Effects of eosinophils on nerve cell morphology and development: the role of reactive oxygen species and p38 MAP kinase

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Kingham, Paul J., W. Graham McLean, Marie-Therese Walsh, Allison D. Fryer, Gerald J. Gleich, and Richard W. Costello. Effects of eosinophils on nerve cell morphology and development: the role of reactive oxygen species and p38 MAP kinase. Am J Physiol Lung Cell Mol Physiol 285: L915–L924, 2003. First published June 6, 2003; 10.1152/ajplung.00094.2003.—The adhesion of eosinophils to nerve cells and the subsequent release of eosinophil products may contribute to the pathogenesis of conditions such as asthma and inflammatory bowel disease. In this study we have separately examined the consequences of eosinophil adhesion and degranulation for nerve cell morphology and development. Eosinophils induced neurite retraction of cultured guinea pig parasympathetic nerves and differentiated IMR32 cholinergic neuroblastoma cells. Inhibition of eosinophil adhesion to IMR32 cells attenuated this retraction. Eosinophil adhesion to IMR32 cells led to tyrosine phosphorylation of a number of nerve cell proteins, activation of p38 MAP kinase, and generation of neuronal reactive oxygen species (ROS). Inhibition of tyrosine kinases with genistein prevented both the generation of ROS in the nerve cells and neurite retraction. The p38 MAP kinase inhibitor SB-239063 prevented neurite retraction but had no effect on the induction of ROS. Thus eosinophils induced neurite retraction via two distinct pathways: by generation of tyrosine kinase-dependent ROS and by p38 MAP kinase. Eosinophils also prevented neurite outgrowth during differentiation of IMR32 cells. In contrast to their effect on neurite retraction, this effect was mimicked by medium containing products released from eosinophils and by eosinophil major basic protein. These results indicate that eosinophils modify the morphology of nerve cells by distinct mechanisms that involve adhesion and released proteins.

THE ACCUMULATION AND ACTIVATION of eosinophils and their subsequent interaction with other cells are critical in determining the severity of a number of allergic and inflammatory conditions (28, 35). We have previously shown a direct cell-to-cell contact between eosinophils and airway nerves in patients with asthma, accompanied by release of the cationic protein major basic protein (MBP) (5). A similar association of eosinophils with nerves in the gastrointestinal tract has been seen in subjects with inflammatory bowel disease (9, 15). In animal models, eosinophil MBP is associated with the development of vagally mediated hyperreactivity of cholinergic nerves; this is due to inhibition by MBP of protective M3 muscarinic autoreceptor activity (4).

We have also recently shown that primary cultures of parasympathetic nerves and differentiated IMR32 cholinergic neuroblastoma cells express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1, to which eosinophils adhere (29). Engagement of these neural adhesion molecules by eosinophils leads to the generation of reactive oxygen species (ROS) within the nerve cells through an NADPH oxidase-dependent mechanism. Adhesion is also associated with activation and degranulation of eosinophils (18).

