

Identification of Selective Basophil Chemoattractants in Human Nasal Polyps as Insulin-Like Growth Factor-1 and Insulin-Like Growth Factor-2¹

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In a search for novel leukocyte chemoattractants at sites of allergic inflammation, we found basophil-selective chemoattractant activity in extracts of human nasal polyps. The extracts were fractionated by reverse phase HPLC, and the resulting fractions were tested for leukocyte-stimulating activity using sensitive shape change assays. The basophil-selective activity detected was not depleted by a poxvirus CC-chemokine-binding protein affinity column. This activity was further purified by HPLC, and proteins in the bioactive fractions were analyzed by tandem electrospray mass spectrometry. Insulin-like growth factor-2 (IGF-2) was identified in these HPLC fractions, and the basophil-stimulating activity was inhibited by an anti-IGF-2-neutralizing Ab. Recombinant IGF-2 induced a substantial shape change response in basophils, but not eosinophils, neutrophils, or monocytes. IGF-2 stimulated chemokinesis of basophils, but not eosinophils or neutrophils, and synergized with eotaxin-1/CCL11 in basophil chemotaxis. IGF-2 also caused up-regulation of basophil CD11b expression and inhibited apoptosis, but did not stimulate degranulation or Ca²⁺ flux. Recombinant IGF-1 exhibited similar basophil-selective effects as IGF-2, and both growth factors were detected in nasal polyp extracts by ELISA. This is the first demonstration of chemokinetic factors that increase the motility of basophils, but do not act on other granulocytes or monocytes. IGF-1 and IGF-2 could play a role in the selective recruitment of basophils in vivo. *The Journal of Immunology*, 2004, 173: 6448–6457.

Basophils are the rarest granulocyte in the peripheral blood, normally accounting for <1% of total leukocytes, but they accumulate in significant numbers in certain inflammatory responses (1, 2). In addition to being important effector cells in host defense responses to ectoparasitic ticks (3) and prominent in late-phase cutaneous allergic reactions (4), basophils contribute to the cellular infiltrate in the asthmatic lung, where numbers increase after experimental allergen challenge (5). Unlike eosinophils and neutrophils, basophils contain only small amounts of cytotoxic proteins and proteases, but are able to release an array of proinflammatory mediators, including histamine, in response to FcεR1 cross-linking and other stimuli. In particular, basophils can rapidly release large quantities of IL-4 and IL-13 (6–10). Consequently, they are thought to be one of the key cell types involved in biasing an immune response toward a Th2 phenotype and are

likely to play a role in perpetuating allergic inflammatory responses such as asthma (11, 12).

The kinetics of basophil recruitment to sites of allergic inflammation are often distinct from those of other leukocytes. For example, in allergen-induced, late-phase cutaneous reactions, basophil accumulation peaks at 24 h postchallenge, whereas eosinophil infiltration is maximal at 6 h, and basophil numbers do not correlate with the expression of eotaxin-1/CCL11, eotaxin-2/CCL24, RANTES/CCL5, MCP-3/CCL7, or MCP-4/CCL13 (13, 14). Furthermore, basophils can be the major leukocyte type seen in an inflammatory infiltrate, such as in contact dermatitis (15) and responses to ticks (16), suggesting selective recruitment of basophils from the blood. Despite these clinical findings, the mechanisms by which basophils might be recruited selectively from the blood into tissues are not well defined.

Basophils have a large number of receptors for soluble chemoattractants and adhesion molecules on their surface, all of which are also expressed by eosinophils, neutrophils, or monocytes (17, 18). Basophils express several chemokine receptors, many of which are shared with eosinophils (CCR3 and CCR1) and others that are shared with neutrophils (CXCR1 and CXCR2) and monocytes (CCR2). Therefore, it is unclear how basophils might be recruited selectively to sites of allergic inflammation. In a recent study we showed that responses of basophils to certain CC-chemokines were different from those of other leukocytes as a result of their unique expression of multiple chemokine receptors, which can function cooperatively or sequentially (19). However, there may be more specific mechanisms or mediators that bring about the selective recruitment of basophils to sites of allergic inflammation.

We have developed a novel flow cytometric assay of cell stimulation that quantifies the increase in forward scatter of leukocytes that occurs as cells in suspension change shape in response to agonists (20). This assay, which has been adapted to measure

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Received for publication February 9, 2004. Accepted for publication September 8, 2004.

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¹ This work was supported by the Wellcome Trust (Program Grant 038775/Z/96/A; to A.H. and D.M.C.), Asthma U.K. (to T.J.W. and P.J.J.), the Austrian Science Fund FWF (Grant P15453; to A.H.), the Royal Society (Grant HA/ESEP/JP 20527; to A.H. and A.He.), the Arthritis Research Campaign (to R.W.), the Medical Research Council (to R.W.), and the Kennedy Institute trustees (to R.W.).

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responses of human basophils (19), in addition to eosinophils, neutrophils, and monocytes (20), is highly sensitive, detecting responses to <100 pM eotaxin-1/CCL11, and is therefore a useful tool for identifying leukocyte-stimulating activity in extracts of inflamed tissue. We have used this assay to investigate nasal polyp tissue, using a similar strategy to that used previously to identify eotaxin-1/CCL11 (21). Nasal polyps share many of the histopathological features of asthma and allergic rhinitis, including elevated numbers of inflammatory cells such as eosinophils and basophils (22–24). In analyzing HPLC fractions of human nasal polyp extracts for novel leukocyte-stimulating activities, we identified a non-CC-chemokine activity that exhibited high efficacy and selectivity for basophils. This activity was identified as insulin-like growth factor-2 (IGF-2).⁴ In addition, IGF-1, which signals through the same receptor as IGF-2, was found to be a potent and selective basophil stimulator and was also detected in nasal polyps. Our data suggest that IGF-1 and IGF-2 may play roles in the selective recruitment of basophils *in vivo*.

Materials and Methods

Reagents

BSA, glucose, Histopaque 1077, cytochalasin B, fluo-3 acetoxymethyl ester, probenecid, Pluronic F-127, C5a, and anti-human HLA-DR mAb (clone HK14) conjugated to FITC or Quantum Red (QR) were obtained from Sigma-Aldrich (Poole, U.K.). Trifluoroacetic acid (TFA; peptide synthesis grade) was purchased from Rathburn (Walkerburn, U.K.) and acetonitrile (ACN; far UV grade for HPLC) was obtained from Merck (Poole, U.K.). PBS, RPMI 1640, and HEPES were purchased from Invitrogen Life Technologies (Paisley, U.K.). PE-conjugated anti-human CDw123 (clone 7G3), FACSflow, and CellFix were obtained from BD Biosciences (Oxford, U.K.). Anti-CD63-FITC was purchased from Autogen Bioclear (Calne, U.K.), and anti-CD11b-FITC was obtained from DakoCytomation (Ely, U.K.). Human recombinant eotaxin-1/CCL11, IL-8/CXCL8, MCP-1/CCL2, IGF-1, IGF-2, IL-3, and rabbit anti-human IGF-2 (500-P12) were purchased from PeproTech (London, U.K.). Goat anti-human IGF-2 Ab (AF-292-NA) and anti-CCR3-FITC were obtained from R&D Systems (Oxford, U.K.). Kits for negative selection of eosinophils and basophils were purchased from StemCell Technologies (Vancouver, Canada).

Protein purification

Nasal polyp tissue was collected from patients undergoing elective polypectomy. The tissue was surgically removed, placed in PBS at 4°C, transported on ice to the laboratory, cleaned of surrounding tissue, snap-frozen, and stored at –70°C. For analytical experiments, 6 g of nasal polyp tissue was homogenized at pH 2 in 9 vol of 0.2% TFA and 1 M NaCl and centrifuged to remove precipitated material. The supernatant was desalted and concentrated by use of two C₁₈ Environmental Sep-Pak cartridges in series (Waters, Watford, U.K.), eluted with ACN, and freeze-dried. The extract was applied to a wide pore (300 Å) Vydac C₁₈ reverse-phase column (4.6 × 250 mm; HPLC Technology, Cheshire, U.K.) and eluted with a linear gradient of ACN (0–80%, over 80 min) in 0.08% TFA at 1 ml/min. Fractions (1 min) were collected, and aliquots were lyophilized with carrier protein (0.01% BSA) before bioassay for leukocyte-stimulating activity and ELISA.

