Eotaxin: A Potent Eosinophil Chemoattractant Cytokine Detected in a Guinea Pig Model of Allergic Airways Inflammation

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Summary

Eosinophil accumulation is a prominent feature of allergic inflammatory reactions, such as those occurring in the lung of the allergic asthmatic, but the endogenous chemoattractants involved have not been identified. We have investigated this in an established model of allergic inflammation, using in vivo systems both to generate and assay relevant activity. Bronchoalveolar lavage (BAL) fluid was taken from sensitized guinea pigs at intervals after aerosol challenge with ovalbumin. BAL fluid was injected intradermally in unsensitized assay guinea pigs and the accumulation of intravenously injected $^{111}$In-eosinophils was measured. Activity was detected at 30 min after allergen challenge, peaking from 3 to 6 h and declining to low levels by 24 h. 3-h BAL fluid was purified using high performance liquid chromatography techniques in conjunction with the skin assay. Microsequencing revealed a novel protein from the C-C branch of the platelet factor 4 superfamily of chemotactic cytokines. The protein, "eotaxin," exhibits homology of 53% with human MCP-1, 44% with guinea pig MCP-1, 31% with human MIP-1α, and 26% with human RANTES. Laser desorption time of flight mass analysis gave four different signals (8.15, 8.38, 8.81, and 9.03 kD), probably reflecting differential O-glycosylation. Eotaxin was highly potent, inducing substantial $^{111}$In-eosinophil accumulation at a 1–2-pmol dose in the skin, but did not induce significant $^{111}$In-neutrophil accumulation. Eotaxin was a potent stimulator of both guinea pig and human eosinophils in vitro. Human recombinant RANTES, MIP-1α, and MCP-1 were all inactive in inducing $^{111}$In-eosinophil accumulation in guinea pig skin; however, evidence was obtained that eotaxin shares a binding site with RANTES on guinea pig eosinophils. This is the first description of a potent eosinophil chemoattractant cytokine generated in vivo and suggests the possibility that similar molecules may be important in the human asthmatic lung.

The accumulation of eosinophil leukocytes is a characteristic feature of IgE-mediated allergic reactions such as allergic asthma, rhinitis, and eczema. Eosinophil accumulation also occurs in nonallergic asthma. The immediate bronchoconstriction in response to a provoking stimulus in the asthmatic involves mast cell activation and the release of constrictor mediators. This is followed after several hours in some individuals by a late bronchoconstrictor response associated with a massive influx of eosinophils (1). Repeated provocation results in chronic inflammation in the airways and a marked hyperresponsiveness to constrictor mediators. The magnitude of both the late response and the chronic hyperresponsiveness correlates with the numbers of eosinophils present in the lung (2, 3). Further, in experimental studies in allergic monkeys, suppression of eosinophil accumulation in the lung by interference with adhesion mechanisms inhibits bronchial hyperresponsiveness (4).

As in allergic asthmatic patients, exposure of sensitized guinea pigs to aerosolized allergen results in an immediate phase of bronchoconstriction with associated mast cell degranulation followed, in some individuals, by a late phase of bronchoconstriction and airway hyperresponsiveness (5–8). Although no one model mimics all the features of the human disease, the guinea pig model shares common features with the asthmatic response in humans and has been extensively used to investigate possible mechanisms (8). In particular, in both guinea pigs and humans, the immediate response to allergen triggers the subsequent accumulation in the lung of high numbers of eosinophils. The mediators inducing eosinophil accumulation in the lung are unknown. Experiments
were designed to detect the appearance of chemoattractants in the lung that may be responsible for the accumulation of eosinophils. A strategy was employed using a combination of in vivo generation and HPLC purification coupled with in vivo detection of eosinophil chemoattractant activity, as previously applied to the identification of neutrophil chemoattractants in inflammatory exudates (9–11).

