Eosinophil adhesion to cholinergic nerves via ICAM-1 and VCAM-1 and associated eosinophil degranulation

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1Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3GE, United Kingdom; 2Department of Environmental Health Sciences, School of Hygiene and Public Health and 3Division of Pulmonary and Critical Care Medicine, Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205; and 4Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland

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Sawatzky, Deborah A., Paul J. Kingham, Emma Court, Bharathy Kumaravel, Allison D. Fryer, David B. Jacoby, W. Graham McLean, and Richard W. Costello. Eosinophil adhesion to cholinergic nerves via ICAM-1 and VCAM-1 and associated eosinophil degranulation. Am J Physiol Lung Cell Mol Physiol 282: L1279–L1288, 2002.—In vivo, eosinophils localize to airway cholinergic nerves in antigen-challenged animals, and inhibition of this localization prevents antigen-induced hyperreactivity. In this study, the mechanism of eosinophil localization to nerves was investigated by examining adhesion molecule expression by cholinergic nerves. Immunohistochemical and functional studies demonstrated that primary cultures of parasympathetic nerves express vascular cell adhesion molecule-1 (VCAM-1) and after cytokine pretreatment with tumor necrosis factor-α and interferon-γ intercellular adhesion molecule-1 (ICAM-1). Eosinophils adhere to these parasympathetic neurones after cytokine pretreatment via a CD11/18-dependent pathway. Immunohistochemistry and Western blotting showed that a human cholinergic nerve cell line (IMR-32) expressed VCAM-1 and ICAM-1. Inhibitory experiments using monoclonal blocking antibodies to ICAM-1, VCAM-1, or CD11/18 and with the very late antigen-4 peptide inhibitor ZD-7349 showed that eosinophils adhered to IMR-32 cells via these adhesion molecules. The protein kinase C signaling pathway is involved in this process as a specific inhibitor-attenuated adhesion. Eosinophil adhesion to IMR-32 cells was associated with the release of eosinophil peroxidase and leukotriene C4. Thus eosinophils adhere to cholinergic nerves via specific adhesion molecules, and this leads to eosinophil activation and degranulation; this may be part of the mechanism of eosinophil-induced vagal hyperreactivity.

IN THE LUNGS, pulmonary parasympathetic nerves in the vagus provide the dominant innervation of airway smooth muscle (17). Stimulation of these nerves releases ACh, which binds to M3 muscarinic receptors on the airway smooth muscle, causing its contraction. Control over the release of ACh is mediated by neuronal M2 muscarinic receptors located on the parasympathetic nerves (12). In antigen-challenged animals and in some humans with asthma, M2 muscarinic receptors are dysfunctional, leading to vagally mediated hyperreactivity (13).

In vivo studies in antigen-challenged animals have shown that M2 muscarinic receptor dysfunction is prevented by inhibiting eosinophil localization to the airways (3, 7, 9). The eosinophil protein major basic protein (MBP) is an antagonist at M2 muscarinic receptors in vitro (15); neutralizing MBP prevents both M2 receptor dysfunction and antigen-induced hyperreactivity in vivo (8). Furthermore, eosinophils and extracellular MBP are found closely associated with airway parasympathetic nerves in antigen-challenged guinea pigs and rats as well as in humans with asthma (4). Thus it is likely that the localization of eosinophils to airway nerves and the subsequent release of MBP on M2 muscarinic receptors are mechanisms for vagally mediated hyperreactivity.

Inflammatory cells, such as eosinophils, selectively localize to specific sites within inflamed tissue through interactions between adhesion molecules on their surface and counterligands on tissue structures (21). Eosinophils express the integrin adhesion molecules CD11/18 complex and very late antigen-4 (VLA-4), which interact with intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), respectively (23). It is also recognized that the process of adhesion to some integrin adhesion molecules is important for cell activation (18). One mechanism whereby eosinophils localize to and subsequently release MBP on M2 muscarinic receptors may