For purification of the basophil-selective activity, 100 g of nasal polyp tissue was extracted in 0.2% TFA and 0.75 M NaCl; concentrated with C₁₈ Sep-Paks; freeze-dried; dissolved in 10 mM sodium phosphate buffer containing 0.5 M NaCl, 10 mM EDTA, and 0.1% azide, pH 7.4; and depleted of CC chemokines by affinity chromatography. For the affinity column, a 35-kDa poxvirus CC chemokine-binding protein-Fc fusion protein was expressed in Chinese hamster ovary cells (25), purified on protein A-Sepharose, and covalently attached to this matrix with 20 mM dimethylpiperimidate (26). Five percent of the affinity column effluent was kept for analytical reverse phase HPLC (RP-HPLC), and the remainder was dialyzed in 3.5 kDa *M_r* cutoff tubing (Mediatech International, London, U.K.) against 20 mM sodium phosphate buffer, pH 7.4, then passed sequentially through 4-ml columns of carboxymethyl-Sepharose and DEAE-Sepharose

previously equilibrated in the same buffer. The cation and anion eluates (2 M NaCl in 20 mM phosphate buffer, pH 7.4) were adjusted to 0.5 M NaCl and pH 2 with TFA and applied to RP-HPLC as described above. The basophil-selective RP-HPLC fraction from the DEAE-Sepharose eluate was lyophilized for anion exchange HPLC on a Protein-Pak DEAE 5PW 8 × 7-mm column (Waters) in 20 mM Tris-HCl buffer, pH 8.0, at 1 ml/min, using a linear gradient of 0–2 M NaCl over 40 min. Aliquots of the 1-ml fractions were mixed with carrier BSA (0.01%) and desalted using 3 kDa *M_r* cutoff centrifugal filters (Microcon YM-3; Millipore, Watford, U.K.) for bioassay.

Mass spectrometry

In-gel trypsinolysis was performed using an Investigator Progest (Genomic Solutions, Huntingdon, U.K.) robotic digestion system, as previously described (27). The resulting mixtures of peptides were characterized by tandem electrospray HPLC mass spectrometry on a Micromass Q-ToF spectrometer interfaced to a Waters CapLC chromatograph (28). Mass spectra were searched against SwissProt/TREMBL as previously described (28).

ELISA

Eotaxin-1 (CCL11) was measured by sandwich ELISA as described previously (29). Matched pairs of monoclonal capture and biotinylated detector Abs from R&D Systems (Abingdon, U.K.) were used for all other chemokines and for IGF-1. In-house assays with polyclonal Abs, biotinylated for the detector application, were used for IGF-2 and C5a. Biotinylated Abs were quantitated with neutravidin-conjugated HRP (Perbio Science, Cheshire, U.K.) and enhanced K-blue TMB substrate (Neogen, Lexington, KY).

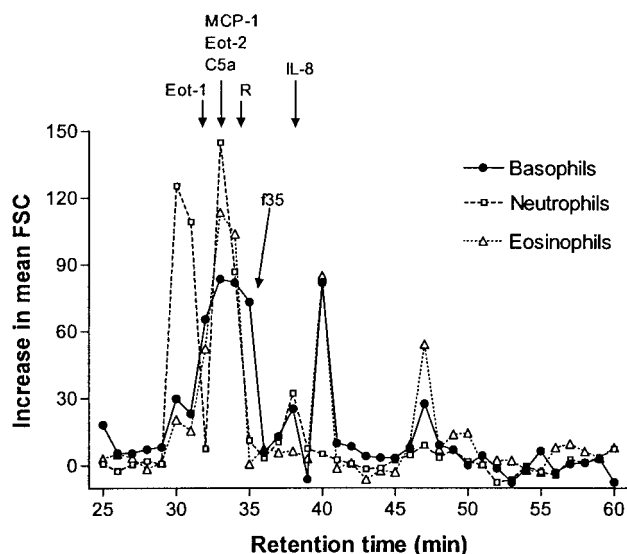


FIGURE 1. Leukocyte shape change responses to nasal polyp extract (6 g) fractionated by RP-HPLC. The reverse phase column was eluted with a linear gradient of acetonitrile (0–80%, over 80 min) in 0.08% TFA at 1 ml/min. Aliquots of the 1-ml HPLC fractions were lyophilized, reconstituted to the original volume in assay buffer, and added to leukocytes at a final dilution of 1/10 to measure shape change responses. Leukocytes were incubated with HPLC fractions for 4 min at 37°C and fixed, and their shape change was measured by flow cytometry as the increase in mean forward scatter (FSC). Basophils and eosinophils exhibited similar profiles of shape change responses; the major region of activity was associated with CC-chemokines such as eotaxin-1/CCL11, eotaxin-2/CCL24, and RANTES/CCL5. The distinct profile of neutrophil responses probably reflects the presence of CXC chemokines. Significantly, fraction 35 (f35; retention time, 34–35 min) stimulated a shape change response in basophils, but not eosinophils or neutrophils. The data shown are the mean of two (basophil) or three (eosinophil and neutrophil) independent experiments, each using cells from different donors. Arrows indicate the fractions containing immunoreactive eotaxin-1 (Eot-1), eotaxin-2 (Eot-2), MCP-1, C5a, RANTES (R), IL-8, and fraction 35 (f35).

⁴ Abbreviations used in this paper: IGF, insulin-like growth factor; ACN, acetonitrile; CC-CKBP, poxvirus-encoded CC chemokine-binding protein; IGF-BP, IGF-binding protein; IGF-R1, type 1 IGF receptor; PMN, polymorphonuclear; QR, Quantum Red; RP-HPLC, reverse phase HPLC; TFA, trifluoroacetic acid.

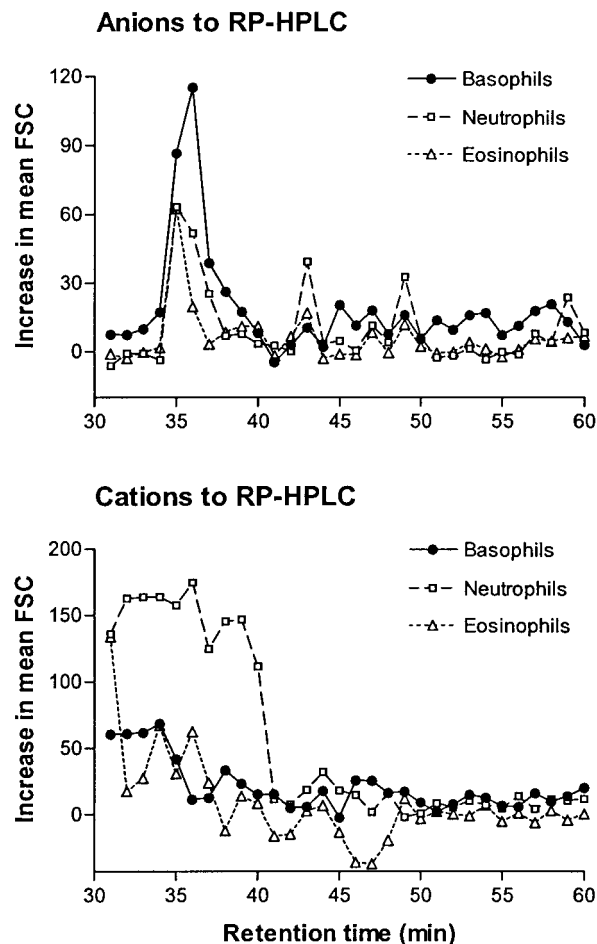


FIGURE 2. Leukocyte shape change responses to nasal polyp extract subjected to ion exchange chromatography, to select anionic and cationic proteins, followed by RP-HPLC. Nasal polyp extract (100 g), depleted of CC-chemokines, was passed sequentially through carboxymethyl- and DEAE-Sepharose columns, and the eluates were applied separately to RP-HPLC. The C_{18} column was eluted with a linear gradient of acetonitrile (0–80%, over 80 min) in 0.08% TFA at 1 ml/min. Aliquots of the 1-ml HPLC fractions were lyophilized, reconstituted to the original volume in assay buffer, and added to leukocytes at a final dilution of 1/10 to measure shape change responses. There was substantial basophil-stimulating activity in RP-HPLC fractions 35 and 36 (retention time, 34–36 min) of the anionic components, but much less stimulating activity for eosinophils or neutrophils (*top panel*). The eluate from the cation column contained bioactivity for all three cell types, neutrophils $\gg$ basophils = eosinophils, consistent with the presence of CXC chemokines and C5a (*lower panel*). A representative graph is shown of two independent experiments, each using cells from different donors.

