

Intracellular localization and release of eotaxin from normal eosinophils

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Abstract Eotaxin is a potent and selective CC chemokine for eosinophils and basophils. We established several monoclonal antibodies (Mabs) allowing the neutralization and measurement of human eotaxin. Using the Mabs as probes, we demonstrated that normal eosinophils contained intracellular granule-associated eotaxin. Quantification of cell-associated eotaxin in different leukocyte subsets revealed that it was principally expressed in eosinophils. Finally, we showed that normal eosinophils released eotaxin upon stimulation with either of two secretagogues, C5a or ionomycin. These findings raise the possibility that eosinophil-derived eotaxin contributes to the local accumulation of eosinophils at the site of inflammation.

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Key words: Eotaxin; Eosinophil; Chemokine

1. Introduction

Eosinophil infiltration is a fundamental trait of parasitic infections and allergic diseases [1]. Through the release of diverse toxic mediators, activated eosinophils contribute to parasite killing and associated tissue damage [2]. In addition, the recent demonstration that eosinophils may release various cytokines suggests that these cells play a regulatory role in inflammation associated with allergic disorders [3–15]. Indeed, there is evidence that these cytokines may regulate the maintenance and progression of allergic inflammation through autocrine and/or paracrine mechanisms.

Although the processes involved in local eosinophilia are not fully understood, accumulating evidence indicates that the recruitment and activation of leukocytes in inflammatory processes are regulated by a large number of CXC and CC chemokines [16,17]. As a specific CC chemokine for eosinophils, eotaxin was purified from the bronchoalveolar lavage fluid of a guinea pig model of allergic lung inflammation by Williams and colleagues [18] in 1993. In animals, eotaxin induces local accumulation of eosinophils in synergy with interleukin (IL)-5 [19–21]. In humans, increased expression of eotaxin was observed in the bronchoalveolar lavage fluid and airway biopsies of asthmatic patients [22,23].

In the present study, we established several monoclonal antibodies (Mabs) to human eotaxin and developed a sensitive enzyme-linked immunosorbent assay (ELISA) system for the

quantification of eotaxin. We demonstrated the localization and storage of eotaxin in normal eosinophils, and the release of eosinophil-associated eotaxin upon stimulation with either C5a or ionomycin.

2. Materials and methods

2.1. Reagents

Recombinant human macrophage chemoattractant protein (MCP)-1, MCP-2, MCP-3, MCP-4, human macrophage inflammatory protein (MIP)-1 α , eotaxin-2 and RANTES were purchased from PeproTech EC (London, UK). The production and purification of recombinant human eotaxin were performed as described previously [24]. Natural eotaxin- α , - β and - γ were purified from the supernatant of IL-4-stimulated dermal fibroblasts as described previously [25]. Bovine serum albumin (BSA), and Tween 20 were obtained from Sigma (St. Louis, MO, USA).

2.2. Hybridoma production

BALB/c mice were injected subcutaneously with 2 μ g of recombinant human eotaxin emulsified in complete Freund's adjuvant (Difco, Detroit, MI, USA). Additional injections of 1 μ g of recombinant human eotaxin emulsified in incomplete Freund's adjuvant (Difco) were administered three more times at 1–2-week intervals. Three days before fusion, mice received an intravenous injection of 1 μ g of recombinant eotaxin. Spleen cells were isolated and fused with P3U1 myeloma cells using 50% (w/v) polyethylene glycol solution (Sigma). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium supplemented with 10% fetal calf serum (Dainippon,