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be that parasympathetic nerves express adhesion molecules recognized by eosinophils. The aim of this study, therefore, was to test the hypothesis that cholinergic nerves express adhesion molecules to which eosinophils adhere and that this subsequently leads to eosinophil degranulation.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Poole, UK) or from BDH-Merck (Poole, UK). The IMR-32 cell line was purchased from ECACC (Salisbury, UK). DMEM, normal goat serum, and FCS were purchased from GIBCO-BRL (Paisley, UK). Medium 199 was obtained from GIBCO-BRL (Rockville, MD). All cell culture plastic materials were from Becton-Dickinson Labware (Oxford, UK). Mouse anti-rat ICAM-1 (IgG1, clone 1A29) and mouse anti-rat CD11/18 (IgG2a, clone WT.1) were purchased from Pharmingen. The anti-rat VCAM-1 antibody (IgG1, clone 5F10) was a gift from Biogen (Cambridge, MA). Goat polyclonal anti-human ICAM-1 (sc-1510), goat polyclonal anti-human VCAM-1 (sc-1504), and affinity-purified mouse and goat IgG1 were obtained from Autogen Bioclear (Calne, UK). Nitrocellulose paper, [3H]sodium chromate, and Percoll were obtained from Amersham Pharmac Biotech (Little Chalfont, UK). Chemiluminescent substrate stable peroxidase solution and chemiluminescent sub- strate luminol/enhancer solution were obtained from Pierce (Rockford, IL). The biotinylated mouse anti-goat and horse anti-mouse secondary antibodies, avidin-biotinylated horseradish peroxidase complex, chromagen Slate Gray (SG) peroxidase substrate, Novo Red, and 3-amino-9-ethylcarbazole (AEC) staining kits were purchased from Vector Laboratories (Petersborough, UK). CD16 MicroBeads and MACS V5+ columns were obtained from Miltenyi Biotech (Bisley, UK). Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) were purchased from PeproTech (London, UK). The cyclic peptide VLA-4 inhibitor (ZD-7349), cyclo-(MePhe-Leu-Asp-Val-D-Arg-D-Arg) acetate salt, an equivalent protein kinase C (PKC) inhibitor rottlerin and mouse anti-rat CD11/18 (IgG2a, clone WT.1) were generously provided by Dr. Duncan Haworth (Astra Zeneca Pharmaceuticals, Macclesfield, UK). The neutralizing anti-human CD11/18 monoclonal antibody (Med Cl285) was obtained from Accurate Scientific Chemicals. The neutralizing mouse monoclonal anti-human antibody to ICAM-1 (RR1/1.1.1) was a kind gift from Dr. Robert Rothlein (Boehringer Ingelheim Pharmaceuticals). The neutralizing monoclonal anti-human VCAM-1 antibody (B-K9, isotype IgG1) was obtained from Lab Vision. The protein kinase C (PKC) inhibitor rottlerin was purchased from Affiniti Research Products (Manhead, UK). Peroxidase-conjugated immunoglobulin standard was obtained from Dako (High Wycombe, UK). The leukotriene C4 (LTc4) EIA kit came from Cayman Chemicals (Ann Arbor, MI).

Culture of tracheal parasympathetic nerves. Two different species of animal were used because of the current availability of appropriate antibodies. Specific pathogen-free female Dunkin-Hartley guinea pigs and Sprague-Dawley rats (both 180–200 g) were housed in high-efficiency particulate-filtered air and were fed a normal diet. Animals were handled in accordance with the standards established by the United States Animal Welfare Acts set forth in the National Institutes of Health guidelines and the Policy and Procedures manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee. The animals were killed with a lethal intraperitoneal dose of pentobarbital sodium. The technique of cell culture was essentially the same for the two species, and the methods have been described previously (10). Briefly, the trachealis muscle was excised, cut into small sections, and suspended in 0.2% (wt/vol) collagenase solution at 4°C overnight. The pellet was resuspended in DMEM containing 10% (vol/vol) FCS and penicillin (100 U/ml), plated on a petri dish, and incubated overnight at 37°C in 5% CO2,95% air. Nonadherent cells were collected, resuspended in serum-free medium, and plated on poly-L-lysine- and Matrigel-coated four-well glass Lab-Tek chamber slides. After 24 h, the medium was replaced with serum-free medium containing 5 μM cytosine arabinoside. In prior studies, it had been shown that after 7 days these cells have extensive neurite outgrowth and demonstrate a cholinergic phenotype (10).

Culture of IMR-32 human neuroblastoma cells. The human cholinergic neuroblastoma cell line IMR-32 was cultured in 25-cm2 flasks in DMEM with 10% (vol/vol) FCS, 100 U/ml (wt/vol) penicillin, 0.1 mg/ml (wt/vol) streptomycin, and 10 μg/ml (vol/vol) gentamicin at 37°C in 5% CO2,95% air. Confluent, adherent cells were removed by application of medium, centrifuged at 100 g for 10 min, and resuspended in fresh medium. IMR-32 cells were plated on 48-well flat-bottom plates at a density of 2 x 104 cells/well in 400 μl of the above medium. After 24 h, the medium was removed and replaced with serum-free medium containing 1 mM dibutyryl-cAMP (DbcAMP). They were then incubated for 7 days to allow differentiation and neurite outgrowth. In preliminary studies, it was shown that these cells develop neurites and develop a cholinergic phenotype including expression of M2 muscarinic receptors, hemicholinium-dependent uptake of choline, and release of ACh in response to chemical and electrical stimuli.