Preparation of human leukocytes

Blood was obtained from healthy volunteers, after obtaining informed consent, according to local ethics committee-approved protocols. Leukocytes from citrated whole blood were separated into a polymorphonuclear (PMN) cell population (containing eosinophils and neutrophils) and a PBMC population (containing basophils, monocytes, and lymphocytes) by dextran sedimentation and centrifugation on Histopaque 1077 as described previously (19, 20, 30, 31). For chemotaxis experiments, basophils and eosinophils were further purified from PBMC or PMN preparations, respectively, by negative magnetic selection using Ab mixtures from Stem-Cell Technologies, according to the manufacturer's protocol. Basophil purity was $>85\%$, and eosinophil purity was $>90\%$ by Kimura staining (32) or immunofluorescence, the contaminating cells being lymphocytes in both cases.

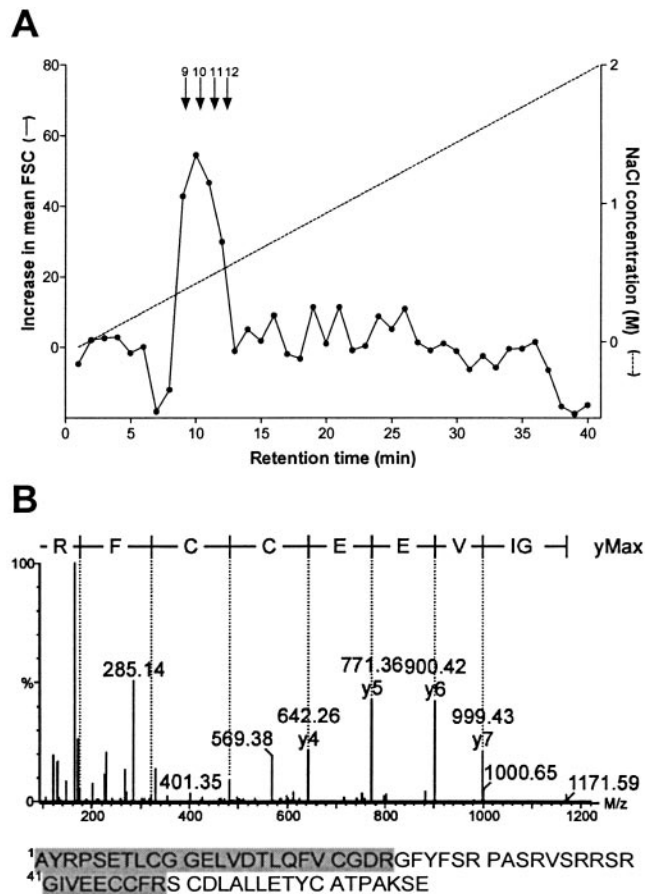


FIGURE 3. A, Basophil shape change response to nasal polyp extract fractionated by anion exchange HPLC after purification on DEAE-Sepharose and RP-HPLC. RP-HPLC fraction 36 of the crude anionic preparation, containing basophil-stimulating activity, was subjected to a second anion exchange step, this time using HPLC, and eluted with a linear gradient of NaCl (0–2 M, over 40 min) at 1 ml/min. Aliquots of the 1-ml fractions were desalted using 3 kDa cutoff centrifugal filters and were assayed at a final dilution of 4/5. A single peak of basophil-stimulating activity was detected in fractions 9–12 (indicated by the arrows), eluting at ~ 0.4 – 0.6 M NaCl. B, Tandem mass spectrum of the doubly charged ion at m/z 585, from which the amino acid sequence G(L/I)VEECCFR (residues 41–49 of IGF-2) was deduced. The full amino acid sequence of the processed IGF-2 protein is shown below; the shaded regions correspond to the two peptides sequenced by tandem mass spectrometry.

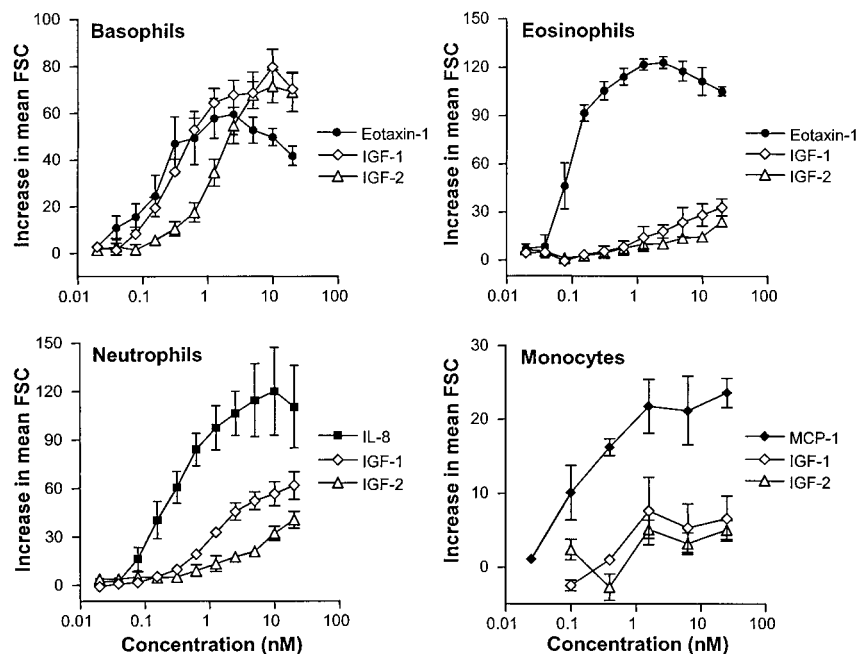
Leukocyte shape change

Eosinophil, neutrophil, basophil, and monocyte shape change was assayed as described previously (19, 20). PMN or PBMC preparations were resuspended in assay buffer (PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$, supplemented with 0.1% BSA, 10 mM HEPES, and 10 mM glucose, pH 7.4) at 1×10^7 cells/ml, and 25- μl aliquots were mixed with 25 μl of agonists and incubated for 4 min at 37°C . To stop the reaction, samples were transferred to ice and fixed with 100 μl of fixative solution ($1 \times$ CellFix diluted 1/4 with FACSFlow). Eosinophils were distinguished from neutrophils by their higher autofluorescence (20). For basophil shape change, PBMC were stained with anti-HLA-DR-FITC and anti-CD123-PE (1/50 and 1/100 dilutions of the Abs, respectively) for 10 min at room temperature and washed before use; basophils were identified as CD123 $^{\text{pos}}$ /HLA-DR $^{\text{neg}}$ cells (19). For monocyte shape change, PBMC were stained with anti-CD14-FITC (1/100 Ab dilution) for 10 min at room temperature, washed, and used (20). Samples were analyzed immediately on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA), and data were displayed as the increase in mean forward scatter compared with that in samples treated with buffer alone.