In animal models of inflammatory bowel disease, the associated increase in vagally mediated gut motility is followed by a period of diminished cholinergic nerve activity suggestive of a reduction in nerve function (6). We hypothesized that this may be due to the effect of eosinophil localization and adhesion to nerves. Eosinophils could influence nerve morphology or survival via the release of factors; some of these are already known to be toxic to epithelium (23) and muscle (30), whereas others, e.g., nerve growth factor (NGF), can induce cholinergic nerve growth (19). Alternatively, adhesion of eosinophils to neural cell adhesion molecules and the consequent induction of ROS could interfere with microtubule structure and thus induce neurite retraction (14).
In this study we have therefore further developed the in vitro cell culture model to investigate the consequences of eosinophil adhesion and degranulation for cholinergic nerve cell morphology.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were purchased from Invitrogen (Paisley, UK), and tissue culture plastic ware was from Corning (Schenectady, New York). The IMR32 nerve cell line was obtained from ECACC (Salisbury, UK). Percoll was purchased from Amersham Biosciences (Little Chalfont, UK). CD16 immunomagnetic beads and LS+ VarioMacs columns were from Miltenyi Biotech (Bieles, UK). Diff-Quik Fix was from Dade (Munich, Germany). The Dc protein assay kit was purchased from Bio-Rad (Hemel Hempstead, UK). Sodium butyrate, penicillin/streptomycin solution, gentamicin, PBS, paraformaldehyde, Coomassie brilliant blue G, dihydrodorhodamine 123 (DHR), Hoechst 33258 (bis-benzimide), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-formyl-Met-Leu-Phe (fMLP) peptide, diphenylhexatrienyl iodum chloride (DPI), genistein, poly-L-lysine, SDS-PAGE protein molecular weight markers, CDP Star chromaminescent substrate, and all common buffer constituents were obtained from Sigma (Poole, UK). The Rho kinase inhibitor Y-27632 was from Tocris (Bristol, UK). Mouse anti-human CD18 MAb (685A5, isotype IgG2A) was purchased from Cymbus Biotechnology (Chandlers Ford, UK), mouse anti-human phosphotyrosine PY20 MAb was from Autogen Bioclear (Calne, UK), and rabbit anti-human phospho-p38 MAP kinase antibody was from Cell Signaling Technology (Beverly, MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Cyclo(MePhe-Leu-MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Cyclo(MePhe-Leu-MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Cyclo(MePhe-Leu-MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA). Nitro-Block II substrate compound for alkaline phosphatase was from Tropix (Bedford, MA).

Guinea pig eosinophil isolation. Eosinophils were prepared from the blood of healthy human volunteers as previously described (18). Briefly, 15 ml of blood were layered onto a 1.086 g/ml Percoll solution and centrifuged at 400 g for 20 min to yield a pellet containing granulocytes. Containing red blood cells were removed by two hypotonic lysis treatments. Granulocytes were then incubated with immunomagnetic anti-CD16 antibody-conjugated beads (1 μl of beads/106 cells) for 30 min at 4°C before depletion of CD16+ neutrophils through a magnetic separation column (MACS system, Miltenyi Biotech). Cytospins of the eluted cell population stained with Diff-Quik indicated the purity to be ~98% eosinophils. The eluted cells were then washed once with PBS plus EDTA to remove the remaining beads. The cell pellet was resuspended in 1 ml of PBS and centrifuged at 4°C for 5 min to yield a pellet containing eosinophils. Eosinophils (10,000–50,000/well) were added directly to differentiated IMR32 cells in their culture medium. In some experiments, the medium from coincubated eosinophils and IMR32 cells was removed after 2 h of coincubation, centrifuged to remove cells, and added to IMR32 cells. Conditioned medium was also taken from equivalent numbers of eosinophils stimulated for 2 h with 1 μM fMLP and then added to IMR32 cells. Nerves were then fixed in 4% (wt/vol) paraformaldehyde for 20 min at room temperature, stained with Coomassie blue stain [0.6% (wt/vol) Coomassie brilliant blue G in 10% (vol/vol) acetic acid, 10% (vol/vol) methanol, and 80% (vol/vol) PBS] for 10 min, and then washed once with PBS and twice with distilled H2O. Fixed and stained cells were viewed by light microscopy (Zeiss Axiosvert 35M) linked by a video camera to a Kontron Vidas 2.0 image analyzer. The average length of neurites (in pixels) per cell was then calculated automatically from 200 randomly chosen cells as previously described (22). Where appropriate, drugs or enzyme inhibitors were added to IMR32 cells for various times before the addition of eosinophils or conditioned media. Experimental treatments were expressed as percentages of their appropriate control.

Coculture of eosinophils with undifferentiated IMR32 cells and measurement of neurite outgrowth. Eosinophils or their conditioned media were added to IMR32 nerve cells at the initiation of differentiation, and neurite outgrowth was subsequently measured as above. The length of neurites in cells maintained in proliferation medium (and therefore producing few neurites) was subtracted from the length of neurites in differentiating cells. The net neurite length in cells subjected to experimental treatment was then expressed as a percentage of that in control cells.