Immunohistochemical detection of the adhesion molecules VCAM-1 and ICAM-1 on tracheal parasympathetic nerves. Primary cultures of tracheal parasympathetic nerves were cultivated on four-well Lab-Tek chamber slides as described above for 7 days. In some cases, cells were incubated with the cytokines TNF-α (2 ng/ml) and IFN-γ (1,000 U/ml) for 48 h (days 5–7). The cells were fixed in methanol-acetone (50:50 vol/vol) for 5 min, washed three times with PBS, and preincubated for 30 min with 5% (vol/vol) mouse serum. The rat tracheal cells were incubated with either mouse anti-rat VCAM-1 antibody (mAbs5F10) or an IgG1, mouse antibody (both at 1:50 for 30 min), whereas the guinea pigs were pretreated with a mouse anti-rat ICAM-1 antibody or an IgG1 mouse antibody (both at 1:100 for 2 h). After a wash with PBS, the cells were incubated for 30 min at room temperature with a biotinylated horse anti-mouse secondary antibody added for 30 min. The cells were then washed with PBS before the addition of the avidin-biotinylated horseradish peroxidase complex (ABC Elite) for 30 min. Immunoreactivity was detected with a peroxidase-dependent chromagen, either SG or ABC.

Isolation of guinea pig eosinophils. Female Dunkin-Hartley guinea pigs (0.75–1 kg) were pretreated with a solution of 1% horse serum weekly for a period of 3 wk to induce peritoneal eosinophilia. After the last treatment (24 h), animals were anesthetized with xylazine (10 mg/kg im) and ketamine (40 mg/kg im). A cannula was inserted into the peritoneal cavity, and lavage with sterile PBS was performed. The eosinophils were purified by centrifugation through a Percoll gradient (specific gravity 1.090 g/ml), and any red blood cells were removed by hypotonic lysis. Cells were used if this technique yielded a population of eosinophils >90% pure and >95% viable.
**Eosinophils Adhere to Cholinergic Neuronal Cells**

Coculture of guinea pig eosinophils and tracheal parasympathetic nerves. Eosinophils were then coincubated with the cytokine-stimulated parasympathetic cholinergic nerves for 30 min (5 x 10^4 eosinophils/well). In some experiments the eosinophils were also incubated with a mouse monoclonal anti-rat antibody to CD11/18 (1:1,000 dilution). Nonadherent eosinophils were removed by washing the cocultured cells three times with medium 199. The cells were then fixed with 3.7% (wt/vol) formaldehyde, and the eosinophils were detected using their endogenous peroxidase to catalyze a reaction, which in the presence of exogenous hydrogen peroxide yielded the red chromagen Vector Novo Red. Nerves were counterstained with diate hematoxylin.

Immunohistochemical detection of the adhesion molecules VCAM-1 and ICAM-1 on IMR-32 cells. IMR-32 cells cultured on four-well Lab-Tek chamber slides were fixed in 4% (wt/vol) paraformaldehyde and incubated in 3% (vol/vol) hydrogen peroxide for 10 min to quench endogenous peroxidase activity. The cells were then incubated in 5% goat serum (wt/vol) for 30 min at room temperature and then with a 1:100 dilution of a goat polyclonal anti-human ICAM-1 antibody for 1 h at room temperature, goat polyclonal anti-human VCAM-1 antibody (1:50 dilution for 30 min at room temperature), or a goat IgG; at 1:50 dilution. After a wash with PBS, the cells were incubated for 30 min at room temperature with a biotinylated mouse anti-goat secondary antibody added for 30 min. The cells were washed further with PBS before the addition of the avidin-biotinylated horseradish peroxidase complex (ABC Elite) for 30 min. Immunoreactivity was detected with a peroxidase-dependent chromagen, either SG or AEC.