Chemotaxis

Twenty microliters of purified cells, at $1 \times 10^6/\text{ml}$ in RPMI 1640 with 25 mM HEPES and 0.1% BSA, was placed on top of 96-well chemotaxis

FIGURE 4. Leukocyte shape change responses to rIGF-1 and rIGF-2. Leukocytes were incubated with agonists for 4 min at 37°C and fixed, and their shape change was measured by flow cytometry as increase in mean forward scatter (FSC). IGF-2 stimulated a shape change response in basophils, but not eosinophils, neutrophils, or monocytes, with a maximal response equal to that of eotaxin-1/CCL11, but with ~5-fold lower potency. IGF-1 also stimulated basophil shape change, with a potency similar to that of eotaxin-1/CCL11. Eosinophils and monocytes did not respond to IGF-1, whereas neutrophils responded weakly at higher IGF-1 concentrations. Data are the mean $\pm$ SEM ($n = 7$ (basophils) or $n = 3$ (eosinophils, neutrophils, and monocytes)); each experiment used cells from different donors.



chambers with 5- μ m pore size polycarbonate filters (NeuroProbe, Gaithersburg, MD), and 30 μ l of agonist or buffer was placed in the bottom wells. The plates were incubated at 37°C in a humidified CO₂ incubator for 1 h, and the membrane was removed carefully. Cells that had migrated into the lower chamber were removed, the wells were washed with 20 μ l of assay buffer, and 100 μ l of diluted CellFix was added to the cells before counting by flow cytometry, as previously described (33). Migrated eosinophils and neutrophils were distinguished from lymphocytes according to their forward and side scatters. To distinguish basophils from lymphocytes, cells were stained by adding anti-HLA-DR-FITC and anti-CD123-PE (1/50 and 1/100, respectively) and incubating for 10 min at room temperature before diluting with fixative. In some experiments basophils were mixed with various concentrations of agonist immediately before being applied to the chemotaxis chamber, and migration toward buffer was determined (chemokinesis). In each chemotaxis plate, migration in response to agonist was calculated as a ratio of the migration in response to the buffer control (chemotactic index). To investigate possible synergism between IGFs and eotaxin-1/CCL11, basophils were mixed with various concentrations of IGF-1 or IGF-2 before being applied to the chemotaxis chamber and migration toward 1 nM eotaxin-1/CCL11 determined. For these experiments migration in response to IGF alone was expressed as a ratio of migration in the absence of IGF, as before.

CCR3 expression

PBMC preparations were resuspended in assay buffer at 5×10^6 cells/ml and were incubated with IGF-2 (100 nM) alone for 1 or 4 h or for 30 min, followed by the addition of eotaxin-1/CCL11 (10 nM) for an additional 30 min at 37°C. The cells were then washed in ice-cold staining buffer (PBS containing 0.1% sodium azide and 0.5% BSA) and incubated with anti-CCR3-FITC together with anti-HLA-DR-QR and anti-CD123-PE on ice. After washing, the cells were analyzed immediately by flow cytometry. Data were expressed as the percent change from a control sample incubated with buffer for 1 h.

CD11b expression

PMN and PBMC preparations were resuspended in assay buffer at 5×10^6 cells/ml together with agonists and incubated for 30 min at 37°C. The cells were washed in ice-cold staining buffer and incubated with anti-CD11b-FITC together with anti-CD16-PE for PMN or anti-HLA-DR-QR and anti-CD123-PE for PBMC on ice. After washing, the cells were analyzed immediately by flow cytometry. Data were expressed as the percent change from a control sample incubated with buffer alone.

CD63 expression

Basophil degranulation was assayed by flow cytometric detection of the granule-associated marker CD63. PBMC preparations were labeled with

anti-HLA-DR-QR and anti-CD123-PE for 6 min at room temperature, washed in PBS without Ca²⁺/Mg²⁺, and resuspended in assay buffer at 5×10^6 cells/ml in the presence of anti-CD63-FITC (1/100 dilution). Cells were then pretreated with cytochalasin B (5 μ g/ml) for 5 min at 37°C, and 50- μ l aliquots were mixed with 50 μ l of agonists and incubated for 30 min at 37°C. To stop the reaction, samples were transferred to ice, washed in cold PBS without Ca²⁺/Mg²⁺, and fixed with 250 μ l of fixative solution. Samples were analyzed immediately by flow cytometry. Basophils were identified as CD123^{pos}/HLA-DR^{neg} cells. Data were expressed as the percent change from a control sample incubated with buffer alone.

Calcium flux

Changes in intracellular calcium levels were analyzed by three-color flow cytometry, as described previously (34). Briefly, PBMC were labeled with

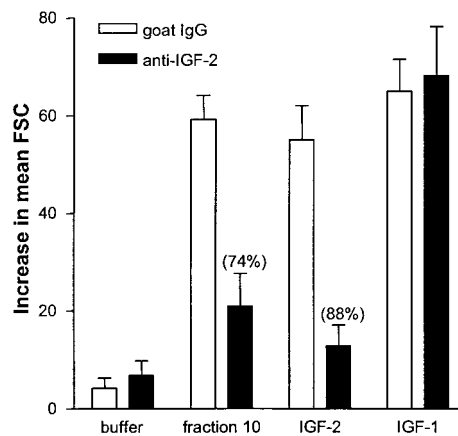


FIGURE 5. Inhibition of the basophil-stimulating activity purified from nasal polyp extract by a neutralizing Ab to IGF-2. Fraction 10 from the final anion exchange HPLC purification was preincubated with goat anti-IGF-2 or goat IgG as a control at 1 μ g/ml for 15 min at room temperature before assaying for basophil shape change activity. The presence of goat IgG increased the baseline forward scatter slightly (buffer). A substantial increase in mean FSC occurred in response to fraction 10 (2/5 dilution; ~16 nM IGF-2), IGF-1 (5 nM), and IGF-2 (5 nM). The response to fraction 10 was inhibited by 74% in the presence of anti-IGF-2 compared with control goat IgG. Similarly, the response to IGF-2 was inhibited by 88%, whereas the IGF-1-stimulated response was unaffected. Data are the mean $\pm$ SEM ($n = 3$); each experiment used cells from different donors.

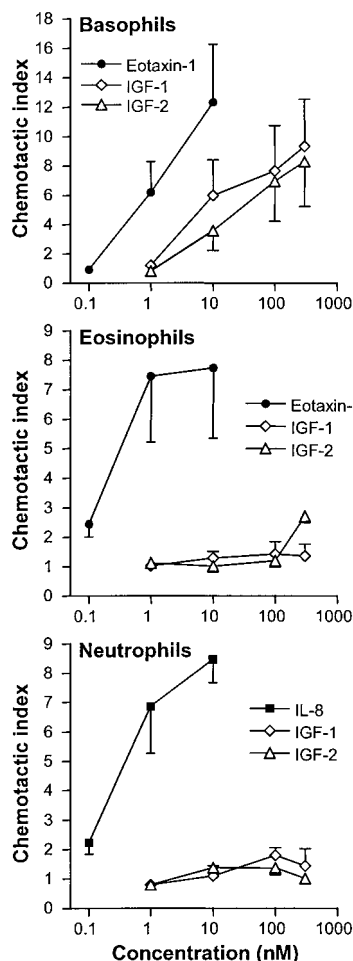


FIGURE 6. Chemotaxis of leukocytes in response to IGF-1 and IGF-2. Purified cells were placed onto the top of 96-well chemotaxis chambers with agonists in the wells below. After incubation at 37°C for 1 h, migrated cells were counted by flow cytometry. In each chemotaxis plate, migration in response to agonists was calculated as a ratio of the migration in response to the buffer control (chemotactic index). IGF-1 and IGF-2 stimulated basophil migration, but were not as potent as eotaxin-1/CCL11. Eosinophils and neutrophils did not migrate in response to IGF-1 or IGF-2, but showed a good chemotactic response to eotaxin-1/CCL11 and IL-8/CXCL8, respectively. Data are the mean $\pm$ SEM ($n = 4-6$ (basophils) or $n = 3$ (eosinophils and neutrophils)).