Measurement of neuronal apoptosis. The number of apoptotic IMR32 cells was assessed by Hoechst 33258 (bis-benzimide) staining. Cells were fixed in 4% (wt/vol) paraformaldehyde for 20 min at room temperature and then stained with Hoechst 33258 (5 μg/ml) in PBS for 10 min. Nuclear morphology was viewed under a fluorescence microscope (Zeiss Axiovert 35M), and apoptotic cells were defined as those that exhibited bright-condensed nuclear (17). Cells were counted in eight randomly chosen fields.

MTT assay. IMR32 nerve cells, eosinophils, or cocultures were incubated with MTT (1 mg/ml) at 37°C for 4 h. The formazan product formed was then solubilized in 20% (vol/vol) Triton X-100 for 30 min with constant agitation. Absor-
bance was then measured at 570 nm in a microplate spectrophotometer.

Measurement of neuronal ROS production. IMR32 nerve cells differentiated for 4–6 days were incubated with 10 μM DHR, an oxidant-sensitive probe (21), for 30 min at 37°C and then washed twice with fresh culture medium. The IMR32 cells were then pretreated with a number of inhibitors before the addition of 5 × 10⁶ eosinophils. Alternatively, an equal number of paraformaldehyde-fixed eosinophils was used. The conversion of DHR to rhodamine 123 was detected in a microplate fluorescence reader (Bio-Tek FL600), with excitation filters at 485 ± 20 nm and emission filters at 530 ± 20 nm.

Gel electrophoresis and Western blotting. After coincubation of eosinophils with IMR32 nerve cells on 35-mm tissue culture dishes, the cultures were washed once with ice-cold PBS and lysed in 100 μl of Triton X-100 cell lysis buffer [1% (vol/vol) Triton X-100, 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 150 μM sodium orthovanadate, and 0.5 mM dithiothreitol]. Alternatively, eosinophils, nerves, or cocultures were treated with 4% paraformaldehyde for 20 min, and lysates were prepared in the same way. All lysates were then incubated at 4°C for 15 min and centrifuged at 12,000 g for 15 min at 4°C, and the supernatant was assayed for protein with the DC protein assay kit (Bio-Rad). Twenty micrograms of protein from each live cell lysate was boiled for 5 min in sample buffer [125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% (wt/vol) bromphenol blue, and 2% (vol/vol) 2-mercaptoethanol]. Proteins were separated by SDS-PAGE in a 10% polyacrylamide resolving gel overlaid with a 4% stacking gel, electrophoresed at 200 V for 45 min, and blotted onto nitrocellulose membranes in transfer buffer [25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol] at 30 V for 2 h. The nitrocellulose membranes were then added to blocking buffer consisting of 1% (wt/vol) BSA in TBS/T [10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% (vol/vol) Tween 20] for 2 h and then incubated overnight at 4°C with mouse anti-human phosphotyrosine PY20 MAb (1:1,000) diluted in blocking buffer. After three 10-min washes in TBS/T, the membranes were incubated for 2 h at room temperature with goat anti-mouse IgG peroxidase conjugate (1:10,000) diluted in blocking buffer. Finally, the membranes were washed in TBS (3 × 10 min) and then exposed to Chemiluminescent substrate for alkaline phosphatase (19:1) for 5 min at room temperature. Blots were then exposed to X-OMAT lightsensitive film to obtain an image.