**Gel electrophoresis and Western blotting to detect the adhesion molecules VCAM-1 and ICAM-1 on IMR-32 cells.** IMR-32 cells were lysed with Tris-buffered saline (TBS) containing 10 mM EDTA, 0.5% (vol/vol) Nonidet, 0.5% (wt/vol) deoxycholate, 8 M urea, 2% (wt/vol) SDS, and 1% (wt/vol) bromphenol blue (pH 7.1) at a concentration of 2 x 10^6 cells/ml. Samples were boiled for 5 min, and 2-mercaptoethanol was added to a final concentration of 5% (vol/vol). The samples were then separated on 8% SDS-polyacrylamide gels with a 4% polyacrylamide stacking gel, and the proteins were transferred to nitrocellulose paper, as described previously (20). The resulting Western blots were incubated with 5% (wt/vol) BSA for 1 h and then incubated overnight at 4°C with a 1:1,000 dilution of goat polyclonal anti-human ICAM-1 or VCAM-1 antibody. Controls, to which no antibody was added, were maintained in 5% (wt/vol) BSA. Blots were then washed three times with 0.05% Tween 20 in TBS and incubated with 1:10,000 dilution of peroxidase-conjugated sheep anti-goat immunoglobulin for 2 h at ambient temperature. After further washing, the chemiluminescent substrate stable peroxi- dase and substrate luminol/enhancer solutions were added. Blots were then applied to Kodak X-Omat film, and immunoreactive proteins were visualized on the developed film.

Isolation of human eosinophils. Eosinophils were isolated with the VarioMACS magnetic cell separation system (MACS; Miltenyi Biotech). Peripheral venous blood (60 ml), donated from healthy volunteers, was suspended in an equal volume of PBS containing 1,000 U/ml heparin sulfate, layered over 12 ml of Percoll solution (specific gravity 1.090 g/ml), and centrifuged at 400 g for 20 min at room temperature. The resulting supernatant and monocyte layer were carefully aspirated and discarded, and red blood cells were removed by hypotonic water lysis. The remaining granulocytes were washed in 10% (wt/vol) PIPES (pH 7.4), 1% (wt/vol) glucose, and 0.0003% (vol/vol) human serum albumin. The cells were then coincubated with anti-human CD16 microbeads (1 μl/10^6 cells) and an equal volume of MACS buffer [0.5% (wt/vol) BSA and 2 mM EDTA in PBS] at 6°C for 45 min. The eosinophils were then separated from contaminating neutrophils by passing the solution through the VarioMACS magnetic separation apparatus. Cell viability was determined by Trypan blue exclusion, and eosinophil purity was determined by Diffquick staining. Only populations of eosinophils that were >98% pure and >95% viable were used.

**Eosinophil adhesion assay.** Freshly isolated eosinophils, suspended in IMR-32 culture medium (see above), were incubated with [51Cr]sodium chromate (37 kBq/ml; sp act >9.25 GBq/mg chromium) for 1 h at 37°C. The eosinophils were then washed three times with culture medium. Eosinophils (5 x 10^4/well) were then coincubated on 48-well plates with differentiated IMR-32 cells (2 x 10^5 cells/well) at 37°C for 30 min. In some experiments, the eosinophils were pretreated with antibodies to CD11/18 at 4°C with or without the VLA-4 inhibitor ZD-7349 with an equipotent concentration of the nonbasic VLA-4 peptide inhibitor cyclo-(MePhe-Leu-Asp-Val-d-Ala-d-Ala), with the inactive control cyclic peptide cyclo-(Ile-Leu-Asp-Val-β-Ala), or with the specific PKC inhibitor rottlerin at 37°C for 30 min. Alternatively, the differentiated IMR-32 cells were pretreated with antibodies to VCAM-1 or ICAM-1 for 30 min or with TNF-α and/or IL-1β for 24 h at 37°C (see results for drug concentrations and antibody dilutions). In some experiments after coincubation with IMR-32 cells, eosinophils were stained with the Vector AEC stain for 5 min followed by three washes with medium and were viewed by light microscopy.

Nonadherent eosinophils were removed from the wells by three gentle washes with medium. The cells were lysed with 0.025% (vol/vol) Triton X-100, and the released radioactivity, which was a measure of adherent eosinophils, was measured in a liquid scintillation analyzer (model 1500; Packard Tri-Carb, Pangbourne, UK). The conditions of this assay were optimized by experiments that involved varying the density of IMR-32 cells (from 2 x 10^5 to 2 x 10^6 cells/well) and varying the number of washes required to eliminate residual nonadherent eosinophils (from 1 to 6 washes). In preliminary experiments, it was also determined that measured radioactivity was directly proportional to eosinophil number at eosinophil concentrations between 2 x 10^5 and 5 x 10^6 cells/ml and that ~30% of added cells adhered to IMR-32 cells under optimal conditions. Specific eosinophil adhesion was calculated by subtracting eosinophil adhesion in the absence of neuronal cells from that in the presence of neuronal cells.