Materials and Methods

Animals. Male Dunkin Hartley guinea pigs (300–400 g), purchased from Harlan Olac Ltd. (Bicester, Oxon, UK), were used for the in vivo generation and assay of eosinophil chemoattractant activity. Female exbreeder guinea pigs (600–800 g) from the same source were used as eosinophil and neutrophil donors.

Materials. Human recombinant MCP-1, MIP-1α, and RANTES were purchased from Pepro Tech Inc. (Rocky Hill, NJ). A generous gift of RANTES from Dr. T. J. Schall (Genentech Inc., South San Francisco, CA) was also used, which exhibited identical activity to the commercially obtained product.

Generation of Eosinophil Chemoattractant Activity In Vivo Time-Course Studies. Male Dunkin Hartley guinea pigs (300–400 g) were sensitized with intraperitoneal ovalbumin (1 mg) on day 1 followed by exposure to aerosolized antigen (2% OVA for 5 min using an ultrasonic nebulizer) on day 8 (7). On days 15–21, animals were pretreated with an antihistamine to prevent acute fatality (pyrilamine; 10 mg/kg i.p.) and challenged by exposure to aerosolized allergen (1% OVA for 20 min). In a limited number of experiments, the antihistamine was added and antigen challenge was stepwise (10-min exposure to each of the following: 0.01, 0.1, 1.0, 5.0, and 10.0 mg/ml OVA). At different times after allergen challenge, animals were treated with atropine (0.06 mg/kg i.p.) and challenged by exposure to aerosolized allergen (1% OVA for 20 min). In a limited number of experiments, the antihistamine was added and antigen challenge was stepwise (10-min exposure to each of the following: 0.01, 0.1, 1.0, 5.0, and 10.0 mg/ml OVA). At different times after allergen challenge, animals were treated with atropine (0.06 mg/kg i.p.) and challenged by exposure to aerosolized allergen (1% OVA for 20 min). In a limited number of experiments, the antihistamine was added and antigen challenge was stepwise (10-min exposure to each of the following: 0.01, 0.1, 1.0, 5.0, and 10.0 mg/ml OVA).

Purification of Eosinophil Chemoattractant Activity. BAL fluid collected from 25 sensitized guinea pigs (each lavaged with 4 ml, followed by 2 × 10 ml, saline) 3 h after allergen challenge (1% OVA, 3 min exposure) was adjusted to pH 5.5 and applied to a cation exchange HPLC column (Ultrapac TSK555CM 7.5 × 150 mm; Pharmacia Fine Chemicals, Piscataway, NJ). The column was eluted with a linear gradient of 0.3–2.0 M ammonium acetate, pH 5.5. Eosinophil chemoattractant activity eluted as a single peak at ≈1.4 M ammonium acetate, pH 5.5. After lyophilization, the active fractions were applied to size exclusion HPLC (Ultrapac TSK columns SWP, 7.5 × 75 mm; G4000SW, 7.5 × 600 mm; and G2000SW, 7.5 × 600 mm, in series, equilibrated in 0.08% TFA). Activity eluted at 0.7–14 kD. This was applied to a wide pore (300 Å) Vydac C18 reversed phase column (4 × 250 mm; HPLC Technology, Cheshire, UK) in 0.08% TFA, eluted with a linear gradient of acetonitrile (ACN; 0–80% ACN in 0.08% TFA, over 80 min, at 1 ml/min) and 0.5–min fractions were collected. The in vivo bioassay was used to confirm the chemoattractant activity. Aliquots of each fraction were lyophilized in the presence of carrier protein (BSA, <0.1 ng endotoxin/mg) and resuspended in saline for testing in the skin bioassays for 111In-eosinophil and 111In-neutrophil accumulation over 2 h (n = 4 assay guinea pigs).

SDS-PAGE Analysis. 2% aliquots of each fraction from reversed phase HPLC were lyophilized, resuspended in 10 μl SDS buffer, heated (95°C, 5 min), and 0.3 μl was run on 8–25% gradient gels in a Phast System (Pharmacia Fine Chemicals). Gels were visualized with silver staining.