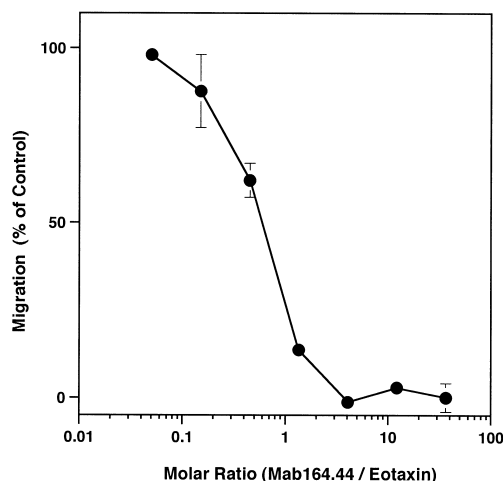


Fig. 1. Inhibition of eosinophil chemotaxis by Mab 164.44. After preincubation of recombinant human eotaxin with various concentrations of Mab 164.44 for 1 h, eotaxin-induced eosinophil chemotaxis was performed using 96-well modified Boyden microchemotaxis chambers, as described in Section 2.

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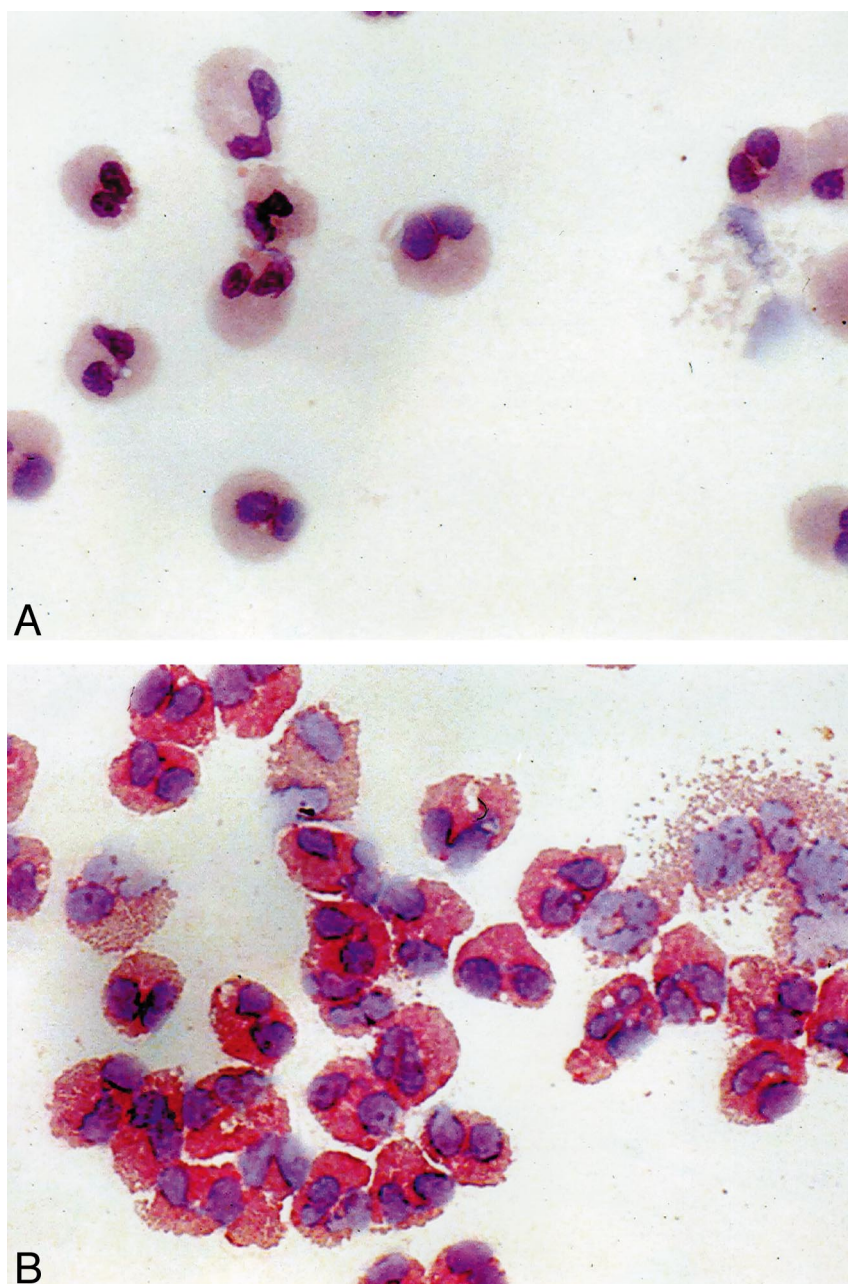


Fig. 2. Light micrographs ($\times 400$) of peripheral blood eosinophils stained with normal mouse IgG1 (A) or monoclonal anti-eotaxin antibody (Mab 174.4) (B) and counterstained with Myer's hematoxylin.