Statistical analysis. Values are expressed as means ± SE. The statistical significance of differences between control and treated samples was evaluated by one-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Eosinophils induce neurite retraction in guinea pig parasympathetic nerve cultures and the IMR32 cholinergic nerve cell line. When eosinophils were added to guinea pig parasympathetic nerves in culture, there was a noticeable reduction in the length of neurites (Fig. 1). Due to the limited number of nerves obtained from the primary cultures, we subsequently analyzed this phenomenon using the IMR32 nerve cell line. These cells were induced to differentiate into nerve-like cells by the reduction of serum and addition of 2 mM sodium butyrate. Cells treated under these conditions for 5 days display an extensive neurite network (Fig. 2A, i) and other neuronal characteristics such as muscarinic receptor expression (unpublished observations). After 48-h exposure to eosinophils, there was a noticeable reduction in the length of neurites (Fig. 2A, ii). We quantified this effect using computerized image analysis (22) of fixed and Coomassie blue-stained cocultures to investigate the time course of neurite retraction. There was a steady time-dependent decline in the length of neurites with a maximal reduction to 46.13 ± 7.35% of control

Alternatively, when the antiphospho-p38 MAP kinase antibody was used, membranes were incubated in blocking buffer consisting of TBS/T with 5% (wt/vol) nonfat dry milk for 1 h at room temperature. Membranes were then washed 3 × 5 min in TBS/T at room temperature and then incubated overnight at 4°C with rabbit anti-human phospho-p38 MAP kinase antibody (1:1,000) in TBS/T containing 5% (wt/vol) BSA. After 3 × 5-min washes in TBS/T, the membranes were incubated for 2 h at room temperature with goat anti-rabbit IgG alkaline phosphatase conjugate (1:10,000) diluted in blocking buffer. Membranes were then washed in TBS/T (3 × 5 min) and exposed to CDP Star chemiluminescent substrate solution plus Nitro-Block II chemiluminescent substrate compound for alkaline phosphatase (19:1) for 5 min at room temperature. Blots were then exposed to X-OMAT lightsensitive film to obtain an image.

Fig. 1. Guinea pig parasympathetic nerves display shorter neurites in the presence of eosinophils. Parasympathetic nerves were isolated from the trachealis muscle of adult guinea pigs and maintained as described in the MATERIALS AND METHODS and then either cultured alone (A) or in the presence of 1 × 10⁶ eosinophils (B) for a period of 24 h before fixation and staining with dilute hematoxylin. Bar = 80 μm.
at 72 h (Fig. 2B). To determine whether this effect was a consequence of general toxicity due to the eosinophils, we measured both apoptosis and MTT metabolism. There was no significant increase in the number of apoptotic cells as determined by Hoechst 33258 staining up to 72 h, although this did increase to 12.36 ± 2.52% at 96 h (Fig. 2C). There was a small reduction in the level of MTT metabolism in cocultures, falling to 79.97 ± 4.30% of control at 72 h. In contrast, as a positive control, IMR32 cells were deprived of serum, a common model to induce cell death (20). This resulted in a 74% fall in MTT metabolism within 24 h (Fig. 2C).

The effect of eosinophils on neurite retraction was dose dependent (Fig. 3A). Under the conditions of coculture, eosinophils may mediate their effects either via contact or via the release of eosinophil products, since IMR32 nerve cells induce eosinophil degranulation (18). Therefore, we investigated whether adhesion per se or the consequent release of eosinophil products into the medium was responsible for neurite retraction. To generate conditioned medium containing eosinophil products, eosinophils were either incubated with IMR32 cells for 2 h to induce degranulation (18) or treated with 1 μM fMLP, and the medium was collected and transferred to differentiated IMR32 cells for 24 h. There was no significant change in the length of neurites in cultures treated with either set of media, suggesting that the effects of eosinophils on differentiated IMR32 nerve cells were a direct consequence of contact between the cells (Fig. 3, B and C).