Microsequencing. 5% aliquots of each bioactive fraction (51, 52, and 54 shown in Fig. 2) were applied directly to automated NH2-terminal sequence analysis using fast cycles on a pulsed liquid automated Edman sequencer (model 477A; Applied Biosystems Inc., Foster City, CA) containing a microcartridge essentially as described (14). The NH2-terminal 37, 35, and 29 residues were obtained for fractions 51, 52, and 54, respectively. No differences between corresponding positions were found. The apparent initial yields of these three analyses were all ≥7–8 pmol. Thus, fractions 51, 52, and 54 contained ≥200 pmol each, assuming 70–80% sequencing yields. Gels were found at positions 8, 9, and 33, consistent with the presence of cysteine residues at these positions. Approximately 30 pmol of fraction 54 was reduced and alkylated by sequential treatment with 1 M dithiothreitol for 5 min at 50°C and then 10 mM acrylamide for 30 min at 37°C before digestion with alkylated trypsin (Promega Biotech, Madison, WI), 20 mM Tris/HCl, pH 8.8 containing 0.5% chymotrypsin. Peptides were separated using a ReliaSIL C18 (300 Å, 5 μm) column (1 × 150 mm) developed with a linear acetonitrile concentration gradient in 0.08% TFA at 50 μl/min on a Microm HPLC system (Microm Biorcesource Inc., Pleasanton, CA). Purified peptides were subjected to NH2-terminal sequence analysis as above, but all cysteine residues were positively identified as the phenylthiohydantoin-cys-5-S-prolineamide derivative (15).

Binding Assays. Guinea pig eosinophils were prepared as above. Because red blood cells have been reported to bind related chemokines (16), the eosinophils were routinely subjected to a red blood cell lysis step (0.2% NaCl for 30 s) to exclude any possible contamination before use. RANTES (2.5 μg) was iodinated with Na111.
counts and then expressed as B/B0 i.e., with respect to binding centrifuged through 100 #1 silicone oil. Cell-bound radioactivity was calculated using the ratio of the two fluorescence readings and a Kd 380 nm and emission wavelength 510 nm. [Ca2+]i levels were monitored at 37~ using a spectrometer (LS50; Perkin-Elmer Corp., CA). Human and guinea pig eosinophils (107 cells/ml in Ca2+/Mg2+-free PBS containing 10 mM Heps/0.25% BSA/10 mM glucose, pH 7.4. Aliquots were dispensed into quartz cuvettes and the external [Ca2+]i adjusted to 1 mM with CaCl2. Changes in fluorescence were monitored at 37°C using a spectrometer (LS50; Perkin-Elmer Corp., Beaconsfield, Bucks, UK) at excitation wavelengths 340 nm and 380 nm and emission wavelength 510 nm. [Ca2+]i levels were calculated using the ratio of the two fluorescence readings and a Kd for Ca2+ binding at 37°C of 224 nM (18).

Eosinophil Chemotaxis. Human eosinophils were purified (>98%) as described above. Eosinophil locomotion was assessed using 48-well microchemotaxis chambers (Neuro Probe, Inc., Cabin John, MD) as previously described (19). Nitrocellulose membranes (8 #m thick) were coated with 40 #g/cm2 bovine serum albumin and incubated at 37°C for 30 min. Cells were resuspended in Ca2+/Mg2+-free PBS (pH 7.4) containing 10 mM Heps/0.1% BSA/100 mM glucose, and added to the chambers at 106 cells/ml. After 30 min, cells were fixed in 100% ethanol and stained with acidified hematoxylin (Chromotrope 2R).

Histology. Guinea pigs (n = 3) were injected intradermally with either saline (200 #l) or eosinophil chemoattractant activity in BAL fluid (4 pmol). Skin sites were examined 30 min after injection for eosinophil and neutrophil infiltrate and stained sections (3/skin site) were examined.