Osaka, Japan) and 5% BriClone (BioResearch Ireland, Dublin, Ireland). Hybridomas were screened by ELISA, and positive hybridomas were subcloned by limiting dilution. For screening of anti-eotaxin antibody-producing hybridomas, 96-well microplates (Maxisorp, Nunc, Denmark) were coated with 75 μ l per well of eotaxin (5 μ g/ml in bicarbonate buffer, pH 9.0) overnight. The plates were washed with PBS containing 0.1% Tween 20. Hybridoma supernatants were added and incubated at 4°C overnight. The plates were washed, and bound antibodies were detected with horseradish peroxidase (HRPO)-labeled anti-mouse Ig antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 3 h. After additional washing, the reaction was developed using a TMB microwell peroxidase substrate system (Kirkengard and Perry, Gaithersburg, MD, USA). Absorbance was measured using an ELISA reader (Bio-Rad, Melville, NY, USA) at 450 nm.

2.3. Antibody isotyping and purification

The class and subclass of monoclonal antibodies were determined

by ELISA. Ascites was generated by injecting 10^6 hybridoma cells into the peritoneal cavity of pristane-primed BALB/c mice. The ascites was clarified by centrifugation, and the Mab was purified by using a protein A affinity chromatography kit (Bio-Rad) according to the manufacturer's instructions.

2.4. ELISA for eotaxin

A double-sandwich ELISA for measurement of human eotaxin was

Table 1

Measurement of cell-associated eotaxin in freshly isolated peripheral blood eosinophils, PMNs and MNCs from normal individuals

Cell	Eotaxin (pg/ 10^6 cells)
Eosinophils ($n = 5$)	19.53 ± 3.53^a
PMNs ($n = 4$)	0.80 ± 0.83
MNCs ($n = 4$)	2.09 ± 0.77

^aMean \pm S.D.; eotaxin was determined by ELISA after cell lysis.

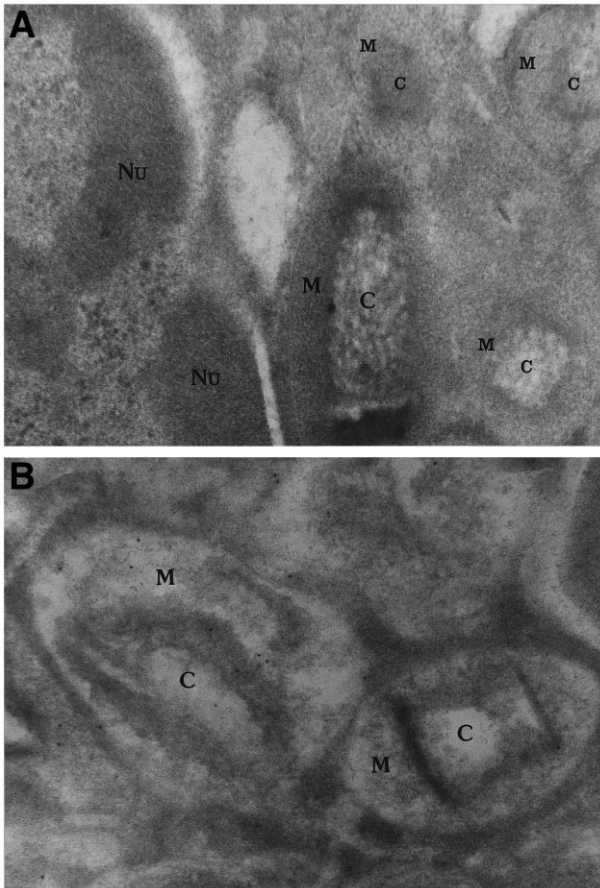


Fig. 3. Immunoelectron micrographs of human eosinophils labeled with normal mouse IgG1 or monoclonal anti-eotaxin antibody (Mab 174.4) followed by 10 nm gold particle-conjugated anti-mouse IgG(H+L). Negative-control labeling of specific granules (SG) in an eosinophil using normal mouse IgG1 substituted for the primary antibody (A) or with Mab 174.4 (B). C: central core of SG; M: matrix of SG; Nu: nucleus.