Kinase activation and ROS generation in IMR32 nerves mediate neurite retraction. It is established that the processes of neurite retraction and outgrowth can involve protein tyrosine kinase activity (2). To determine whether or not eosinophil-IMR32 cell interactions involved changes in protein tyrosine phosphorylation, we subjected cell extracts to Western blotting and probed them with an antiphosphotyrosine antibody (PY20). To ensure that any observed changes

Fig. 2. Eosinophils induce neurite retraction in differentiated IMR32 nerve cells. A: nerve cells were differentiated for 5 days with medium containing 2 mM sodium butyrate and 2% FCS. Cells were either untreated (i) or exposed to eosinophils (20,000/well) for 48 h (ii) and then fixed in 4% paraformaldehyde and stained with Coomassie brilliant blue G. Bar = 40 μm. B: length of neurites (●) was measured by computerized image analysis for 0–96 h after the addition of eosinophils. Values were expressed as the mean percentage of the neurite length of control nerves (in the absence of eosinophils as described in MATERIALS AND METHODS) ± SE. C: number of apoptotic cells (○) was counted after Hoechst 33258 staining. Cells exhibiting a condensed nucleus when viewed with a fluorescence microscope were scored as apoptotic. Cell viability was also assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (●). Values were expressed as the percentage of control nerves after subtraction of the MTT values for eosinophils alone. Nerve cells were also switched to serum-free medium as a positive control for cell death (— — — ). The graphs show mean of 4 independent experiments.
occurred in the nerves and not the eosinophils, we added paraformaldehyde-fixed eosinophils (which adhere as effectively as live eosinophils) to the IMR32 nerve cells. There was a time-dependent increase in the phosphorylation of a number of proteins in the molecular mass range 30–45 kDa (Fig. 4A). The phosphorylated protein bands were absent when lysates of paraformaldehyde-fixed eosinophils and/or paraformaldehyde-fixed nerves were electrophoresed, providing further evidence that the phosphorylation was occurring in the nerves (Fig. 4B). We also probed Western blots of cell extracts for the activated phosphorylated form of p38 MAP kinase, and this revealed a rapid, transient phosphorylation of the enzyme at 5 min (Fig. 4C).

We have previously shown that eosinophil adhesion via CD18 leads to the production of ROS within the

![Fig. 3. Eosinophils in coculture but not conditioned media influence neurite retraction. A: length of neurites in cultures exposed to various numbers of eosinophils (10,000–50,000/well) was quantified by computerized image analysis at 24 h and expressed as a percentage of the control neurite length as above. Alternatively, the eosinophils were either cultured with differentiated IMR32 nerve cells or treated with N-formyl-Met-Leu-Phe (fMLP, 1 μM) for 2 h, and the resulting conditioned medium was centrifuged to produce a cell-free extract. Differentiated IMR32 nerve cells were then treated with the conditioned medium from eosinophils cultured with nerves (B) or the conditioned medium from eosinophils treated with fMLP (C), and neurite length was measured after 24 h. **P < 0.01 significantly different from control, n = 4.](http://ajplung.physiology.org/)