**Assessment of the activation and degranulation of human eosinophils.** The release of eosinophil peroxidase (EPO) and LTC_4 was used as an index of degranulation and activation, respectively. After incubation with [51Cr]sodium chromate for simultaneous cell adhesion assays (see above), isolated eosinophils were resuspended in IMR-32 culture medium and 5 x 10^4 eosinophils incubated with 2 x 10^5 IMR-32 cells, which had been differentiated for 7 days with DBcAMP on 48-well culture plates. Eosinophils were also cultured alone (5 x 10^4 cells/well) to determine background levels of degranulation. Aliquots of medium were then removed from the wells at various time periods after eosinophil adhesion, and degranulation was terminated by centrifugation of the aliquots at 5,400 g for 5 min. The supernatants were stored at ~80°C until assayed.

A homovanillic acid (HVA) oxidation assay, as previously described (16), was used to assess EPO activity. Supernatant aliquots (100 μl) were transferred to a 96-well plate and diluted with 100 μl of 0.1 M glycine buffer (pH 10.5) containing 0.3% (vol/vol) hydrogen peroxide. The reaction was initi-
ated by the addition of HVA (8 mM final concentration), and the samples were incubated for 1 h at 37°C. At the end of the incubation, fluorescence was measured with a microplate fluorescence reader (model FL600; Bio-Tek) with excitation filters at 360 ± 40 nm and emission filters at 485 ± 20 nm. Fluorescence values were converted to units of peroxidase activity by comparison with a peroxidase-conjugated immunoglobulin standard.

Supernatants from eosinophils cultured alone or in the presence of IMR-32 cells were also measured for the production of LTC4. Production of LTC4 was determined with a commercially available LTC4 EIA kit according to the manufacturer's instructions.

Statistics. All values are shown as means ± SE from the number of experiments indicated. The effects of agents inducing or inhibiting eosinophil adhesion or degranulation were compared with control values using ANOVA with Dunnett's post hoc test.

RESULTS

Experiments in primary cultures of parasympathetic nerve cells. Immunohistochemical staining of rat tracheal nerve cells demonstrated that VCAM-1 was expressed by these cells (Fig. 1A), and this expression was not altered by pretreatment with the cytokines TNF-α and IFN-γ (Fig. 1B); no staining was detected when an isotype control antibody was used (Fig. 1C). In contrast, staining of untreated cultured guinea pig nerve cells did not reveal evidence of expression of ICAM-1 (Fig. 2A). However, when these nerve cells were preincubated with the cytokines TNF-α and IFN-γ, immunostaining of ICAM-1 (Fig. 2B) was detected on the cell bodies and along the neurites. In contrast, there was no staining of nerve cells incubated in the presence of cytokines when an isotype-matched mouse IgG1 antibody was used in place of the anti-ICAM-1 antibody (Fig. 2C). Coincubation of guinea pig eosinophils with primary cultures of parasympathetic nerves revealed that eosinophils only adhered to these cells after preincubation with the cytokines TNF-α and IFN-γ (compare untreated neurones in Fig. 2E with cytokine-pretreated neurones Fig. 2D). Eosinophil adhesion was attenuated by pretreatment with an antibody to eosinophil CD11/18 (Fig. 2F).

Expression of VCAM-1 and ICAM-1 by IMR-32 cells. Immunohistochemical staining of IMR-32 neuroblastoma cells demonstrated the expression of both VCAM-1 (Fig. 3A) and ICAM-1 (Fig. 3C) on the cell bodies, and along the neurites of these cells no staining was apparent when an isotype-matched antibody was used. The expression of these adhesion molecules was detected in the absence of cytokine pretreatment. Western blotting studies also demonstrated that IMR-32 cells constitutively expressed both VCAM-1 and ICAM-1 cell adhesion molecules (Fig. 4).

Adhesion of eosinophils to cholinergic cells in culture: dependence on cell adhesion molecules. When freshly isolated eosinophils were cocultured with IMR-32 cells, eosinophils adhered to the cells even after extensive washing to remove nonadherent cells (Fig. 5A). This adhesion was prevented when the experiments were performed on ice or in the presence of 10 mM EDTA (results not shown). The number of eosinophils that adhered to IMR-32 cells depended on the duration of incubation. Because adhesion was well established, but not maximal, after 30 min, this time point was used for subsequent studies to investigate the factors that inhibited or augmented adhesion (Fig. 5B). After treatment of differentiated IMR-32 cells with either 10 nM TNF-α or 10 nM IL-1β for 24 h, there was a significant increase in the number of eosinophils adhering to the cells compared with control nonstimulated IMR-32 cells (Fig. 6).