anti-HLA-DR-QR and anti-CD123-PE (1/20 dilution of the Abs) for 6 min at room temperature, washed, and resuspended in assay buffer without $\text{Ca}^{2+}/\text{Mg}^{2+}$ at 1×10^7 cells/ml. The cells were then treated with $2 \mu\text{M}$ of the acetoxymethyl ester of fluo-3 in the presence of 0.02% Pluronic F-127 for 20 min at room temperature, washed in assay buffer without $\text{Ca}^{2+}/\text{Mg}^{2+}$ containing probenecid, and resuspended in the same buffer at 5×10^6 cells/ml. Aliquots (950 μl) of the leukocyte suspension were removed and treated with 50 μl of PBS containing Ca^{2+} (36 mM) and Mg^{2+} (20 mM) for 5 min. Changes in intracellular free calcium levels of basophils ($\text{CD123}^{\text{pos}}/\text{HLA-DR}^{\text{neg}}$) were detected by flow cytometry as the increase in fluo-3 fluorescence intensity in fluorescence channel-1. Time kinetics of calcium flux were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Apoptosis assay

Apoptosis of basophils was determined as described previously (35). Purified basophils were resuspended at 10^5 cells/ml in RPMI 1640 containing 100 U/ml penicillin and 100 mg/ml streptomycin, and 500- μl aliquots were incubated at 37°C in a humidified CO_2 incubator in the absence or the presence of IGF-2 (20 nM) and/or IL-3 (10 pM). At various time points (0–48 h), aliquots were removed and washed twice in PBS, and the cells were resuspended in binding buffer. Basophils were then stained with an-

nexin V-FITC (1/100) and propidium iodide (1/50) in the dark for 10 min at room temperature and immediately analyzed by flow cytometry. Each sample was acquired for 1 min, and the total number of basophils gated on a forward scatter/side scatter plot and the percentage of nonapoptotic cells (annexin $\text{V}^{\text{neg}}/\text{propidium iodide}^{\text{neg}}$), early apoptotic cells (annexin $\text{V}^{\text{pos}}/\text{propidium iodide}^{\text{neg}}$), and necrotic cells (annexin $\text{V}^{\text{pos}}/\text{propidium iodide}^{\text{pos}}$) were recorded.

Results

Nasal polyp tissue was homogenized in 1 M NaCl at pH 2, and the supernatant was subjected to RP-HPLC. The presence of basophil-, eosinophil-, and neutrophil-stimulating activity in the RP-HPLC fractions was determined by measuring shape change responses (Fig. 1). A similar profile of shape change activity was seen for basophils and eosinophils; the major peak of activity was associated with a broad peak containing several CC chemokines (including eotaxin-1/CCL11, eotaxin-2/CCL24, RANTES/CCL5, and MCP-1/CCL2), whereas the profile of neutrophil shape change activity was distinct, probably reflecting responses to CXC chemokines. Significantly, one fraction (fraction 35; retention time, 34–35 min) adjacent to the major peak of CC chemokines, stimulated basophils, but not eosinophils, neutrophils (Fig. 1), or monocytes (data not shown). Of the chemokines measured, only RANTES/CCL5 (the majority of which eluted in fraction 34) was detected in fraction 35, and its concentration in the shape change assay (<0.1 nM) was insufficient to account for the basophil response. To test the possibility that the activity was due to a basophil-stimulating CC chemokine not measured by ELISA, such as MCP-3/CCL7 or MCP-4/CCL13, basophil shape change was measured after stimulation with fraction 35 in the presence of a pox-virus-encoded, 35-kDa CC-chemokine-binding protein (CC-CKBP) that blocks the functional activity of eotaxin-1/CCL11 and other CC-chemokines (25). The bioactivity was not blocked by the CC-CKBP (data not shown), suggesting that it was unlikely to be due to a CC-chemokine.

To purify this basophil-selective activity, 100 g of nasal polyp tissue was extracted and depleted of CC chemokines using a CC-CKBP affinity column. The flow-through from the affinity column was passed over cation and anion exchange columns, and the two eluates were fractionated by RP-HPLC as before. A basophil-selective activity was found in the eluate from the anion exchange column (fraction 36; retention time, 35–36 min), whereas the eluate from the cation column contained bioactivity predominantly for neutrophils (Fig. 2). The activity in RP-HPLC fraction 36 was not sufficiently pure for identification; therefore, this fraction was subjected to gradient anion exchange HPLC, followed by bioassay (Fig. 3A). Fractions 9–12, which eluted at $\sim 0.4-0.6$ M NaCl, contained the basophil-stimulating activity and were analyzed by SDS-PAGE and mass spectrometry.

SDS-PAGE of fraction 11 revealed a complex pattern of silver-stained bands, of which 24 were excised, subjected to in-gel trypsinolysis, and analyzed by tandem mass spectrometry, enabling identification of >20 discrete proteins. Most of these could be eliminated as candidates for the biological activity, because 10 were also present in inactive fractions (fractions 8 and 13); of the remainder, only IGF-2 was detected in the corresponding band of fraction 9. The identification of this protein was based on the tandem MS sequencing of two peptides, AYRPSETLCGGELVDTLQFVCGDR and GIVEECCFR, which correspond to the N-terminal 24 residues and residues 41–49 of IGF-2, respectively (Fig. 3B).

Subsequent to the identification of IGF-2 in nasal polyp extracts, rIGF-2 was tested and found to stimulate a substantial shape change response of basophils, but had little or no effect on eosinophils, neutrophils, and monocytes (Fig. 4). IGF-2 was ~ 5 -fold less potent than eotaxin-1/CCL11 in the basophil shape change