![Fig. 4. Kinase activation and reactive oxygen species (ROS) generation in IMR32 nerves. A: time course of protein tyrosine phosphorylation and p38 MAP kinase activation. IMR32 cells were cultured in the presence of paraformaldehyde-fixed eosinophils (eos) for the time indicated, the cells were lysed, and 20 μg protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with an antiphosphotyrosine MAb (PY20). The apparent molecular masses of the phosphorylated proteins can be compared against the markers indicated. B: lysates of paraformaldehyde-treated nerves and eosinophils either alone or together after 15-min coincubation were also immunoblotted with the same antibody. C: alternatively, IMR32 cells were cocultured with eosinophils for the indicated times and then probed with antibody to the phosphorylated form of p38 MAP kinase. D: differentiated IMR32 nerves were loaded with the oxidant-sensitive probe dihydorhodamine 123 (DHR, 10 μM) for 30 min and then washed twice in culture medium. Live eosinophils or paraformaldehyde-fixed eosinophils were added, and fluorescence measurements were taken after 2 h i n a plate reader with excitation 485/20 nm and emission 530/20 nm. They are expressed as the means ± SE of 3 independent experiments. ***P < 0.001 significantly different to the absence of eosinophils; ns, not significantly different from live eosinophils. E: DHR-loaded IMR32 cells were also pretreated for 30 min with the tyrosine kinase inhibitor genistein (50 μM), the inactive analog daidzein (50 μM), or the p38 MAP kinase inhibitor SB-239063 (10 μM); eosinophils were added, and fluorescence measurements were recorded after 2 h. Data are expressed as the mean percentage ± SE (n = 6) reduction in fluorescence intensity. **P < 0.01 significantly different from the fluorescence in the absence of inhibitor.](http://ajplung.physiology.org/)
nerve cells, which can be prevented with the NADPH oxidase inhibitor DPI, and that IMR32 cells express the p47phox subunit of NADPH oxidase (18). Nerves were loaded with DHR, an oxidant-sensitive fluorescent probe, and cocultured with either live or paraformaldehyde-fixed eosinophils (Fig. 4D). Both samples of eosinophils induced a significant increase in fluorescence in the nerves, further indicating that the ROS were of neuronal origin and not diffusing from live eosinophils into the nerves. We also treated cocultures of nerves and eosinophils with kinase inhibitors. The increase in fluorescence associated with ROS production was significantly reduced by 72.52 ± 8.29% in the presence of genistein but not daidzein, the inactive analog of genistein (Fig. 4E). The p38 MAP kinase inhibitor SB-239063 also failed to prevent the increase in neuronal ROS induced by eosinophils. Activation of p38 MAP kinase was unaffected by DPI (data not shown).

These signaling pathways were then examined with regard to neurite retraction. Paraformaldehyde-fixed eosinophils were as effective as live eosinophils in reducing the length of neurites, providing further evidence that these effects were not mediated by released eosinophil factors and were a result of nerve signaling following eosinophil adhesion (Fig. 5A). When eosinophils were treated with MAb against CD18 (2 pg/ml) and the very late antigen (VLA)-4 peptide inhibitor ZD-7349 (10 μM) to prevent adhesion (29), there was a significant attenuation of neurite retraction (Fig. 5A). Treatment of the nerves with DPI (1 μM) also reduced the effect of eosinophils to induce neurite retraction (Fig. 5A), indicating a role for ROS. These ROS were of neuronal origin since treating eosinophils with DPI and washing out of the drug before addition of the eosinophils to the nerves did not reduce the eosinophil-mediated retraction (Fig. 5A). Both genistein and SB-239063 significantly reduced the effects of eosinophils on neurite retraction (Fig. 5B), suggesting at least two separate pathways were in operation: one involving ROS production via tyrosine kinases and the other mediated by p38 MAP kinase activation, which does not involve ROS. A possible downstream target of these pathways is Rho kinase (2). However, although the specific Rho kinase inhibitor Y-27632 (10 μM) induced neurite outgrowth on its own, it had no effect on the retraction induced by eosinophils (Fig. 5B).

None of the kinase inhibitors used in these studies interfered with the adhesion of eosinophils to the nerves following 30-min coculture (data not shown), indicating that their effects were via downstream pathways within the nerve cells.

**Eosinophil products inhibit neurite outgrowth.** We also studied the process of neuronal differentiation in the presence of eosinophils or their conditioned medium by measuring the first 48 h of neurite outgrowth. Freshly plated IMR32 nerve cells were switched from proliferation medium to differentiation medium (in the presence or absence of eosinophils) or to differentiation medium that had been conditioned by eosinophils in contact with nerves or treated with fMLP. There was a significant dose-dependent reduction in the length of neurites in nerves directly in contact with eosinophils (Fig. 6A), in nerves treated with conditioned medium from nerve-stimulated eosinophils (Fig. 6B), and to a lesser extent in nerves treated with conditioned medium from fMLP-treated eosinophils (Fig. 6C). These results contrast with the failure of the same conditioned media to cause neurite retraction (Fig. 3).

