Eotaxin Expression in Sephadex-Induced Lung Injury in Rats

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The CC chemokine eotaxin is a potent and specific eosinophil chemoattractant. Eosinophil-dependent tissue injury has been shown to contribute to airway inflammation such as that in asthma. In the present study, We investigated eotaxin expression in a rat model of pulmonary inflammation (featuring accumulation of eosinophils) induced by intratracheal instillation of cross-linked dextran beads (Sephadex G200). Intratracheal instillation of 5 mg/kg Sephadex caused a time-dependent eosinophil infiltration into the lung, reaching a peak at 24 hours. Eotaxin mRNA in the lung paralleled the eosinophil influx. Eotaxin protein in bronchoalveolar (BAL) fluids and lung homogenates was shown by Western blot and immunostaining to be maximally expressed by 24 hours. Sephadex-induced lung injury, as measured by ¹²⁵I-labeled albumin leakage from the pulmonary vasculature, developed in a time-dependent manner. Intravenous injection of blocking antibody to eotaxin significantly decreased eosinophil infiltration and lung permeability. These data suggest that, in the Sephadex model of lung inflammation, eotaxin up-regulation mediates intrapulmonary accumulation of eosinophils and the development of lung injury. (Am J Pathol 1999, 155:2001–2008)

Eosinophil infiltration into the airway is a characteristic feature in human with allergic asthma. On activation, eosinophils at the site of inflammation may release cationic proteins, proteases and peroxidase, lipid mediators, and oxygen radicals.¹ These reactive substances may cause airway hyperresponsiveness and respiratory epithelial damage.^{2,3} Eosinophils may also regulate the inflammatory process by releasing various cytokines.¹ Accumulating evidence indicates that local accumulation of eosinophils is regulated, at least in part, by a group of chemoattractant cytokines termed chemokines.⁴ Based on the location of the first two conserved cysteines, che-

mokines are divided into four groups: the CXC, CC, C, and CX₃C subfamilies. While the CXC, C, and CX₃C chemokines mainly attract neutrophils or lymphocytes, the CC family members are active toward macrophages, lymphocytes, basophils, and eosinophils.⁵ Several chemokines, including monocyte chemoattractant protein-2 (MCP-2), MCP-3, MCP-4, MCP-5, macrophage inflammatory protein-1 α (MIP-1 α), eotaxin, and eotaxin-2, have been shown to have eosinophil chemotactic activities.^{6–8}

The CC chemokine eotaxin was first identified from bronchoalveolar lavage fluid from allergen-sensitized guinea pigs and was subsequently demonstrated to cause eosinophil infiltration into the guinea pig lung and skin.^{9,10} More recently, eotaxin genes from mice,^{11,12} rats,¹³ and humans^{14,15} have been cloned. Eotaxin exhibits potent and specific chemotactic activity for eosinophils in all of these species both *in vivo* and *in vitro*. When delivered to mice, eotaxin induces a potent and rapid eosinophil recruitment that is enhanced by interleukin-5 (IL-5).^{16,17} Targeted disruption of eotaxin in knock-out mice partially reduces antigen-induced tissue eosinophil accumulation.¹⁸ Eotaxin is constitutively expressed in a number of organs such as intestine, lung, and thymus.¹⁹ The expression of eotaxin mRNA and protein is up-regulated in rodent models of allergic airway inflammation.^{11,13} Elevation of eotaxin mRNA and protein is also observed in bronchoalveolar lavage fluids and in airway tissues obtained from asthmatic patients.^{20,21}

Recently, a rat model of pulmonary inflammation has been developed by intratracheal instillation of cross-linked dextran beads (Sephadex G-200).^{22,23} To understand the pathophysiology of this model, we examined eotaxin expression in this eosinophil-rich lung inflammation. Experiments were performed to analyze 1) the kinetics of lung injury and eosinophil infiltration into the lung after Sephadex treatment, 2) the correlation between the accumulation of eosinophils and expression of eotaxin, and 3) the effects of blockade of eotaxin *in vivo* on the infiltration of eosinophils and lung injury.

