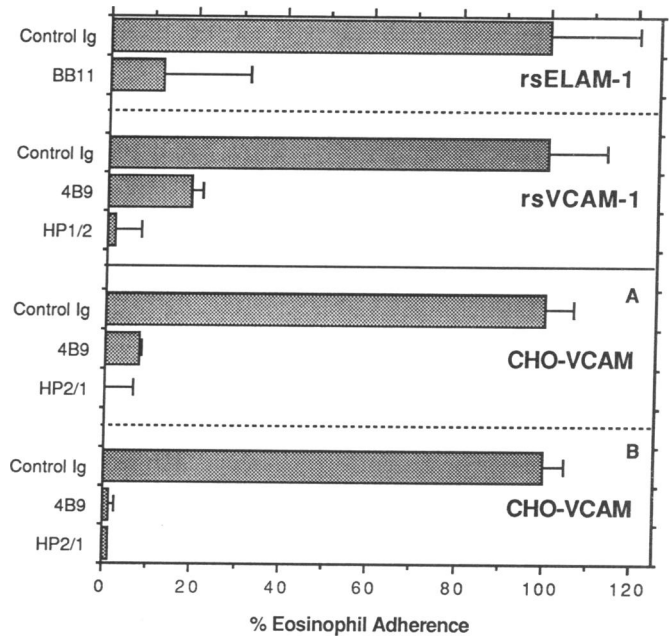

FIG. 1. Eosinophil adherence to plates coated with rsELAM-1 or rsVCAM-1 (*Upper*) and to adherent CHO cells expressing VCAM-1 (*Lower*). Adherence was inhibited with an anti-ELAM-1 (BB11) or an anti-VCAM-1 (4B9) mAb or by prior exposure of eosinophils to anti-VLA-4 mAbs (HP1/2 or HP2/1) as indicated. Eosinophil adherence (mean ± SEM in nine low-power fields), corrected for nonspecific binding to BSA-coated plates or untransfected CHO cells, is expressed relative to that observed with control Ig subclass proteins and is shown for representative experiments with eosinophils from a hypereosinophilic donor (*Upper* and *Lower A*) and a normal donor (*Lower B*).

Eosinophils Express the VCAM-1-Binding Ligand, VLA-4. Expression of the VCAM-1 binding ligand, VLA-4, was evaluated on eosinophil-enriched granulocytes by using differential expression of CD16 ($Fc_\gamma RIII$) to discriminate between neutrophils, which are strongly $CD16^+$, and eosinophils, which express little, if any, CD16 (17, 18). Eosinophils, but not $CD16^+$ neutrophils, were stained with two anti-VLA-4 mAbs, 5-BG10 and HP2/1, as shown for two representative analyses with leukocytes from a normal and a hypereosinophilic donor (Fig. 2). VLA-4 was similarly detected on eosinophils, but not neutrophils, with two additional anti-VLA-4 mAbs, HP1/3 and HP1/2 (not shown). The mAb C_2E_5, which recognizes a cell-surface ligand formed by ELAM-1 ligand fucosyl transferase (9), bound to both eosinophils and neutrophils (not shown). Similar results showing VLA-4 expression on eosinophils, but not neutrophils, and C_2E_5 ligand expression on both eosinophils and neutrophils, were obtained with eosinophil-enriched granulocytes from all three normal and four eosinophilic donors examined (not shown).

Eosinophil Adherence to TNF-α-Stimulated Endothelial Cells. The roles of ELAM-1 and VCAM-1 in mediating adherence of eosinophils to TNF-α-stimulated HUVECs were next evaluated (Fig. 3). With eosinophils from both normal and eosinophilic donors, treatment of eosinophils with mAb 60.3, which blocks CD18-mediated adherence to endothelium (6), partially blocked eosinophil adherence to TNF-α-stimulated endothelial cells—a finding compatible with previous demonstrations that eosinophil adherence to stimulated endothelial cells is partially CD18 dependent (4, 19). With all donors studied, mAbs to either ELAM-1 (BB11) or VCAM-1 (4B9) partially inhibited eosinophil adherence to TNF-α-stimulated endothelial cells. Prior treatment of eosinophils with the anti-VLA-4 mAb (HP2/1) by itself also