Preincubation of eosinophils with a mouse anti-human monoclonal antibody to either CD11/18 or ICAM-1 inhibited eosinophil adhesion to IMR-32 cells. The antibody to CD11/18 significantly reduced the adhesion of eosinophils to IMR-32 cells to a quarter of the control value (25.4 ± 14.8%, P < 0.005, n = 6) at 1:1,000 dilution (Fig. 7A). That this effect was mediated through adhesion to neural ICAM-1 was shown by pretreating the IMR-32 cells with an anti-ICAM-1 antibody. In these studies, it was shown that at a concentration of 20 ng/ml, eosinophil adhesion to IMR-32 cells was 33.5 ± 9.9% of the control value (P < 0.005, n = 5; Fig. 7B). There was no inhibitory effect when a
mouse IgG1 antibody was used as a control (data not shown, n = 3).

There was also a dose-dependent inhibition of eosinophil adhesion to IMR-32 cells in the presence of the VLA-4 peptide inhibitor ZD-7349 (Fig. 8A). Significant inhibition of adhesion to near baseline levels was achieved at concentrations of $10^{-5}$ and $10^{-6}$ M (for example, with $10^{-6}$M ZD-7349 adhesion was reduced to $16.4 \pm 17\%$, $n = 7$, $P < 0.005$). Adhesion was similarly inhibited in the presence of a nonbasic equipotent VLA-4 peptide inhibitor ($10^{-5}$M), whereas an inactive equivalent peptide ($10^{-5}$ M) had no inhibitory effect. An anti-VCAM-1 antibody also significantly inhibited eosinophil adhesion (Fig. 8B).

The specific PKC inhibitor rottlerin reduced the adhesion of eosinophils at concentrations at or above $10^{-5}$ M. At a concentration of $10^{-8}$ M, adhesion was inhibited to $42.2 \pm 12.9\%$ of the control value ($P < 0.05$, $n = 5$; Fig. 9).

Heparin sulfate has recently been shown to be an inhibitor of inflammatory cell adhesion, and this is mediated in part via inhibition of CD11/18 and has been shown to be an inhibitor of allergen-induced hyperreactivity and inflammation (5, 15). Thus the effect of heparin on eosinophil adhesion was assessed. Heparin, at a concentration $\geq 2$ U/ml, inhibited eosinophil adhesion to IMR-32 cells. For example, at a concentration of 10 U/ml, adhesion was inhibited to $28.4 \pm 15.6\%$ of the control value ($P = <0.05$, $n = 3$; Fig. 10).

**Eosinophil degranulation followed adhesion to IMR-32 cells.** To determine the role of eosinophil adhesion to differentiated IMR-32 cells on the process of eosinophil activation and degranulation, EPO release and LTC4 generation were measured. The time course of EPO release showed that degranulation occurred in parallel with eosinophil adhesion, with maximal secretion of EPO after 1 h of coincubation (0.060 $\pm$ 0.006 units of peroxidase activity; $P < 0.01$; $n = 4$; Fig. 11). Spontaneous secretion of EPO from control eosinophil cultures (i.e., in the absence of IMR-32 cells) remained low at that time point (0.010 $\pm$ 0.004 units of peroxidase activity) and throughout the assay period. When adhesion, as opposed to close contact, was prevented by placing Transwell inserts between the IMR-32 cells, eosinophil EPO release was completely inhibited (data not shown). The released EPO represented between 15 and 20% of the total cellular EPO content.

IMR-32 cells also induced the secretion of LTC4 from eosinophils. Background levels of LTC4 in culture medium taken from IMR-32 cells was 36.37 $\pm$ 23.28 pg. After 2 h, the quantity of LTC4 in the supernatant from eosinophils cultured alone was 50.65 $\pm$ 24.81 pg, which...
was increased to 390.72 ± 89.46 pg (P < 0.05; n = 4) from eosinophils cultured in the presence of IMR-32 cells.

DISCUSSION

In previous studies, we have shown that, both in animal models of hyperreactivity and in humans with asthma, eosinophils and MBP are seen in close association with airway cholinergic nerves (4). Furthermore, in vivo, preventing the localization of eosinophils to airway nerves prevents antigen-induced hyperreactivity (9). In this study, we have investigated the mechanism of this localization of eosinophils to airway parasympathetic nerves.