Statistical Analysis. Data are presented as the mean ± SEM of n assay animals or, for in vitro experiments, cell preparations and were tested by analysis of variance. p <0.05 was considered statistically significant.

Results and Discussion

BAL fluid was collected at different intervals after aerosol challenge of sensitized guinea pigs with allergen, and assayed in vivo for eosinophil chemotactic activity after injection into the skin of recipient assay animals. Fig. 1 shows the time-course of appearance of eosinophil chemotactant activity in BAL fluid. Significant activity was observed 30 min after allergen challenge. Activity increased up to 3 h, remained high at 6 h, but was not significant in 24 h samples. Antihistamine pretreatment did not affect the generation of activity (legend to Fig. 1). Control samples (BAL fluid from sham-sensitized/challenged, or sensitized/sham-challenged guinea pigs) taken at 3 h had no significant activity.

Eosinophil chemotactic activity, which we termed eotaxin, was purified from 3-h BAL fluid by sequential cation exchange, size exclusion, and reversed phase HPLC steps, using the in vivo 111In-eosinophil accumulation assay to measure the activity of fractions throughout. The activity eluted as a single discreet peak of bioactivity from both the cation exchange and the size exclusion steps, indicating a strongly cationic protein of 7-14 kD (data not shown). A small amount of activity, three times the control in the skin assay, was detectable in the flow-through from the cation exchange step, but only when 50 ml was concentrated to 500 #l. In comparison, 0.6% of the most active fraction eluted from the column induced a response 31 times the control. Subsequent reversed phase chromatography separated eosinophil chemotactant activity into two peaks (fractions 51 + 52 and fraction 54), which were associated with discreet peaks of protein absorbance (Fig. 2). Selectivity for eosinophils was shown by the lack of significant neutrophil chemotactant activity in these fractions as measured by the accumulation of 111In-neutrophils in the skin assay (Fig. 2 c). C5a des Arg, used as a positive control, was highly active in both the eosinophil and neutrophil accumulation assays (legend to Fig. 2). Histological examination of skin injected with eotaxin (2 pmol) demonstrated that at both 4 and 24 h eosinophils were present particularly around small blood vessels (data not shown). No mononuclear cell infiltrate was observed at either time point.

SDS-PAGE analysis revealed a single protein band in each of fractions 51, 52, and 54 (Fig. 3 a). The protein in fractions 51 and 52 was slightly larger than that in fraction 54. This was confirmed by mass analysis in which the major signals were at ~8.81 and 8.38 kD, respectively (legend to Fig. 3). NH2-terminal sequencing of fractions 51, 52, and 54 revealed identical amino acid sequences (Fig. 3 b). The NH2-terminal 37 residue sequence of eotaxin showed closest ho-
mology (57%) with human monocyte chemotactic protein (MCP-1) (20), otherwise known as MCAF (21) and JE (22). Tryptic peptides of fraction 54 were also sequenced and readily aligned by comparison with human MCP-1 to give the virtually complete sequence of eotaxin with an overall homology of 53% (Fig. 3, b and c). While we cannot exclude differences in the COOH-terminal sequences of fractions 51, 52, and 54, it is likely that the variations in molecular mass reflect differential glycosylation as the four mass signals obtained (two major: 8.38 and 8.81 kD, and two minor: 8.15 and 9.03 kD, legend to Fig. 3) are all different from each other by multiples of ~220 mass units. The sequence contains no N-glycosylation sites, but a potential O-glycosylation at position 70.