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Materials and Methods

Chemical and Reagents

Except where noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal IgG to murine eotaxin was generously provided by Dr. Steven Kunkel (University of Michigan, Ann Arbor, MI). The antibody did not demonstrate cross-reactivity with other known cytokines and chemokines, including human eotaxin.

Animal Model

Male Long-Evans rats (275–300 g, specific pathogen free; Harlan Industries, Rochester, MI) were anesthetized with intraperitoneal injections of ketamine (2.5–5.0 mg/100 g body weight). A suspension of Sephadex G-200 beads (Pharmacia, Piscataway, NJ) was prepared in sterile phosphate-buffered saline (PBS) 2 days before use. Lung injury was induced by intratracheal instillation of 0.3 ml of Sephadex beads (5 mg/kg body weight) through a tracheal cannula during inspiration. Negative controls received the same volume of sterile PBS intratracheally. Immediately thereafter, trace amounts of ^{125}I -labeled bovine serum albumin (BSA) (as a quantitative marker of permeability) were injected intravenously as described.²⁴ Rats were sacrificed at the indicated times, and the amount of radioactivity (^{125}I -labeled BSA) was determined by scintillation counting to quantitate lung injury by measuring vascular permeability. To calculate the permeability index, the amount of radioactivity (^{125}I -labeled BSA) in the PBS-perfused lungs was compared with the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice.

Bronchoalveolar Lavage and Leukocyte Counts

Bronchoalveolar lavage (BAL) was performed as described before²⁴ by gently instilling PBS into the lung via a tracheal catheter followed by withdrawal. This process was repeated five times with 10 ml PBS each time. Eighty percent of the fluid instilled was retrieved at the end of the lavage procedure. The cell pellet was put through a hypotonic lysis step to remove contaminating red blood cells. Total BAL cells counts were determined with a hemacytometer. Slides for differential cell counts were prepared by cytopspin at $700 \times g$ for 7 minutes. Slides were then fixed and stained with Camco Quik Stain (Cambridge Diagnostic Products, Lauderdale, FL) to determine the percentage of eosinophils, monocytes/macrophages, and neutrophils. The total numbers of these cells for each sample were then determined according to the volume of BAL recovered.

Northern Blot Analysis

RNA was extracted from whole lungs, using TRIzol Reagent (Gibco BRL, Gaithersburg, MD) according to the

manufacturer's protocol. Twelve micrograms of total RNA was fractionated electrophoretically in a 1% formaldehyde gel and transferred to a nylon blot (MSI, Westboro, MA). A cDNA probe for rat eotaxin was generated by reverse transcriptase-polymerase chain reaction, using a 5' primer 5'-CGCTTCTATTCCTGCTGCTC-3' corresponding to bp 51–70 of murine eotaxin cDNA and a 3' primer 5'-ACTTCTTCTTGGGGTCAGCA-3' corresponding to bp 253–272.²⁵ The PCR product was verified by DNA sequencing. The rat eotaxin cDNA probe was radiolabeled with [^{32}P]dCTP, using a Redivue labeling kit (Amersham, Little Chalfont, UK). The blot was prehybridized at 42°C for 2 hours in 50% formamide, 5× saline sodium phosphate-EDTA (SSPE), 2% sodium dodecyl sulfate (SDS), 10× Denhardt's solution, and 100 $\mu\text{g/ml}$ salmon sperm DNA solution. Hybridization was performed in the same solution at 42°C overnight with 1.5×10^7 cpm ^{32}P -labeled eotaxin probes. After hybridization, the blot was washed twice at 50°C for 20 minutes in $0.1 \times$ standard saline citrate and 0.1% SDS. The autoradiogram was developed on X-Omat film (Eastman Kodak, Rochester, NY). Equal loading of RNA was confirmed by probing with [^{32}P]dCTP-radiolabeled mouse β -actin (Clontech, Palo Alto, CA). Densitometry was performed with a densitometer (Fotodyne, New Berlin, WI) with AM-BIS software (San Diego, CA). The relative intensity units were defined as the autoradiographic densities of eotaxin normalized to β -actin.