The results of this study show that primary cultures of airway parasympathetic nerves expressed the adhesion molecule VCAM-1, whereas ICAM-1 was expressed only after pretreatment of these cells with TNF-α and IFN-γ. Furthermore, after cytokine stimulation, eosinophils adhered to these cells via a CD11/18-dependent mechanism. The human cholinergic cell line IMR-32 constitutively expressed VCAM-1 and ICAM-1. Human eosinophils adhered to these cells via these adhesion molecules in a time-dependent manner, and this adhesion led to eosinophil activation and degranulation.

For practical reasons, including the limited number of reagents available and the relatively small number of neurones obtained by primary culture of guinea pig and rat tracheal parasympathetic nerves, further studies examining the interactions of eosinophils with neural adhesion molecules were performed using human IMR-32 cells and human eosinophils. In preliminary studies (results not shown), we demonstrated that these cells develop a cholinergic phenotype on differentiation in DBcAMP, with the ability to release ACh in response to electrical stimulation and to express M2 muscarinic receptors. In keeping with the observations in primary cultures of tracheal parasympathetic nerves, IMR-32 cells also expressed VCAM-1 and ICAM-1; however, the expression of

Fig. 3. The neuronal cholinergic cell line IMR-32 expressed the adhesion molecules VCAM-1 (A) and ICAM-1 (C). B and D: control experiments in which IMR-32 cells were studied using an isotype-matched control antibody. Cell culture and immunohistochemistry were performed as described in MATERIALS AND METHODS.

Fig. 4. Western blotting of IMR-32 cells showed constitutive expression of ICAM-1 and VCAM-1. IMR-32 cells were extracted, and proteins were separated on 8% SDS-polyacrylamide gels and blotted on nitrocellulose. The adhesion molecules were detected with goat anti-human antibodies to ICAM-1 or VCAM-1. A peroxidase-conjugated mouse anti-goat antibody was added, and immunoreactivity was detected by enhanced chemiluminescence. Lanes 1 and 2 are controls in which the primary antibody was omitted; lanes 1 and 3 were derived from cells grown in serum-containing medium; lanes 2 and 4 were derived from cells differentiated with dibutyryl-cAMP (DBcAMP) for 7 days.
these adhesion molecules did not require the addition of cytokines.

Using IMR-32 cells, we then investigated whether eosinophils adhered to these cells and whether this was mediated by VCAM-1 or ICAM-1. In these studies, eosinophils adhered to IMR-32 cells in a time-dependent manner. Adhesion appeared to be an active process, since it was completely inhibited in the presence of EDTA or the experiments were performed at low temperature. Although cytokine stimulation was not necessary for expression of ICAM-1 or VCAM-1, pre-treatment with the cytokines TNF-α and IL-1β increased eosinophil adhesion to IMR-32 cells that had been differentiated for 7 days with DBcAMP.

The adhesion molecules mediating adhesion were investigated by means of specific blocking antibodies.
and inhibitors. Eosinophil CD11/18 was inhibited by blocking the common \(\beta\)-family of integrin molecules with a specific antibody, and the dependence of the adhesion on ICAM-1 was investigated with an anti-ICAM-1 antibody. In both cases, eosinophil adhesion to IMR-32 cells was inhibited, and an isotype-matched control antibody had no such inhibitory effect.

The VLA-4 peptide inhibitor ZD-7349, a related cyclic peptide (14), and a specific antibody to VCAM-1 also prevented eosinophil adhesion to IMR-32 cells. We can therefore conclude that eosinophil adhesion involves both ICAM-1 and VCAM-1. It appears from quantitative analysis of the extent of inhibition in each case that the contributions of the two cell adhesion molecules were not additive, since >50% inhibition occurred in the presence of inhibitors of either mole-

**Fig. 8.** The very late antigen (VLA)-4 peptide inhibitor ZD-7349 (A) or MAb against VCAM-1 (B) inhibited adhesion of eosinophils to IMR-32 cells. Eosinophils labeled with \(^{51}\text{Cr}\) were incubated with the VLA-4 peptide inhibitor ZD-7349, the nonbasic peptide equivalent, or the inactive VLA-4 peptide inhibitor ZD-7349 (both at \(10^{-5}\) M) for 30 min at 37°C. In separate experiments, IMR-32 cells differentiated with DBcAMP were pretreated with MAb to VCAM-1 for 30 min at 37°C. Eosinophils were then incubated for 30 min with IMR-32 cells that had been differentiated for 7 days with DBcAMP, and adhesion was measured as described in MATERIALS AND METHODS. Values are means of 3–5 separate experiments. \(**P < 0.01\) vs. untreated controls.**