developed by using two Mabs. ELISA microtiter plates (Covalink NH, F8 Modules, Nunc) were coated with Mab 164.44 by using a crosslinking reagent, disuccinimidyl suberate (Pierce, Rockford, IL, USA). Samples were added to Mab-coated plates, which were washed and incubated with HRPO conjugated-Mab 174.4 Fab' (HRPO-conjugated Fab' was kindly prepared by Dr. A. Morita, Shionogi&Co., Osaka, Japan). The plates were developed with the TMB microwell peroxidase substrate system, and the reactions were stopped with 2 N H_2SO_4 . Absorbance was measured at 450 nm, and a standard curve was generated by using recombinant eotaxin for each assay.

2.5. Cell culture

Eosinophils, polymorphonuclear cells (PMNs) and mononuclear cells (MNCs) were isolated from the peripheral blood of healthy individuals. The purity of eosinophils was more than 95%. Highly purified eosinophils were stimulated with either cytochalasin B (CB; 5 μ g/ml; Sigma) plus C5a (100 nM; Sigma) or ionomycin (1 μ M; Calbiochem-Behring, La Jolla, CA, USA) in PIPES buffer (pH 7.4) containing 0.03% human serum albumin for 60 min at 37°C as described previously [26]. Supernatants were harvested by centrifugation. Cell lysates were prepared by addition of 0.5% Nonidet P-40 and stored at -80°C .

2.6. Eosinophil chemotaxis assay

The neutralizing activity of the Mabs against human eotaxin was tested by the human eosinophil chemotaxis assay. The assay was performed using an assembly consisting of a 96-well modified Boyden microchemotaxis chamber and a polycarbonate filter membrane (pore

size 5 μ m; Nucleopore, Pleasanton, CA, USA), as previously described [27].

2.7. Immunocytochemistry

Smears of eosinophils were prepared with a cytocentrifuge onto poly-L-lysine-coated glass slides. Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 min and treated with 0.1% trypsin. The slides were blocked with Tris-buffered saline, pH 7.6, containing 2% BSA and 5% normal goat serum, for 2 h at room temperature. To detect intracellular eotaxin, the cells were incubated with Mab 174.4 overnight and stained with a commercially available biotin-streptavidin system (LSAB-AP Kit, DAKO, Carpinteria, CA, USA). Isotype-matched mouse IgG was used as negative control. Cells were also counterstained with Myer's hematoxylin.

Immunoelectron microscopy was performed as described previously, with some modifications [28,29]. We used JB-4 resin (Polysciences, Warrington, PA, USA) for indirect immunoelectron microscopic labeling using a postembedding procedure. Eosinophils were fixed with a 4% paraformaldehyde, 0.75% glutaraldehyde, 1 mM $CaCl_2$, 0.1 M phosphate-buffered solution. The sections were incubated with either Mab 174.4 or isotype-matched mouse IgG for 12 h at room temperature. They were then labeled with gold particle-conjugated anti-mouse IgG(H+L) antibody (10 nm; GMHL10, British Biocell International, Cardiff, UK). Finally, the sections were stained with 1% aqueous uranyl acetate followed by a Reynolds' lead citrate solution. The electron microscope used was a Hitachi 1200-EX at an acceleration voltage of 100 kV.