Eosinophils are known to release a number of highly charged cationic proteins such as MBP and eosinophil peroxidase that may affect nerve function (16). We treated IMR32 nerve cell cultures with both MBP and the synthetic cationic molecule poly-L-lysine and found

![Fig. 5. ROS and kinase activation mediate neurite retraction.](image)

**Fig. 5.** ROS and kinase activation mediate neurite retraction. A: differentiated IMR32 nerves were exposed to live eosinophils or paraformaldehyde-fixed eosinophils. Alternatively, eosinophils were pretreated for 1 h with an MAb against CD18 (2 pg/ml) in combination with the very late antigen-4 peptide inhibitor ZD-7349 (10 μM) to prevent adhesion (29). Either nerves or eosinophils were pretreated for 1 h with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI, 1 μM), the drug was washed out, and nerves were exposed to the eosinophils. B: eosinophils were added to either untreated IMR32 nerves or those pretreated for 1 h with the tyrosine kinase inhibitor genistein (10 μM), the p38 MAP kinase inhibitor SB-239063 (10 μM), or the Rho kinase inhibitor Y-27632 (10 μM). Neurite length was measured after 24 h as described in MATERIALS AND METHODS and is expressed as mean percentage of the control neurite length (in the presence of inhibitors alone where appropriate) ± SE of 4 independent experiments. *P < 0.05, significantly different from the neurite length in the absence of inhibitor.
there was a significant dose-dependent reduction in neurite outgrowth (Fig. 7, A and B) but no effect on neurite retraction (Fig. 7, C and D), which is consistent with our above observations on the effects of conditioned media from eosinophils. Neither compound was directly toxic to the IMR32 nerves (data not shown).

**DISCUSSION**

In this study we describe the consequences of the interactions between eosinophils and cholinergic nerves on the morphology and development of neurites. Eosinophils induced neurite retraction in guinea pig parasympathetic nerves and in the IMR32 nerve cell line. Adhesion of eosinophils to IMR32 cells resulted in both the activation of tyrosine kinases that led to the generation of neuronal ROS and the activation of p38 MAP kinase. Blocking either of these pathways significantly attenuated eosinophil-induced neurite retraction. These effects could not be simulated by medium containing released eosinophil products. In contrast, eosinophils, released eosinophil products, and MBP all inhibited neurite outgrowth. Thus eosinophils exert diverse effects on nerve morphology and development.

Tissue sections taken from patients with inflammatory bowel disease and asthma show that eosinophils adhere to cholinergic nerves and degranulate in their presence (5, 9). The long-term effect of these processes on nerve function is unknown. However, studies in an animal model of inflammatory bowel disease have shown that acute inflammation causes a long-lasting reduction in the magnitude of cholinergic nerve-mediated smooth muscle contraction (6). We hypothesized that one mechanism for this inflammation-dependent reduction in cholinergic nerve function may be reflected in a change in the length of nerve processes.

Our data show that eosinophils caused a retraction of neurites of the cholinergic nerve cell line IMR32. To investigate whether this was the consequence of cell death or apoptosis (24, 26), we made measurements of MTT metabolism and cell apoptosis (using Hoechst 33258 staining). MTT metabolism was not affected by prolonged culture with eosinophils, and there was only a small increase in the number of apoptotic nerves. Therefore, eosinophils were not exerting their effect on neurite retraction via either apoptosis or necrosis; so retraction should not be considered a pathological response to eosinophils but, rather, a physiological remodeling process.

Previously, we have shown that eosinophils adhere to IMR32 nerve cells via specific integrin-cell adhesion molecule interactions and that this leads to the generation of neuronal ROS, which in turn leads to eosinophil degranulation (18). We found that blocking adhesion of eosinophils to nerves with an anti-CD18 MAb and a VLA-4 peptide inhibitor attenuated eosinophil-induced neurite retraction. Furthermore, the NADPH oxidase inhibitor DPI also attenuated neurite retraction, suggesting that adhesion-generated ROS were important. The role of ROS in neurite retraction has
recently been explained by the observation that ROS induce reconfiguration of microtubules (14).