**Fig. 9.** Adhesion of eosinophils to IMR-32 cells involved the protein kinase C (PKC) signaling pathway. Eosinophils labeled with \(^{51}\text{Cr}\) were incubated with the specific PKC inhibitor rottlerin for 30 min at 37°C. Eosinophils were then incubated for 30 min with IMR-32 cells that had been differentiated for 7 days with DBcAMP, and adhesion was measured as described in MATERIALS AND METHODS. Values are means of 5 separate experiments. \(*)P < 0.05\) and \(**P < 0.02\) vs. untreated controls.

**Fig. 10.** Heparin sulfate attenuated the adhesion of eosinophils to IMR-32 cells. IMR-32 cells that had been differentiated for 7 days with DBcAMP were pretreated with heparin sulfate for 1 h at 37°C. Eosinophils labeled with \(^{51}\text{Cr}\) were then coincubated with IMR-32 cells for 30 min, and adhesion was measured as described in MATERIALS AND METHODS. Values are means of 3 separate experiments. \(\ast P < 0.01\) vs. untreated controls, \(\ast\ast P < 0.001\) vs. untreated controls.

**Fig. 11.** Adhesion of eosinophils to IMR-32 cells was associated with release of eosinophil peroxidase. Eosinophils were incubated alone (○) or with IMR-32 cells that had been differentiated for 7 days with DBcAMP (●), and the supernatant was removed at various time points. Released eosinophil peroxidase was detected as described in MATERIALS AND METHODS. Values are means of 4 separate experiments.
A synergistic interaction between various integrins and adhesion molecules has been reported with other inflammatory cells; for example, binding of VLA-4 on T cells to VCAM-1 strengthens CD11a/18 adhesion to ICAM-1, mediated by an increase in avidity in the β2-integrin (2). A mechanism for this was proposed by Nagel et al. (19), who showed that binding to VLA-4 integrins releases cytohesin-1, which binds to the cytoplasmic tail of the β2-integrin and promotes β2-integrin clustering. Thus inhibition of the interaction between one set of integrin/cell adhesion molecules can reduce the ability of another set to support adhesion. Our data suggest a possible intracellular link via PKC, which is a known secondary messenger in the CD11/18 signaling pathway (22), since the inhibitor rottlerin inhibited the adhesion of eosinophils to the IMR-32 cells to near baseline levels.

Previous studies in both animal models and in humans with asthma have shown a benefit of heparin in ameliorating antigen-induced hyperreactivity (1, 5, 11). This is thought to be because of the ability of this compound to displace the cationic protein MBP from M2 muscarinic receptors (6). In addition, it has been suggested that heparin may also be of benefit in the treatment of a number of other conditions, including inflammatory bowel disease, because of an anti-inflammatory mechanism of action (6). In these studies, it was shown that pretreatment of IMR-32 cells with heparin completely inhibited eosinophil adhesion to these cells. These findings suggest that some of heparin’s anti-inflammatory properties and benefits in asthma may relate to an inhibition of adhesion of inflammatory cells to nerves.

Adhesion of eosinophils to nerve cells would not in itself constitute a mechanism for parasympathetic nerve hyperreactivity. Therefore, it was necessary to demonstrate that the nerve cells influenced eosinophil function. The release of the eosinophil mediator EPO was increased after incubation with IMR-32 nerve cells under conditions in which adhesion took place. This was not simply an effect on peroxidase activity, since similar enhancement of LTC4 release occurred under the same conditions. Because it is known that eosinophil granule proteins mediate the loss of function of neuronal M2 muscarinic receptors (8), the process of nerve cell-mediated eosinophil degranulation may be critical to this dysfunction. The process of nerve cell-mediated eosinophil degranulation may also account for the apparent reduction in adhesion that follows several hours of incubation of eosinophils with nerves that is seen in Fig. 5. The precise mechanism of how nerve cells lead to eosinophil degranulation is the subject of further current work in our laboratory.

In summary, this study suggests that the expression of the adhesion molecules VCAM-1 and ICAM-1 by airway parasympathetic nerves contributes to the process whereby eosinophils localize to airway nerves. Furthermore, the results suggest that eosinophil adhesion to airway nerves may be central to the development of antigen-induced hyperreactivity since eosinophil degranulation follows adhesion.

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