3. Results

Five Mabs were produced by immunizing mice with recombinant eotaxin. Two of the Mabs, designated Mab 164.44 (IgG2a) and Mab 174.4 (IgG1), were fully characterized in terms of their antigen specificity and neutralizing activity. Their specificity was confirmed by direct-binding ELISA: they did not react with either MCP-1, MCP-2, MCP-3, MCP-4, MIP-1 α , IL-8 or RANTES (data not shown). Furthermore, Mab 164.44 antagonized eotaxin-induced eosinophil chemotactic activity: 50% inhibition was obtained at a molecular ratio (Mab/eotaxin) of approximately 1.0 (Fig. 1).

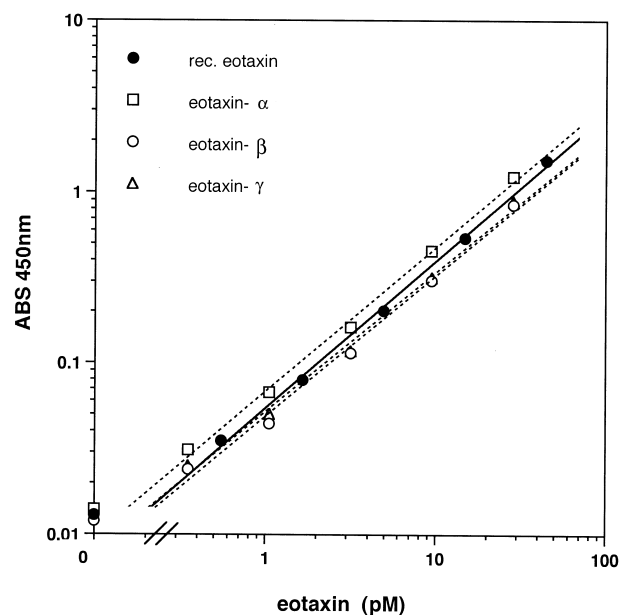


Fig. 4. Detection of recombinant and natural eotaxin by ELISA. Recombinant and dermal fibroblast-derived eotaxin (eotaxin- α , - β and - γ) were measured by sandwich ELISA using two Mabs (Mab 164.44 and Mab 174.4), as described in Section 2.

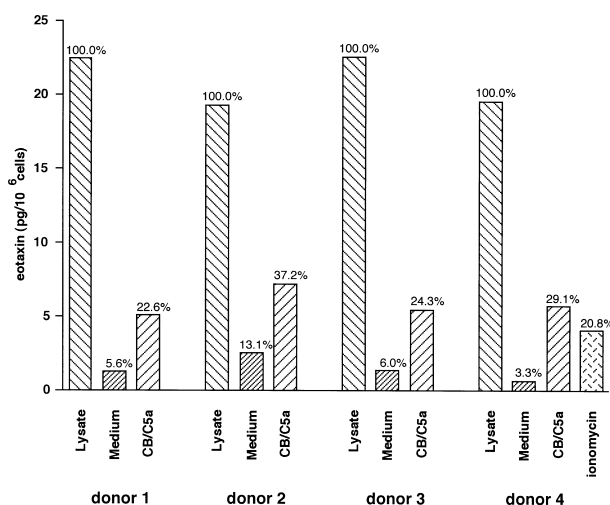


Fig. 5. Release of eotaxin from human eosinophils. Purified peripheral eosinophils were stimulated with either CB plus C5a or ionomycin for 60 min. The cell-associated total eotaxin was determined using the cell lysate.

Similar neutralizing activity was observed with Mab 174.4 (data not shown). To determine the presence of eotaxin in eosinophils, cells were purified from five non-allergic donors and reacted with Mab 174.4. As illustrated in Fig. 2, eosinophils were intensely stained with Mab 174.4. Immunoelectron microscopic analysis further demonstrated the intracellular localization of eotaxin to specific granules with the cytoplasm of eosinophils (Fig. 3).