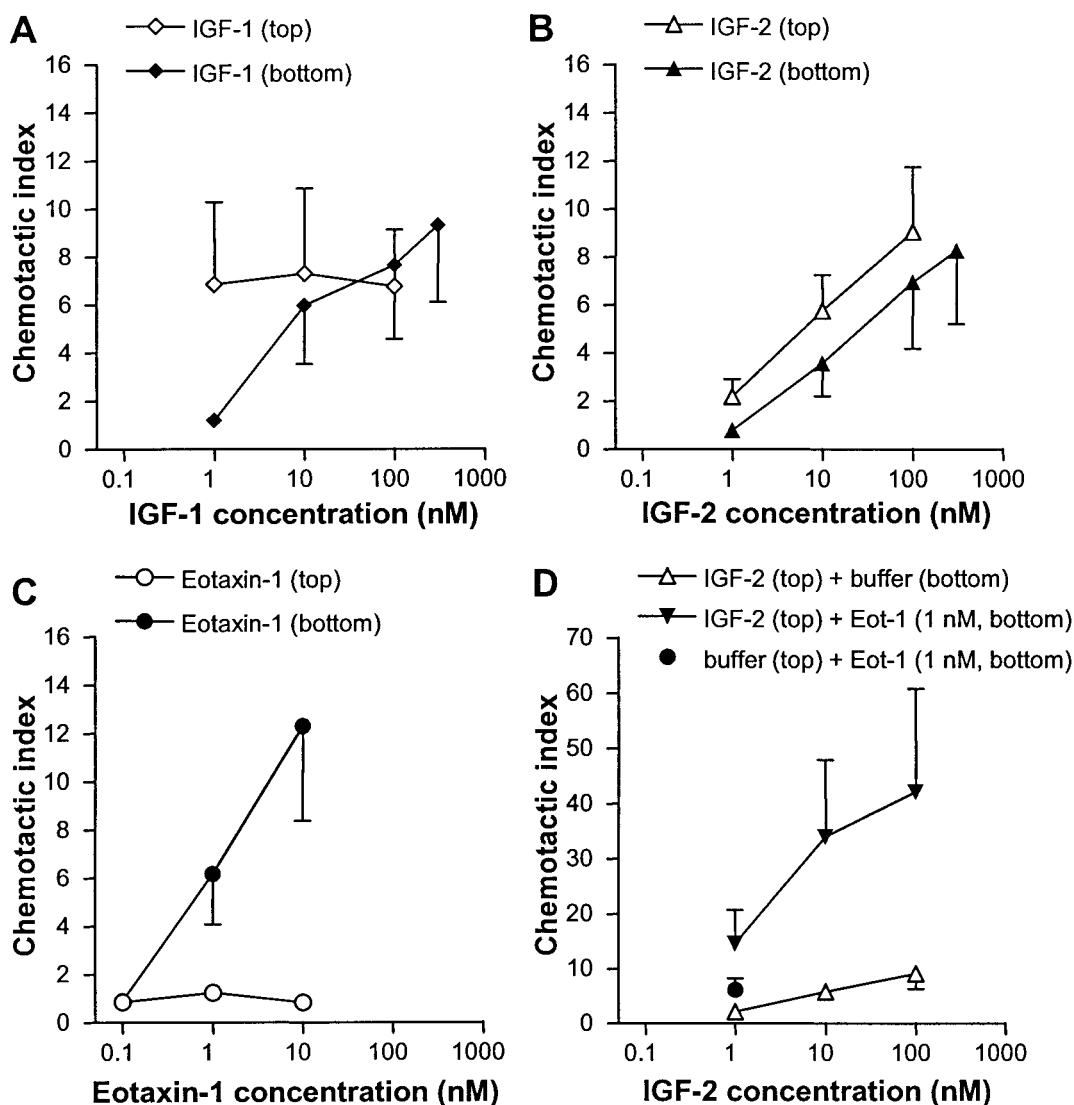


FIGURE 7. IGF-1 and IGF-2 are chemokinetic and enhance basophil chemotaxis toward eotaxin-1/CCL11. Purified basophils were placed on top of 96-well chemotaxis chambers with agonists or buffer in the wells below. After incubation at 37°C for 1 h, migrated cells were counted by flow cytometry. In each chemotaxis plate, migration in response to agonists was calculated as a ratio of the migration in response to the buffer control (chemotactic index). Basophils were either mixed with various concentrations of IGF-1 (*A, top*), IGF-2 (*B, top*), or eotaxin-1 (*C, top*), immediately before being applied to the chemotaxis chamber with assay buffer in the lower wells (chemokinesis) or were mixed with assay buffer and applied above wells containing IGF-1 (*A, bottom*), IGF-2 (*B, bottom*), or eotaxin-1 (*C, bottom*). Basophils were also mixed with various concentrations of IGF-2, and migration toward 1 nM eotaxin-1/CCL11 or buffer in the lower well was quantified (*D*). Data are the mean $\pm$ SEM ($n = 4-6$).

assay; the threshold concentration was ~ 0.5 nM IGF-2 compared with <0.1 nM eotaxin-1/CCL11, but the maximal response was of similar magnitude for both agonists (Fig. 4). Recombinant IGF-1, which signals through the same receptor as IGF-2, also stimulated basophil shape change, but with a potency similar to that of eotaxin-1/CCL11 (Fig. 4). IGF-1 caused a partial response in neutrophils at high concentrations, which was $<50\%$ of the maximal response to IL-8/CXCL8, but was ineffective in eosinophil and monocyte shape change (Fig. 5).

To confirm that the basophil-stimulating activity in the anion exchange HPLC fractions (Fig. 3A) was IGF-2, a neutralizing polyclonal Ab specific for IGF-2 (AF-292-NA; R&D Systems) was preincubated with fraction 10 before performing the shape change assay. This Ab inhibited the basophil shape change response to 5 nM IGF-2 (88% inhibition), but had no effect on the response to 5 nM IGF-1 (Fig. 5). The basophil-stimulating activity in fraction 10, the fraction with maximum activity, was markedly

reduced (74% inhibition) by this neutralizing Ab (Fig. 5) and also by another specific anti-IGF-2 polyclonal Ab (500-P12; Pepro-Tech; data not shown). The basophil-stimulating activity in fraction 12 was similarly inhibited by anti-IGF-2 (69% inhibition; data not shown).

To quantify IGF-2 in the HPLC fractions, a sandwich ELISA was developed using commercially available Abs. The concentration of IGF-2 in the peak anion exchange HPLC fraction (fraction 10; Fig. 3A) was found to be 39.5 nM; therefore, the concentrations of IGF-2 in the bioassays of this fraction were ~ 32 nM (Fig. 3A) and 16 nM (Fig. 5), which are supramaximal concentrations in this assay (Fig. 4). No IGF-1 was detected in fraction 10 by ELISA (detection limit, 0.31 nM). When we measured IGF-2 and IGF-1 for comparison in diluted extracts of individual nasal polyps, we detected both IGF-1 and IGF-2. However, because the quantities were close to the limit of detection, especially for IGF-1, we obtained a better estimate of the relative amounts of these growth

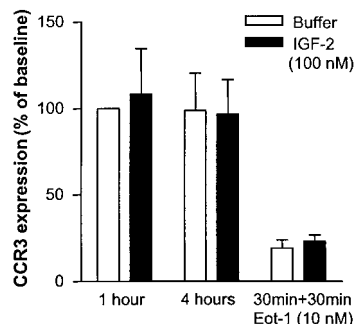


FIGURE 8. Effect of IGF-2 on basophil CCR3 expression. PBMC were incubated with IGF-2 (100 nM) alone for 1 or 4 h or for 30 min, followed by eotaxin-1/CCL11 (10 nM) for an additional 30 min at 37°C, then stained with anti-CCR3-FITC together with anti-HLA-DR-QR and anti-CD123-PE. CCR3 expression on CD123^{pos}/HLA-DR^{neg} basophils was quantified and is represented as the percent change from a control sample incubated with buffer for 1 h. IGF-2 did not have a significant effect on CCR3 expression at either time point and did not alter the down-regulation of CCR3 expression that occurred after incubation with eotaxin-1/CCL11. Data are the mean $\pm$ SEM ($n = 5$); each experiment used cells from different donors.

factors by ELISA of RP-HPLC fractions of a nasal polyp extract before extensive purification (5% of the 100-g extract after depletion of CC chemokines, but without any ion exchange chromatography; see *Materials and Methods*). There was ~ 4.5 -fold more IGF-2 than IGF-1 (17.8 pmol of IGF-2/g tissue vs 4.0 pmol of IGF-1/g tissue) in these fractions, and IGF-2 was found to elute slightly later than IGF-1 in RP-HPLC under the conditions used.

Basophil responses to IGF-1 and IGF-2 were investigated further using recombinant protein. We found that IGF-1 and IGF-2 could stimulate basophil migration in vitro when introduced into the lower chambers of 96-well chemotaxis plates (Fig. 6). IGF-1 and IGF-2 were ~ 10 -fold less potent than eotaxin-1/CCL11. In contrast, IGF-1 and IGF-2 did not stimulate migration of eosinophils or neutrophils (Fig. 6). When IGF-1 or IGF-2 was added to the cells just before applying them above the filter in the chemotaxis assay, migration of basophils into the bottom well was also stimulated, indicating that the chemoattractant effect of IGF-1 and IGF-2 resulted from chemokinesis (Fig. 7, *A* and *B*). This was in contrast to eotaxin-1/CCL11, which was clearly chemotactic, only stimulating the migration of basophils when present in the lower well, not when mixed with the cells (Fig. 7*C*). To investigate whether the chemokinetic effect of the IGFs could enhance chemotaxis of basophils toward a chemokine, basophils were mixed with different concentrations of IGF-1 or IGF-2 just before applying them above the filter and allowing them to migrate toward a low concentration (1 nM) of eotaxin-1/CCL11 in the well below. Significantly, IGF-1 (data not shown) and IGF-2 (Fig. 7*D*, note the scale) dramatically enhanced the chemotaxis of basophils toward eotaxin-1/CCL11. There was a synergistic effect between 1 nM eotaxin-1/CCL11 and IGF-2 at 10 and 100 nM ($p < 0.05$; $n = 6$).