Western Blot Analysis

Lung homogenates and BAL fluid from rats undergoing Sephadex-induced lung injury and murine eotaxin (R&D Systems, Minneapolis, MN) were separated by electrophoresis on 15% SDS-polyacrylamide gels. Homogenate protein levels were determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA), and a total of 150 μg protein for each time point was loaded under reducing conditions. BAL fluids were concentrated 10 times with an Ultrafree-4 Centrifugal Filter Unit (Millipore Co., Bedford, MA), and 25 μl was loaded. The protein recovery rate is about 95–100% by this method, according to the manufacturer's instructions. The separated proteins were transblotted onto nitrocellulose membrane (0.45 μm ; Bio-Rad) for 1 hour at 12 V. The membrane was blocked overnight at 4°C with 5% nonfat dry milk (NFD) in Tris-buffered saline (TBS) (0.15 mol/L NaCl, 0.02 mol/L Tris, pH 7.6) and then washed three times with 0.05% Tween-20 in TBS (TBS-T). The membrane was then incubated with purified rabbit anti-murine eotaxin Ab diluted 1:1000 in TBS containing 1% NFD for 2 hours at room temperature. After three washings in TBS-T, secondary Ab (goat anti-rabbit IgG horseradish peroxidase-conjugated Ab; Bio-Rad) was added at a final dilution of 1/10,000 in 1% NFD-PBS and incubated for 1 hour at room temperature. After washing with TBS-T, the membrane was developed by an enhanced chemiluminescence technique according to the manufacturer's protocol (Amersham). Recombinant murine eotaxin was used as a reference control.

Pathology and Immunohistochemistry

Lungs from control and Sephadex-injured rats were frozen in OCT compound (Miles Co., Elkhart, IN). Sections (4–5 μm) were prepared from the embedded tissue disks and stained with hematoxylin-eosin (H&E). Lung histology was assessed by light microscopy. For immunostaining, samples were fixed in methanol at -20°C for 10 minutes and then stained with biotinylated anti-eotaxin mouse polyclonal antibody in PBS containing 0.1% BSA for 1 hour in a humidified chamber. Slides were then washed three times in PBS and incubated for 1 hour with horseradish peroxidase-streptavidin (Bio-Rad). Eotaxin was visualized using diaminobenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD), and the tissues were counterstained with hematoxylin. BAL cells were also stained for eotaxin by employing similar immunostaining methods after cytospin preparations.

Effects of Anti-Eotaxin Antibody on Sephadex-Induced Lung Injury and Eosinophil Infiltration

For blockade experiments, either 480 μg irrelevant rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or 480 μg anti-eotaxin polyclonal rabbit IgG was infused intravenously or instilled intratracheally 18 hours after Sephadex instillation. Twenty-four hours after Sephadex instillation, lung permeability indices were calculated and eosinophil numbers were enumerated.

Statistical Analysis

All values were expressed as mean $\pm$ SEM. Data were analyzed with a one-way analysis of variance, and individual group means were then compared by Student's *t*-test. Differences were considered significant when $P < 0.05$. For calculations of percentage change, the negative control values were subtracted from the positive control or the treatment group values.

Results

Lung Injury and Leukocyte Infiltration after Intratracheal Instillation of Sephadex

To characterize this rat model of inflammation, 5 mg Sephadex/kg body weight was instilled intratracheally into the lung. Eosinophils, monocytes/macrophages, and neutrophils present in the BAL fluid were enumerated, and the lung injury was determined by lung permeability at different time points (0, 2, 4, 8, 12, 24, 48 hours). Progressive increases in the numbers of eosinophils were detected in BAL fluids (Figure 1A). A significant increase was found at 12 hours, reaching a maximum level at 24 hours. At 48 hours, the eosinophil number decreased but was still significantly higher than the control level. As will be indicated below, many eosinophils were observed in the interstitium of lung tissue at 24 hours. A significant increase in monocytes/macrophages was also observed

Figure 1. Leukocyte infiltration induced by intratracheal instillation of Sephadex in rat lung. Eosinophils (A), monocytes/macrophages (B), and neutrophils (C) were counted at indicated times after 5 mg/kg Sephadex administration. Values represent mean $\pm$ SEM with $N = 4$ for each group. * $P < 0.05$, ** $P < 0.01$ when compared to PBS-injected controls.