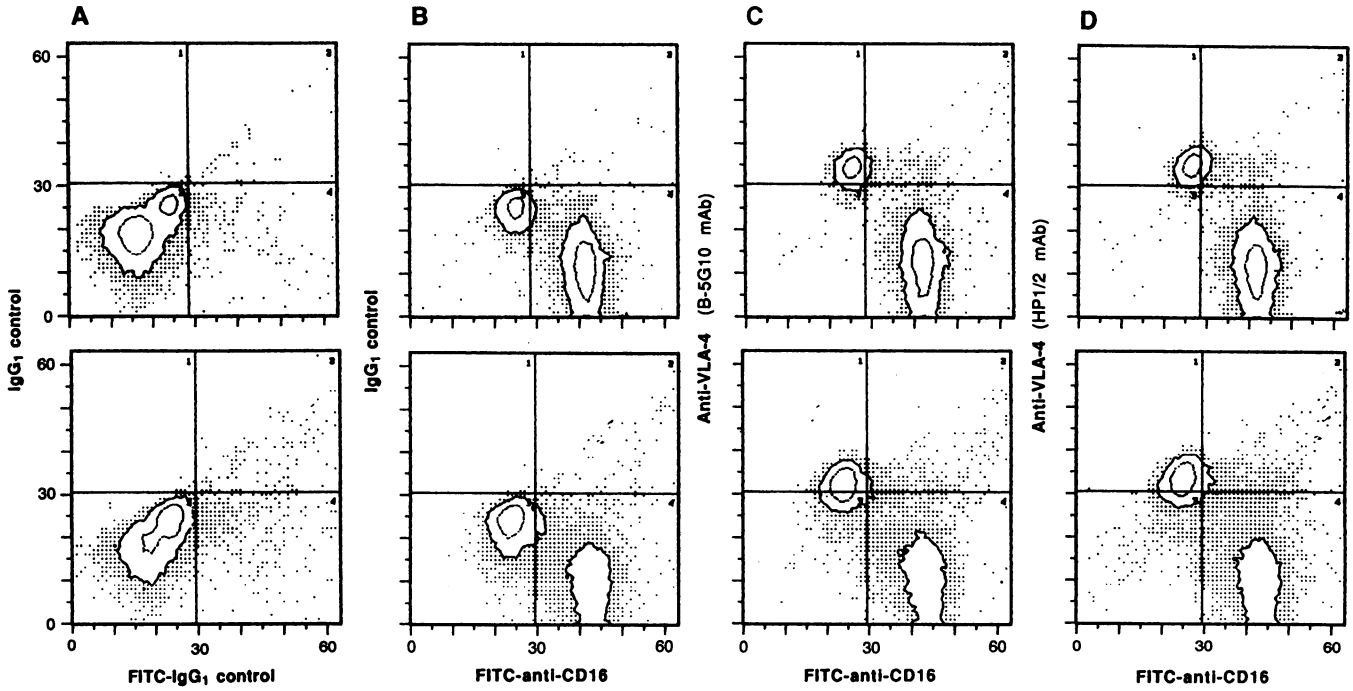

FIG. 2. Flow cytometric analyses of eosinophil and neutrophil expression of the VCAM-1 binding ligand, VLA-4. Eosinophil-enriched granulocytes from a normal donor (30% eosinophils) (*Upper*) and a hypereosinophilic donor (60% eosinophils) (*Lower*) were evaluated by two-color analysis for expression of CD16 and VLA-4. (*A*) Granulocytes were stained with an IgG1 control, a phycoerythrin (PE)-conjugated goat anti-mouse IgG, and a fluorescein isothiocyanate (FITC)-conjugated IgG1 control protein. (*B*) Granulocytes were stained with an IgG1 control, PE-conjugated goat anti-mouse IgG, and FITC-conjugated Leu 11a to identify $CD16^+$ neutrophils. (*C* and *D*) Granulocytes were stained with anti-VLA-4 mAbs B-5G10 (*C*) and HP1/2 (*D*), PE-conjugated goat anti-mouse IgG, and FITC-conjugated Leu 11a. Similar results were found with eosinophils from three normal and four eosinophilic donors.

partially blocked eosinophil adherence to TNF-α-stimulated HUVECs (Fig. 3). Combinations of mAbs concomitantly blocking CD18 and ELAM-1 (60.3, BB11), CD18 and VCAM-1 (60.3 and 4B9 or 60.3 and HP2/1), or all three pathways produced greater inhibition of eosinophil adherence to TNF-α-stimulated HUVECs than did mAb blockade of any single pathway alone. In particular, blockade of both CD18 and VLA-4 integrin pathways with mAbs 60.3 and HP2/1 produced complete inhibition of eosinophil adherence to the TNF-α-treated HUVECs (Fig. 3).