Figure 2. Purification of eotaxin from BAL fluids. (a) Final reversed phase HPLC profile showing absorbance at 214 nm and the acetonitrile gradient. Eosinophil chemoattractant activity measured over 2 h in skin (b) was seen in two peaks, fractions 51 + 52 and fraction 54, which corresponded to discreet peaks of absorbance. No significant neutrophil chemoattractant activity (c) was detected in these fractions. In contrast, guinea pig C5a des Arg (30% zymosan-activated plasma [12], ~10 pmol/site) induced the accumulation of both 11In-eosinophils (5,211 ± 893) and 11In-neutrophils (9,872 ± 473). Fractions 50, 53, 55, and 56 consistently gave little or no activity in the guinea pig skin bioassays of leukocyte accumulation. No protein was detected in the remainder of the gradient (up to 80% acetonitrile). In these assays, 11In-eosinophils (99% pure, 0.5% neutrophils) or 11In-neutrophils (99.4% pure, 0.6% eosinophils) were injected into n = 4 animals for each group.

Figure 3. (a) SDS-PAGE analysis of fractions 50–56. For reference, human IL-8 (72 amino acids, ~8 kD) was run in lanes A, B, and C (12, 2.4, and 0.5 ng/0.3 μl per lane, respectively). Laser desorption time of flight mass analysis gave signals at ~8.81 kD (major) and ~9.03 kD (minor) for each of fractions 51 and 52. Fraction 54 gave signals at ~8.38 kD (major) and ~8.15 (minor). (b) The amino acid sequence of eotaxin was determined by sequencing the intact molecule as well as peptides derived from digestion with trypsin (T). NH2-terminal analyses showed the highest homology with human MCP-1 (57%) and the tryptic peptides were readily aligned by comparison with the human MCP-1 sequence. Position 70 gave no PTH derivative in peptides T6 and T7 and is a probable position of O-glycosylation. The COOH-terminus could not be unequivocally identified. (c) Comparison of the eotaxin sequence with human MCP-1, MCP-2, MCP-3 (26), guinea pig MCP-1 (32), human MIP-1α, MIP-1β, and RANTES (25) showing conserved residues (shaded).
70 has been identified (legend to Fig. 3). Human MCP-1 also exhibits heterogeneity on SDS-PAGE due to differences in the posttranslational modification of O-linked carbohydrate (23).

The relationship between eotaxin and structurally related proteins of the platelet factor 4 superfamily is shown in Fig. 3c. The platelet factor 4 superfamily of chemotactic cytokines, or chemokines, is characterized by four conserved cysteines. The relative position of the two NH2-terminal cysteines allows the subdivision of this superfamily into the C-X-C chemokines (e.g., IL-8 [24]) that are predominantly neutrophil chemoattractants and the C-C chemokines (e.g., MCP-1, RANTES, MIP-1α, and MIP-1β [25]) that are chemotactic for leukocytes other than neutrophils. Eotaxin is a member of the C-C branch of chemokines. Surprisingly, the greatest homology is with human MCP-1 (53%), MCP-2 (54%), and MCP-3 (51%) (26). MCP-1, in the limited in vitro studies to date, has been reported to be inactive on human eosinophils (27, 28). Homology with other human C-C chemokines (Fig. 3c) is: MIP-1β (37%), MIP-1α (31%), and RANTES (26%). The latter two proteins have recently been shown to be potent eosinophil activators in vitro (28-30) whereas MIP-13 activates lymphocytes in vitro (31) but apparently not eosinophils (28). Although eotaxin shows the greatest structural homology with human MCP-1, MCP-2, and MCP-3 we cannot conclusively identify a human counterpart since eotaxin has functional similarities, but relatively low homology, when compared with RANTES and MIP-1α. Eotaxin is clearly a distinct molecule from guinea pig MCP-1; the latter has recently been cloned (32) and has only a 44% homology with the eotaxin sequence (Fig. 3c). Eotaxin also has a 41% homology with a C-C protein whose gene is expressed in mouse mast cells and upregulated 2 h after the interaction between IgE and antigen (33). No functional activity has been reported for this protein but it is distinct (51% homology) from mouse MCP-1/JE (33).