Figure 2. Time course of lung injury induced by intratracheal instillation of Sephadex. The permeability indices were calculated at indicated hours after 5 mg/kg Sephadex administration. Values represent means $\pm$ SEM ($n = 3$ or 4).

at 24 hours (Figure 1B). Neutrophils were maximally increased at 8 hours and then decreased, but remained higher than control levels at 12–48 hours (Figure 1C).

Intratracheal instillation of Sephadex also induced a time-dependent increase in permeability index as measured by albumin leak into the lung (Figure 2). A peak increase was found at 24 hours, with a permeability index of 0.48 ± 0.05 . The permeability index remained at a high level as long as 48 hours.

Eotaxin mRNA Expression in Sephadex-Injured Lungs

Total RNA was extracted from lungs over a range of time (from 0 to 48 hours). Eotaxin mRNA expression was quantitated by Northern blot analysis; the results are shown in Figure 3. Very low levels or constitutive expression of mRNA could be detected in normal lung (time = 0 hours). Eotaxin mRNA expression started to increase at 12 hours (twofold) and increased maximally at 24 hours (sevenfold) (Figure 3B). Sustained up-regulation was still found at 48 hours. Equal loading of RNA was confirmed by probing the same blot to determine β -actin levels (Figure 3A).

Eotaxin Levels in BAL Fluids and Lung Homogenates

Under the same experimental condition as mRNA quantitation, eotaxin levels in BAL fluids and lung homogenates were analyzed by Western blot analysis using anti-eotaxin Ab. The presence of rat eotaxin was indicated by a band at approximately 8 kd. Low levels of eotaxin were revealed in $10\times$ concentrated BAL fluids at 0 hours. Eotaxin levels in BAL maximally increased at 24 hours and remained higher than control levels at 48 hours (Figure 4A). Eotaxin expression in lung homogenates exhib-

Figure 3. Eotaxin mRNA expression in Sephadex-injured lungs. **A:** Total RNA was extracted from whole-lung homogenates after 5 mg/kg Sephadex administration at the times indicated, and Northern analysis was performed according to the description in Materials and Methods. Equal RNA loading was confirmed by probing the same blot with β -actin. **B:** Densitometric quantification of eotaxin mRNA normalized to β -actin RNA. Intensity of control eotaxin/ β -actin signal was defined as 1. Results are representative of two separate experiments.

ited almost the same pattern as that in BAL, with constitutive expression at 0 hours, peak expression at 24 hours, and a high level of expression at 48 hours (Figure 4B).

Immunohistochemical Analysis of Eotaxin in the Lung and BAL Cells

Eotaxin expression in the lung sections and BAL cells was evaluated by immunostaining techniques 24 hours after Sephadex challenge. Infrequent eotaxin-staining cells (appearing as a brown stain) were present at 0 hours (Figure 5A), whereas many more cells with positive staining were found 24 hours after Sephadex instillation (Figure 5B). Similarly, little eotaxin was detected in frozen sections of lungs at 0 hours (Figure 5C), whereas positively staining cells dramatically increased in the peribronchiolar and perivascular areas at 12 hours (data not shown) and 24 hours (Figure 5, D and E).

By comparing the H&E staining and immunostaining of the same sections, many eosinophils were found in interstitial areas (Figure 5G).

(A) BAL Fluids

Time (hours)

Std 0 2 4 6 8 12 24 48

(B) Lung Homogenates

Time (hours)

Std 0 2 4 8 12 24 48

Figure 4. Western blot analysis for eotaxin in 10 × concentrated BAL fluids (A) and whole-lung homogenates (B). Samples were obtained at indicated times after Sephadex instillation. Std, 25 ng eotaxin control. Results are representative of two separate experiments.