In order to quantify the protein levels of human eotaxin, an ELISA system was developed by using Mab 164.44 and Mab 174.4. As shown in Fig. 4, the ELISA system was able to recognize not only recombinant eotaxin but also natural eotaxin- α , - β and - γ purified from supernatants of IL-4-stimulated dermal fibroblasts [25]. The detectable range of eotaxin was 0.6–60 pM (approximately 5–500 pg/ml). There was no crossreaction with eotaxin-2, MCP-1, -2, -3, -4, RANTES, MIP-1 α , pulmonary and activation-regulated chemokine (PARC), thymus and activation-regulated chemokine (TARC), liver and activation-regulated chemokine (LARC), lymphotactin (SCM-1), fractalkine or IL-8 (data not shown). Eosinophils, PMNs and MNCs were separated from healthy donors, and cell-associated eotaxin was measured with this ELISA system. As shown in Table 1, significant amounts of eotaxin were detected in peripheral eosinophils compared to PMNs and MNCs.

Since immunoelectron microscopic examination revealed that eotaxin was associated with specific granules in eosinophils, we next determined whether eotaxin was liberated during the process of degranulation. Purified eosinophils were stimulated with an established secretagogue, C5a, for 60 min. As shown in Fig. 5, the eosinophils released approximately 28.3% (range 22.6–37.2%) of their total intracellular eotaxin in response to C5a. Stimulation with a non-specific secretagogue, ionomycin, also resulted in the release of granule-associated eotaxin (Fig. 5, donor 4).

4. Discussion

Eotaxin is the most potent known chemoattractant for both

eosinophils and basophils [27]. A high-affinity receptor for eotaxin, designated CC chemokine receptor (CCR)3, was identified on eosinophils [24,30], basophils [31] and T-cells of the Th2 subset [32,33], all of which are predominant cells at the sites of ongoing allergic diseases. Other CCR3 ligands [34,35], such as RANTES and MCP-3, are able to bind to other types of CCR [36], and they are thereby active on various cell types. In contrast, eotaxin binds only to CCR3 and displays a selective chemotactic effect on eosinophils and basophils.

The expression of human eotaxin mRNA and/or protein was reported in various types of cells, including fibroblasts [25,37], epithelial cells [38], endothelial cells [39], T cells and macrophages [40]. Studies on biopsy specimens from the upper and lower airways of patients with allergic diseases revealed the expression of eotaxin protein in eosinophils infiltrating the inflammatory sites [22,23]. Using specific Mabs and a sensitive ELISA system, we here provide several lines of evidence that peripheral eosinophils of non-allergic individuals constitutively express eotaxin. Immunohistochemical experiments demonstrated the expression of eotaxin protein at the single-cell level. Quantification of cell-associated eotaxin revealed that eosinophils possess greater amounts of eotaxin compared to other types of cells in the peripheral blood. Most importantly, eotaxin was localized in the matrix of crystalloid granules of eosinophils and was released upon eosinophilic activation.

Freshly isolated eosinophils store several cytokines, including IL-4 [4,18,41], GM-CSF [14], IL-8 [11,13] and RANTES [4]. These preformed cytokines are liberated in response to appropriate stimuli and may contribute to allergic inflammation through autocrine and/or paracrine mechanisms. At present, the physiological significance of eosinophil-derived eotaxin is not fully understood. The amount of eotaxin in eosinophils (~ 20 pg/ 10^6 cells) was below the physiological relevant range (i.e. more than 10 ng/ml of eotaxin was required for chemotaxis of eosinophils in vitro [27]), indicating that eosinophils do not represent a major source of this molecule. We think that one of the major sources of eotaxin is fibroblasts, because we determined that IL-4- plus TNF- α -stimulated dermal fibroblasts released eotaxin in the order of 100 ng/ml into the culture supernatant (unpublished observation). However, the release of eotaxin from eosinophils may represent the autocrine and/or paracrine pathway of local eosinophil accumulation in inflammatory tissues. Although the role of eotaxin inside eosinophils is still unclear, further studies are necessary for the understanding of the presence of eotaxin in the cells.

In summary, we have demonstrated localization and storage of eotaxin in normal human eosinophils. We are currently analyzing preformed eotaxin in eosinophils of patients with allergic diseases. Our results suggest that eosinophils are an important cellular source of eotaxin.

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