Activation of cell adhesion molecules results in their redistribution at the cell surface, an event often controlled by tyrosine phosphorylation of intracellular proteins (32). Thus we examined whether any nerve proteins were activated in this manner, by probing Western blots of extracts from nerve-eosinophil cocultures with a specific antibody directed against PY20 phosphotyrosine. In our experiments the proteins predominantly undergoing tyrosine phosphorylation in response to eosinophils were in the range 30–45 kDa, and our results indicate that the p38 MAP kinase was activated. Ligation of ICAM-1 on endothelial cells leads to the generation of ROS, in a process mediated via the p38 MAP kinase (33, 34). Activation of the p38 pathway has also been associated with the induction of neurite outgrowth (13), but conversely it can mediate ROS-induced apoptosis in neuroblastoma cells (25). Thus we investigated the role of p38 MAP kinase in eosinophil-mediated neurite retraction and ROS production. We found that, by using the p38 MAP kinase inhibitor SB-239063, we could inhibit neurite retraction but not ROS production. Equally, inhibiting ROS production had no influence on p38 MAP kinase activity. In contrast, the tyrosine kinase inhibitor genistein blocked both ROS production and neurite retraction. Together, these data suggest that two separate pathways involving ROS and p38 activation control the retraction of neurites.

The site at which genistein might be acting in our system remains uncertain. Because interaction of eosinophils with ICAM-1 on nerve cells leads to generation of ROS (18) and ICAM-1 is tyrosine phosphorylated (27), this may be where genistein is exerting its effects. Alternatively, it is possible that genistein prevents the activation of the NADPH oxidase complex by inhibiting the translocation of specific subunits such as p67phox or Rac (8, 10) and thus directly controls ROS formation. We and others have shown that nerves express subunits of the NADPH oxidase complex (18, 31). The GTPase family members Rho and Rac normally exist together in an inactive complex. After an activation signal, Rac dissociates from Rho and translocates to the membrane where it mediates the effects of the NADPH oxidase complex, leading to ROS production (7). This leaves Rho free to activate Rho kinase, which can lead to neurite retraction (1). It therefore occurred to us that genistein could act by preventing the downstream activation of Rho kinase (2). However, although the specific Rho kinase inhibitor Y-27632 induced neurite outgrowth in its own right (data not shown), it was unable to inhibit the effects of eosinophils on neurite retraction, indicating that Rho kinase was not involved.

From our experiments on differentiated nerves, we established that eosinophils induced neurite retraction through a process of cell adhesion but that the effect was independent of eosinophil degranulation, since medium from fMLP-conditioned eosinophils did not induce retraction. We have previously shown that fMLP-treated eosinophils release significant quantities of degranulation and activation products (18). However, the release of products from eosinophils did affect another aspect of nerve function, viz. neurite extension. This was demonstrated by the experiments in which we differentiated IMR32 cells in coculture with either eosinophils or their conditioned media. In both
cases, there was a reduction in the ability of IMR32 cells to extend neurites during differentiation, indicating that released eosinophil products were at least partly responsible. This phenomenon may be specific to eosinophils since neutrophils had the opposite effect, promoting neurite outgrowth (data not shown). In particular, we found that eosinophil MBP could inhibit neurite outgrowth in a dose-dependent manner. The mechanism of this effect is unknown, but part of the action of MBP may be related to its cationic charge, since poly-L-lysine had a similar effect. In contrast to our observations, it has recently been shown that IgA-immune complex induces the release of NGF from eosinophils, which could promote neurite outgrowth (19). However, the study used NGF-dependent PC12 cells, and the eosinophils in our experiments were not activated in the same manner.

In conclusion, our observations have implications for the development and morphology of peripheral autonomic cholinergic nerves in conditions in which there is tissue eosinophilia (3). Although our in vitro model has identified some potential consequences of nerve-immune cell interactions, ultimately further investigation in vivo is required to determine their functional significance.

DISCLOSURES

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