Effect of Anti-Eotaxin Ab on Eosinophil Accumulation Induced by Sephadex Administration

A time point of 24 hours was selected based on the time of peak increase in eosinophil number in BAL fluids (Figure 1A) and vascular permeability (Figure 2).

Eighteen hours after Sephadex instillation, rats received either 480 μ g of irrelevant rabbit IgG or 480 μ g of rabbit IgG anti-eotaxin intravenously. Intravenous injection of anti-eotaxin resulted in a significant reduction of eosinophils in BAL retrieved from Sephadex-injured rats at 24 hours (Figure 6A). The BAL eosinophil counts for negative and positive controls treated with 480 μ g of irrelevant IgG were $0.44 \pm 0.26 \times 10^5$ cells and $3.88 \pm 0.43 \times 10^5$ cells, respectively. A 64% reduction (to $1.66 \pm 0.14 \times 10^5$ cells, $P < 0.05$) in BAL eosinophils was observed when anti-eotaxin was intravenously administered 18 hours after Sephadex instillation. Intratracheal anti-eotaxin administration resulted in a slight but insignificant reduction in BAL eosinophils (to $3.49 \pm 0.29 \times 10^5$ cells, $P > 0.05$) (data not displayed).

Effects of Anti-Eotaxin Antibody on Lung Injury Induced by Sephadex

A rabbit anti-murine eotaxin antibody was administered to evaluate the role of eotaxin in the lung injury. The effect on pulmonary vascular permeability at 24 hours was determined after Ab treatment (infused intravenously 18

hours after Sephadex administration). The administration of 480 μ g anti-eotaxin 18 hours after Sephadex instillation resulted in a significant decrease (53%, $P < 0.05$) in the permeability index when compared to controls (Figure 6B). The permeability index was not significantly changed by intratracheal administration of anti-eotaxin antibody (data not shown).

Discussion

Intratracheal instillation of Sephadex beads causes a marked inflammatory reaction in rats. This reaction is characterized by peribronchitis/bronchiolitis with a large accumulation of eosinophils in the airways and peribronchial tissue.²² Perivascular and peribronchial edema also develop after airway administration of Sephadex beads.²² These features correspond to some of the pathological characteristics of human asthmatics.^{22,23} In contrast to human asthma, a granulomatous alveolitis has also been observed in this animal model.²³ Asthma in humans is a recurrent inflammatory process, whereas the animal model used in the current studies is only a short-term inflammatory response caused by the tissue response to the presence of Sephadex beads. Obviously, this model only partly represents patterns of inflammation characteristics of human asthma. Nevertheless, the model is useful for defining mediator pathways involved in eosinophil recruitment.

Eosinophils are considered to be the major proinflammatory leukocytes involved in asthma. Eosinophil products may directly damage airway epithelium by the release of cationic proteins, major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP).²⁶ The number of activated eosinophils in human asthma has been reported to correlate with the degree of airway hyperreactivity.²⁷ In this study, the lung injury induced by intratracheal instillation of Sephadex parallels the number of eosinophils in the BAL fluids over a 12–24-hour time period; the suppression of eosinophil accumulation in the lung by anti-eotaxin reduces lung injury. These results support the link between the eosinophil recruitment and tissue injury. However, there is a lack of correlation between the lung injury and eosinophil influx before 12 hours. As shown in Figure 1C, neutrophil influx tended to increase at 4 hours (although not statistically significantly) and dramatically increased at 8 hours and 12 hours. These data suggest that the early injury occurring at 4–12 hours might be due to other chemotactic factors and neutrophil-mediated injury.