To determine whether IGF-2 enhanced chemotaxis of basophils toward eotaxin-1/CCL11 by altering the expression of CCR3, basophils were incubated with IGF-2 (100 nM) for 1 or 4 h, and CCR3 expression was measured. Treatment with IGF-2 did not have a significant effect on basophil CCR3 expression even after 4 h (Fig. 8). Additional experiments, in which basophils were incubated with IGF-2 (100 nM) for 30 min followed by eotaxin-1/CCL11 (10 nM) for 30 min, showed that IGF-2 did not alter the down-regulation of CCR3 expression that occurred in the presence

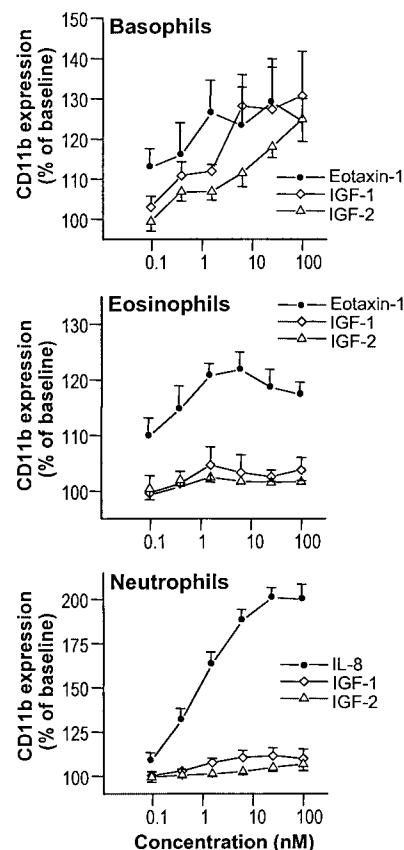


FIGURE 9. Effects of IGF-1 and IGF-2 on leukocyte CD11b expression. PBMC and PMN were incubated with agonists for 30 min at 37°C, then stained with anti-CD11b-FITC in combination with anti-HLA-DR-QR and anti-CD123-PE, or anti-CD16-PE, respectively. CD11b expression on CD123^{pos}/HLA-DR^{neg} basophils, CD16^{neg} eosinophils, or CD16^{pos} neutrophils was quantified and is represented as a percentage of baseline expression. IGF-1 and IGF-2 caused up-regulation of CD11b expression on basophils; the maximal response was similar to that of eotaxin-1/CCL11. In contrast, eosinophil and neutrophil CD11b expression was unaffected by IGF-1 and IGF-2, whereas eotaxin-1/CCL11 and IL-8/CXCL8 stimulated appreciable increases, respectively. Data are the mean $\pm$ SEM ($n = 3$); each experiment used cells from different donors.

of ligand (Fig. 8). Thus, modulation of CCR3 expression or internalization does not appear to be the mechanism for IGF-2 enhancement of chemotaxis to eotaxin-1/CCL11.

In addition to stimulating basophil shape change and migration, IGF-1 and IGF-2 caused up-regulation of CD11b expression on basophils (Fig. 9). The maximal up-regulation of CD11b expression stimulated by IGF-1 and IGF-2 was similar to the response to eotaxin-1/CCL11, but, as in the chemotaxis assay, both IGFs were less potent than eotaxin-1/CCL11. In contrast, no effect of IGF-1 or IGF-2 on eosinophil or neutrophil CD11b expression was observed, whereas eotaxin-1/CCL11 and IL-8/CXCL8 stimulated appreciable increases, respectively (Fig. 9). When purified basophils were cultured in the presence of IGF-2 (20 nM), a significant inhibition of apoptosis was observed at both 24 and 48 h (Fig. 10*A*; $p < 0.05$; $n = 9$) compared with buffer control. In addition, the presence of IGF-2 in combination with IL-3 (10 pM) was more effective at inhibiting apoptosis than IL-3 alone at 48 h (Fig. 10*A*; $p < 0.05$; $n = 9$). In contrast, IGF-1 and IGF-2 were found to be ineffective at stimulating basophil degranulation (CD63 expression; Fig. 10*B*) or calcium flux (Fig. 10, *C* and *D*).

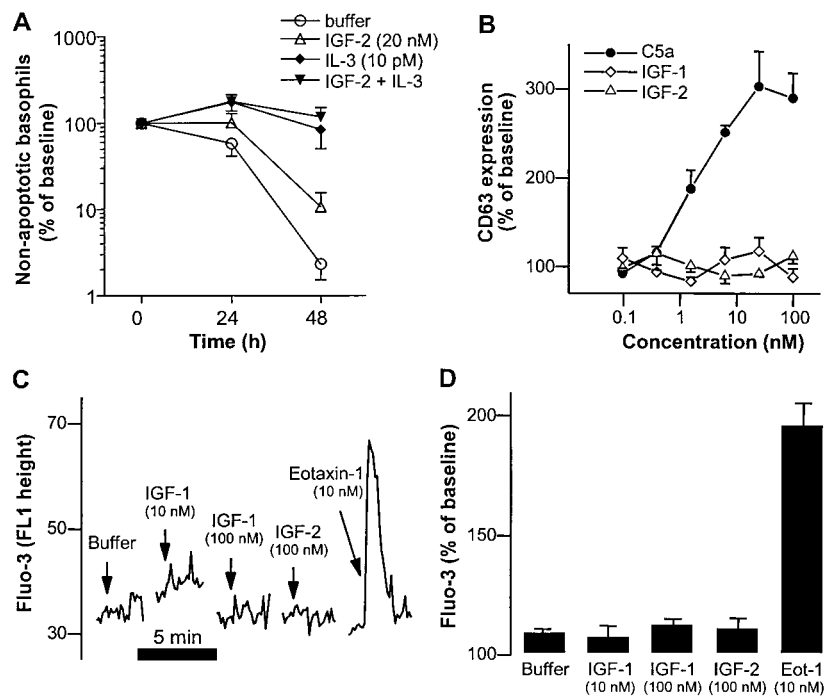


FIGURE 10. Effects of IGF-1 and IGF-2 on basophil apoptosis, CD63 expression, and Ca^{2+} flux. **A**, Purified basophils were incubated in the absence or the presence of IGF-2 (20 nM) and/or IL-3 (10 pM). At various time points (0–48 h) aliquots were stained with annexin V-FITC (1/100) and propidium iodide (1/50) and immediately analyzed by flow cytometry. Data are expressed as a percentage of the baseline number of cells. IGF-2 inhibited apoptosis at 24 and 48 h, as did IL-3, and enhanced the effect of IL-3 at 48 h. Data are the mean $\pm$ SEM ($n = 9$); each experiment used cells from different donors. **B**, PMNC were labeled with anti-HLA-DR-QR and anti-CD123-PE, washed, and resuspended in assay buffer in the presence of the anti-CD63-FITC. Cells were pretreated with cytochalasin B (5 $\mu\text{g}/\text{ml}$) for 5 min at 37°C and then incubated with agonists for 30 min at 37°C , transferred to ice, washed, fixed, and immediately analyzed by flow cytometry. Data are expressed as the percent change from a control sample incubated with buffer alone. C5a caused a dose-dependent increase in CD63 expression, but no increase occurred in the presence of IGF-1 or IGF-2. Data are the mean $\pm$ SEM ($n = 3$); each experiment used cells from different donors. **C**, Plots illustrating calcium flux, as measured by flow cytometry. PBMC were labeled with anti-HLA-DR-QR and anti-CD123-PE, loaded with Fluo-3, and stimulated with IGF-1, IGF-2, or eotaxin-1/CCL11. Changes in intracellular free calcium levels were detected as the increase in fluorescence intensity of the calcium-sensitive dye Fluo-3 in fluorescence channel-1 (FL1). **D**, Change in intracellular calcium level of CD123^{pos}/HLA-DR^{neg} basophils represented as a percentage of the baseline. Eotaxin-1/CCL11 stimulated a substantial rise in intracellular free calcium, whereas IGF-1 and IGF-2 were ineffective. Data are the mean $\pm$ SEM ($n = 3$); each experiment used cells from different donors.