The effects of pure eotaxin in vivo and in vitro are shown in Fig. 4. Eotaxin was a pool of both peaks of the protein from reversed phase HPLC (Fig. 2). Guinea pig eotaxin was potent as an inducer of eosinophil accumulation in vivo, 1.6 pmol/skin giving a significant response at 30 min and 4 h (Fig. 4a). In contrast, recombinant human RANTES and MIP-1α, perhaps because of species specificity, did not induce eosinophil accumulation over 4 h at doses of 1–100 pmol/site (Fig. 4a). Recombinant human MCP-1 was also ineffective in inducing eosinophil accumulation at the same doses (Fig. 4a). In all experiments in which pure eotaxin was tested, 1–2 pmol/site gave a 730 ± 140% response (mean ± SEM, n = 18 guinea pigs) compared with saline/BSA-injected sites. The rapid action of eotaxin (within 30 min) suggests that it has a direct chemoattractant effect on eosinophils in vivo and does not depend on the synthesis of secondary cytokines (e.g., by monocytes) for activity. This is supported by experiments on guinea pig and human eosinophils in vitro.

Guinea pig eotaxin and human RANTES had similar activities on human eosinophils in vitro. Both elevated intracellular calcium levels (Fig. 4b) and induced chemotactic responses of similar magnitude (over the range of 0.1–3.0 nM, data not shown). In contrast, only eotaxin elevated intracellular calcium levels in guinea pig eosinophils (Fig. 4c). Human RANTES, at doses up to 100 nM, did not elevate intracellular calcium levels in guinea pig eosinophils (Fig. 4c) even though these cells bound 125I-RANTES. This binding was inhibited by unlabeled RANTES and similar concentrations of eotaxin, suggesting that eotaxin and RANTES share a common binding site on guinea pig eosinophils (Fig. 4a, inset). These results are consistent with the recent reports of receptor sharing by different C-C chemokines and the dissociation between receptor binding and the ability to elevate...
intracellular calcium levels (34–38). Our data suggest that eotaxin may have functional similarities to RANTES on human eosinophils in vitro whereas human RANTES binds to, but does not activate, guinea pig eosinophils. Human MCP-1, despite its sequence similarity with guinea pig eotaxin, was only weakly active on human eosinophils in vitro (Fig. 4 b and legend) and did not induce \(^{11}\)In-eosinophil accumulation in vivo (Fig. 4 a).

Eosinophils contain an armory of chemicals necessary for killing parasites. These chemicals have been implicated in the damage to airway epithelium that occurs in asthma and may relate to the observed changes in airway function (4, 19). Eotaxin may be an important signal that induces eosinophil accumulation in the lung. We do not yet know the source of eotaxin in the lung: macrophages, lymphocytes, mast cells, and airway epithelial cells are likely candidates. Platelets may also have a role as it has been shown that they can release C-C chemokines (29). Further, it has been suggested that an early platelet deposition may be involved in the subsequent eosinophil accumulation in vivo (39, 40) and there is evidence that platelet-activating factor induces the synthesis of an unidentified eosinophil chemoattractant protein in vivo (41). In addition, it is of interest that platelet-derived growth factor can induce gene expression of C-C chemokines in fibroblasts (42). The C-C chemokines have also been implicated in wound healing (25) which may be important in the subepithelial basement membrane fibrosis that is a prominent feature of the asthmatic lung.

This is the first report of an eosinophil chemoattractant cytokine generated in vivo. Eotaxin is potent in vivo, having significant activity at doses of 1–2 pmol. Further, eotaxin exhibits selectivity, inducing eosinophil but not neutrophil accumulation. We suggest that eotaxin should be considered as a potentially important endogenous mediator of eosinophil accumulation in vivo. In particular, eotaxin and related molecules may be involved in both eosinophil accumulation and in chronic structural changes in the asthmatic lung.

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