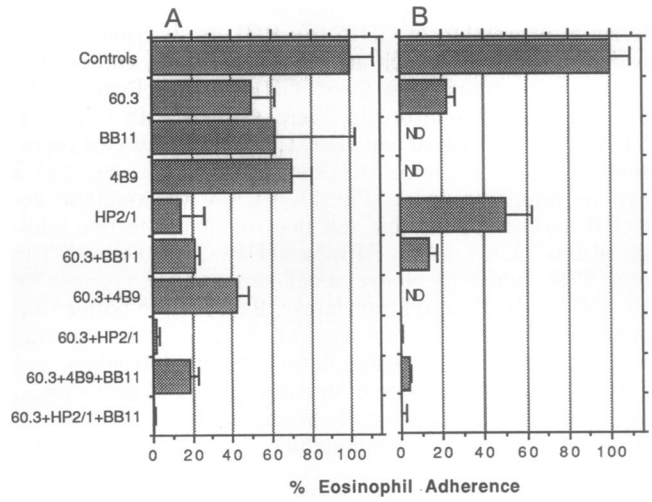

FIG. 3. Binding of eosinophils from two donors to TNF-α-stimulated HUVECs. Eosinophils were pretreated with Ig subclass control myeloma proteins or mAbs 60.3 against CD18 and HP2/1 against VLA-4 to block pathways of binding to ICAM-1 and VCAM-1, respectively. HUVECs were treated with Ig subclass control myeloma proteins or mAbs BB11 against ELAM-1 and 4B9 against VCAM-1. Eosinophil adherence (mean ± SEM in 9–18 low-power fields), corrected for binding to unstimulated HUVECs, is expressed relative to that observed in the presence of control Ig subclass proteins and is shown for representative experiments with eosinophils from a hypereosinophilic donor (*A*) and a normal donor (*B*). Similar results were obtained with four other donors, one with hypereosinophilia, two with mild eosinophilia, and one normal. ND, not done.

DISCUSSION

Our results show that eosinophils were capable of binding to the cell adhesion molecules ELAM-1 and VCAM-1 expressed on cytokine-activated endothelial cells. Although eosinophils can bind to ICAM-1 and ELAM-1 (4, 19), these adhesion molecules also bind neutrophils and would not provide a means for preferential recruitment of eosinophils. The accumulation of eosinophils without neutrophils in specific tissue sites occurs both in some inflammatory and immune reactions and in instances of deficiencies of CD18 integrins when eosinophils are present without mobilized neutrophils (3). Eosinophils from both normal and eosinophilic donors expressed VLA-4, which was not detectable on neutrophils, and the VLA-4 on eosinophils mediated their adhesion to recombinant VCAM-1, both in the soluble form immobilized to plates and in the form expressed on transfected CHO cells. Anti-VLA-4 mAbs completely blocked eosinophil binding to VCAM-1, partially inhibited binding to TNF-α-stimulated HUVECs, and in combination with anti-CD18 mAb, completely blocked eosinophil adherence to the activated HUVECs.

The common expression by human eosinophils as well as lymphocytes and other mononuclear leukocytes of VLA-4 (20) and its role in mediating their adhesion to VCAM-1 and to fibronectin (20) could contribute to the concomitant accumulation of eosinophils and lymphocytes noted in some immune reactions, such as in airway tissues of those with

asthma (21) and in experimentally elicited late-phase airway (22) and cutaneous reactions (23). Moreover, selective expression of VCAM-1—e.g., with interleukin 4 (IL-4) induction of VCAM-1 expression on vascular endothelial cells (24, 25) or at sites of inflammation, including cutaneous delayed hypersensitivity reactions and insect bite sites (26), may contribute to eosinophil localization at specific inflammatory or immune sites without neutrophil infiltration. Recent studies have shown that IL-4 in concert with TNF or IL-1 increases VCAM-1 expression but inhibits ELAM-1 and ICAM-1 expression on endothelial cells *in vitro* (25, 27). It is intriguing to note that IL-4 expression *in vivo* in either transplanted tumors or in transgenic animals has resulted in striking tissue infiltrations of eosinophils (28, 29).

That cell adhesion proteins play an important role in the mobilization and expression of functional responses of eosinophils is evidenced by the demonstration that mAbs to ICAM-1, when administered to primates prior to inhalational antigen challenges, could diminish both eosinophil infiltration and bronchial hyperreactivity induced in this primate model of asthma (30). The ability of combinations of mAbs to block completely the adherence of eosinophils to TNF-stimulated endothelial cells suggests that such mAb combinations—e.g., against CD18 and VLA-4—could be highly effective in inhibiting eosinophil recruitment *in vivo*.

We thank Drs. Martin Hemler (B-5G10), Francisco Sanchez-Madrid (HP1/2, HP1/3, and HP2/1), and John Harlan (60.3 and 4B9) for providing mAbs; Claudia Cabral for assistance with flow cytometric analyses; and Janet Buhlmann, Tonya Barrett, Diane Leone, and Stefan Luhowskyj for assistance with leukocyte isolation, HUVEC growth, and adherence studies. This work was supported in part by National Institutes of Health Grant AI20241, a Fellowship in Immunology from the Irvington Institute for Medical Research (to T.H.R.), and the MWV Leukocyte Research Fund.

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