Discussion

Basophils as well as eosinophils are associated with allergic reactions in the skin (4), lung (5), and nose (36), and their numbers increase in the late phase response after experimental allergen challenge. However, the kinetics of accumulation in tissues are different for basophils and eosinophils (14), and in certain inflammatory reactions basophils are the predominant leukocyte type (3, 15). This suggests the existence of unidentified mechanisms for the recruitment of basophils from blood into tissues. While screening HPLC fractions of nasal polyp extracts for novel chemoattractants using sensitive assays for different leukocyte types, we found an activity that stimulated a shape change response in basophils, but not eosinophils, neutrophils, or monocytes. Purification and mass spectrometric protein sequencing revealed that this activity was associated with IGF-2. This was confirmed by inhibiting the basophil shape change activity in the HPLC fractions with a neutralizing Ab to IGF-2.

Recombinant IGF-2 and the related growth factor, IGF-1, also induced a shape change response in basophils, but not eosinophils, neutrophils, or monocytes. In addition, rIGF-1 and rIGF-2 stimulated migration of basophils in a chemotaxis assay and were found to be chemokinetic. Interestingly, IGF-1 and IGF-2 dramatically enhanced basophil chemotaxis toward eotaxin-1/CCL11 by a mechanism independent of CCR3 expression levels. Thus, in addition to directly promoting random migration, IGF-1 and IGF-2 were able to increase the responsiveness of basophils to eotaxin-

1/CCL11. Therefore, because eotaxin-1/CCL11 is approximately equipotent for eosinophils and basophils in vitro, the presence of IGF-1 or IGF-2 and low levels of chemokines such as eotaxin-1/CCL11 in vivo would be expected to result in the preferential recruitment of basophils. IGF-1 and IGF-2 also stimulated up-regulation of basophil expression of CD11b, an integrin associated with cell adhesion, migration, and activation. In contrast, IGF-1 and IGF-2 did not cause basophil degranulation, measured by CD63 surface expression, or Ca^{2+} flux. This is consistent with our previous observation that stimulation of Ca^{2+} flux in basophils is more closely associated with degranulation than shape change responses (34). Significantly, IGF-2 also inhibited basophil apoptosis and enhanced the antiapoptotic effect of IL-3. Therefore, IGF-2 may influence basophil accumulation in tissues by enhancing both their recruitment and their survival.

Our study is the first to implicate IGF-1 and IGF-2 in enhanced basophil migration; however, two other studies have indicated that functional IGF receptors are expressed by this cell type. A study by Hirai et al. (37) demonstrated that IGF-1 and IGF-2 are able to enhance basophil histamine release stimulated by anti-IgE, calcium ionophore, or phorbol ester, but not by FMLP or C5a. They found that this enhancement was mediated by the type 1 IGF receptor (IGF-R1), and that IGF-1 was 10-fold more potent than IGF-2. Similarly, Ochensberger et al. (9) found that IGF-1 could enhance the release of IL-4 and IL-13 from basophils in combination with anti-IgE or C5a, although the

effect was weak compared with that of IL-3. Therefore, IGF-1 and IGF-2 can enhance, although not directly stimulate, secretory responses of basophils. However, our findings that these growth factors are able to stimulate the migration of basophils in vitro, an effect that is selective compared with eosinophils and neutrophils, and dramatically enhance chemotaxis of basophils toward eotaxin-1/CCL11 point to a significant role for IGFs in basophil recruitment into tissues.

To complement the functional studies, we quantitated IGF-1 and IGF-2 in the nasal polyp extract by ELISA. IGF-2 was found to be almost 5-fold more abundant than IGF-1, the latter having previously been detected in human nasal polyps by immunohistochemistry (38). IGF-1 was present at a concentration of 4.0 pmol/g tissue, which is similar to the amount of eotaxin-1/CCL11 detected (D. M. Conroy, unpublished observations), whereas 17.8 pmol of IGF-2/g tissue was measured. The quantities measured suggest a potentially significant role for both IGF-1 and IGF-2. IGF-1 eluted slightly earlier than IGF-2 on RP-HPLC and may have contributed to the basophil stimulatory activity found in the analytical RP-HPLC fractions (Fig. 1). However, because IGF-1 has a higher pI than IGF-2 (IGF-1 pI 7.8; IGF-2 pI 6.5), it is unlikely to have bound to the anion exchangers used, with the result that only IGF-2 was identified after these stages of purification.

IGF-2 is a 67-aa monomeric protein of 7.5 kDa (39, 40) that shares 70% identity with IGF-1. These IGFs exert both local and systemic biological effects, mediated by the IGF-R1. IGF-R1 is structurally similar to the insulin receptor and has tyrosine kinase activity. A major consequence of IGF-R1 activation is stimulation of cell growth, i.e., cell proliferation, differentiation, and survival. Fibroblasts are a significant source of IGFs, as well as being responsive to these growth factors in terms of proliferation and collagen production (41). IGF-1 is a growth and differentiation factor for myocytes and can also stimulate proliferation and chemotaxis of vascular and airway smooth muscle cells (42). IGF-1R is expressed by T cells (43–45) and other leukocytes, and IGF-1 has been shown to promote T cell proliferation and chemotaxis (43). IGF-1 also inhibits apoptosis of lymphocytes (46) and neutrophils (47) and up-regulates or primes other functional responses of these cells. IGFs in plasma and other extracellular environments are bound to specific IGF-binding proteins (IGF-BPs) (40). The IGF-BPs are a family of six distinct secreted proteins that specifically bind IGF-1 and IGF-2. These binding proteins act as carrier proteins in the bloodstream, prolonging the half-lives of IGFs, and control the efflux of IGFs from extravascular spaces. Some IGF-BPs enhance the binding of IGFs to their receptor, whereas others are inhibitory. Thus, locally expressed IGF-BPs may regulate the binding of IGFs to specific cell types, and the relative levels of IGFs and IGF-BPs must be taken into account when considering IGF action. Increases in IGFs or alterations in IGF-BPs have been associated with different inflammatory conditions, particularly where fibrosis is associated with chronic disease (48, 49). The IGF-BPs are cleaved by a number of proteases, including kallikreins, cathepsins, and matrix metalloproteinases, thereby reducing their affinity for IGFs. Proteolysis of IGF-BPs by matrix metalloproteinase-1 has been proposed as a mechanism involved in the induction of airway smooth muscle hyperplasia and airway obstruction in asthma (50). The release of proteases, by mast cells, for example, could enhance the activity of IGFs present at sites of allergic inflammation, leading to the recruitment of basophils and amplification of the allergic response.

Like eosinophils, basophils respond to IL-3, IL-5, and GM-CSF. Whereas IL-5 is the most potent of these cytokines for eosinophils, IL-3 is the most potent for basophils, reflecting the high level of expression of the respective receptors on these two cell types (51).

IL-3 is able to prime basophil degranulation (52) and leukotriene C₄ generation, inhibit apoptosis (53), and stimulate basophil migration in vitro (54). Although IL-3 and the IGFs may stimulate similar functional responses in basophils, the IGFs, unlike IL-3, are apparently selective for basophils, particularly with regard to migratory responses. Therefore, IGF-1 and IGF-2 may account for the preferential accumulation of basophils observed in certain inflammatory responses, although in vivo studies will be required to confirm this.

Histologically, nasal polyps are benign outgrowths of the mucosa, characterized by proliferation of epithelial cells, glandular hyperplasia, thickening of the basement membrane, edema, fibrosis, and leukocyte infiltration. IGF-1 and IGF-2, as growth factors, may play an important role in the extensive tissue remodeling that occurs. We show in this study that IGF-1 and IGF-2 have selective chemokinetic effects for basophils that may be important for their recruitment in vivo, particularly in the presence of chemokines acting via CCR3. Despite its long history, the precise function of the basophil is ill-defined. These results raise the intriguing possibility that basophils may have a hitherto unrecognized function related to the actions of IGF-1 and IGF-2 as growth factors.

Acknowledgments

We are grateful to Dr. J. A. Symons for recombinant CC-CKBP, to G. F. Martin for performing the IGF ELISAs, and to Drs. Y. S. Bakhle and I. Sabroe for helpful discussions.

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