The evidence for roles of eosinophils in asthma and allergic disease has stimulated considerable interest in molecular mechanisms regulating eosinophil trafficking. The recruitment of eosinophils to the inflammatory sites during allergic inflammation is a complex process that is potentially regulated by a number of cytokines such as interleukin (IL)-1 β , IL-3, IL-4, IL-5, IL-12, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNF- α),^{28–31} and CC chemokines, including MCP-2, MCP-3, MCP-4, MCP-5, MIP-1 α , eotaxin, and eotaxin-2.^{6–8} Eotaxin and IL-5 appear to be

Figure 5. Immunohistochemical staining of eotaxin in Sephadex-injured lungs and BAL cells. Samples were obtained from control (A, C) or 24 (B, D, E, F) hours after Sephadex instillation, stained with biotinylated anti-eotaxin, and counterstained with hematoxylin as described in Materials and Methods. A lung section (24 hours) was stained with hematoxylin-eosin and examined by light microscopy (G). C, D, F, and G: bronchiole; E: venuole. Magnification: A–E, $\times 40$; F and G, $\times 100$.

more importantly responsible for the development of eosinophilia. IL-5 mobilizes eosinophils from the bone marrow¹⁷ and functions with eotaxin to regulate eosinophil homing and migration to sites of allergic inflammation.³² In guinea pigs and mice, eotaxin is expressed in the lung after antigen challenge, and its expression parallels eosinophil infiltration.^{25,33,34} In human allergic asthma patients, eotaxin up-regulation correlates with the numbers of activated eosinophils.³⁵ In the current rat model of allergic airway inflammation, eotaxin mRNA and protein are expressed in lung and correlate with the number of

eosinophils in BAL fluids. Anti-eotaxin antibody administration inhibits eosinophil infiltration by 64% (Figure 6). This results suggest an integral role for eotaxin in regulating eosinophil homing and tissue recruitment. It is well known that adhesion of leukocytes to vascular endothelium is essential for their migration into inflamed tissues. *In vivo* eosinophil accumulation induced by eotaxin is dependent on α_4/β_2 integrin and vascular cell adhesion molecule-1 (VCAM-1) as well as β_2 integrin/intercellular adhesion molecule-1 (ICAM-1) pathways.^{36,37} Eotaxin up-regulates the expression of ICAM-1 and VCAM-1 on

Figure 6. Effects of anti-eotaxin antibody on Sephadex-induced lung injury and eosinophil infiltration. Polyclonal anti-eotaxin mouse IgG or control IgG (480 μ g) was intravenously administered 18 hours after Sephadex instillation. Six hours later or 24 hours after Sephadex instillation, eosinophil counts in BAL fluids (**A**) and vascular permeability (**B**) were measured. All values represent means $\pm$ SEM ($n = 4$).

endothelial cells and CD11b/CD18 on eosinophils.^{38,39} The stimulation of eosinophils with eotaxin results in increased adhesion to human lung microvascular endothelial cells pretreated with TNF- α .⁴⁰ These findings suggest that eotaxin promotes the adherence of circulating eosinophils to vascular walls and their emigration through vascular walls into extravascular space. Interestingly, eotaxin itself also induces a rapid release of eosinophils and their progenitors from the bone marrow.⁴¹ In the blockade experiments, intravenous injection of anti-eotaxin Ab is more effective than intratracheal injection in the inhibition of eosinophil infiltration. This indicates that the development of eosinophil accumulation in lung is an integral process that may require both local and systemic eotaxin.

Many cell types are responsible for eotaxin production, including macrophages, T lymphocytes, bronchial epithelial cells, endothelium, and eosinophils themselves.^{20,21,42,43} In the model described above, immunostaining of lung sections (12 hours and 24 hours) demonstrated that eotaxin was predominantly found in areas featuring the presence of eosinophils. This suggests that eosinophils may be a source of eotaxin in this model. The release of eotaxin from eosinophils may represent an autocrine and/or paracrine pathway of local

eosinophil accumulation in inflammatory tissues. However, further experiments need to be used to identify the cell types responsible for eotaxin secretion.

In summary, this study demonstrates that eotaxin expression correlates with the eosinophil infiltration in the rat lung after airway challenge with Sephadex beads. Anti-eotaxin antibody attenuates eosinophil infiltration and lung injury. Our results provide further evidence of the pivotal role of eotaxin in the development of lung eosinophilia. A potent eotaxin antagonist could be a useful therapeutic strategy in the modulation of eosinophil functional responses in allergic diseases.

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