Regulatory Effects of Eotaxin on Acute Lung Inflammatory Injury

Ren-Feng Guo, Alex B. Lentsch, Roscoe L. Warner, Markus Huber-Lang, J. Vidya Sarma, Tom Hlaing, Michael M. Shi, Nicholas W. Lukacs and Peter A. Ward

J Immunol 2001; 166:5208-5218; doi: 10.4049/jimmunol.166.8.5208
http://www.jimmunol.org/content/166/8/5208

References
This article cites 52 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/166/8/5208.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulatory Effects of Eotaxin on Acute Lung Inflammatory Injury

Ren-Feng Guo,* Alex B. Lentsch,† Roscoe L. Warner,* Markus Huber-Lang,* J. Vidya Sarma,* Tom Hlaing,* Michael M. Shi,** Nicholas W. Lukacs,* and Peter A. Ward*

Eotaxin, which is a major mediator for eosinophil recruitment into lung, has regulatory effects on neutrophil-dependent acute inflammatory injury triggered by intrapulmonary deposition of IgG immune complexes in rats. In this model, eotaxin mRNA and protein were up-regulated during the inflammatory response, resulting in eotaxin protein expression in alveolar macrophages and in alveolar epithelial cells. Ab-induced blockade of eotaxin in vivo caused enhanced NF-κB activation in lung, substantial increases in bronchoalveolar lavage levels of macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (CINC), and increased MIP-2 and CINC mRNA expression in alveolar macrophages. In contrast, TNF-α levels were unaffected, and IL-10 levels fell. Under these experimental conditions, lung neutrophil accumulation was significantly increased, and vascular injury, as reflected by extravascular leak of 125I-albumin, was enhanced. Conversely, when recombinant eotaxin was administered, the increased levels of MIP-2 and CINC mRNAs were markedly diminished, whereas MIP-1α, MIP-1β, TNF-α, and IL-1β expression of mRNA and protein was not affected. These data suggest that endogenous eotaxin, which is expressed during the acute lung inflammatory response, plays a regulatory role in neutrophil recruitment into lung and the ensuing inflammatory damage. The Journal of Immunology, 2001, 166: 5208–5218.

T he CC chemokine, eotaxin, was originally described as the predominant eosinophil chemoattractant present in bronchoalveolar lavage (BAL) fluids of guinea pigs during allergic airway inflammation (1). Additional data have suggested a pivotal role of this chemokine in eosinophil presence in both human and animal models of “allergic inflammation” (2–4). Eotaxin is constitutively expressed in a number of organs such as intestine, lung, and thymus (5). Up-regulation of eotaxin mRNA and protein in BAL fluids and in airway tissues has been found in asthmatic individuals and in rodents undergoing allergic airway inflammation. The expression of eotaxin correlates with the influx of eosinophils into the airways (3, 4, 6, 7). Eotaxin exerts its effects via CC chemokine receptor-3 (CCR3). CCR3 expression has been demonstrated on eosinophils, basophils, and Th2-type lymphocytes (8–11). Human neutrophils stimulated with IFN-γ expressed CCR3 and showed chemotactic responsiveness to eotaxin (12), suggesting that in some conditions eotaxin may be involved in the mobilization of neutrophils. Low levels of CCR3 were shown to be expressed on human alveolar macrophages and served as a coreceptor for HIV entry (13). In addition to the importance of eotaxin in allergic inflammation, this chemokine may be important in acute lung inflammation. An increase in eotaxin mRNA in the lung has been found by using a mouse model of acute lung inflammation induced by inhalation of LPS (14). This model is associated with neutrophil infiltration.

The accumulation of neutrophils in lung is an important component in the pathogenesis of a large number of pulmonary diseases. We have used a rat model of lung inflammatory injury induced by intrapulmonary deposition of IgG immune complexes (IC) to elucidate the mediator pathways that lead to the accumulation of neutrophils in lung. The requirement of cytokines and chemokines for inflammatory responses has been defined in this model (15, 16). Alveolar macrophages appear to be one of the major sources for proinflammatory cytokines and chemokines in this model (17–19). The “early response” cytokines, TNF-α and IL-1, cause vascular adhesion molecule expression (ICAM-1 and E-selectin), setting the stage for in vivo adhesive interactions between neutrophils and endothelial cells (20, 21). The CXC chemokines, macrophage inflammatory protein (MIP)-2 and cytokine-inducible neutrophil chemoattractant (CINC), play important roles in neutrophil recruitment in this inflammatory model (19). In addition, the CC chemokines, MIP-1α and MIP-1β, also play important functions in the lung recruitment of neutrophils (22, 23). The most important endogenous mediators that negatively regulate these NF-κB-dependent inflammatory responses include IL-10, IL-13, and IL-1 receptor antagonists (24–27). In this study we unexpectedly obtained evidence that endogenous eotaxin appears to regulate neutrophil influx and subsequent lung vascular injury induced by IgG-IC deposition in rat lungs. This report provides...
evidence that eotaxin may be involved in regulating the in vivo recruitment of neutrophils into the lung.

Materials and Methods

Reagents

Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Components for the CINC, MIP-1α, and MIP-1β ELISA and recombinant mouse IgG were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal IgG anti-mouse eotaxin was prepared as described elsewhere (28). This Ab has been shown to be nonreactive with other CXC and CC chemokines (28–30). The Ab is cross-reactive with rat eotaxin as described below. Preimmune IgG or anti-eotaxin IgG (400 µg) was instilled i.v. or intratracheally at the initiation of lung injury, as indicated below.

IgG-IC-induced alveolitis

Pathogen-free male Long-Evans rats (275–300 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with ketamine hydrochloride (150 mg/kg i.p.). Rabbit polyclonal IgG anti-BSA (2.5 mg; ICN Biomedicals, Costa Mesa, CA) in a volume of 0.3 ml in PBS (pH 7.4) were instilled intratracheally during inspiration. For one set of experiments involving in vivo evaluation of NF-κB activation, the dose of anti-BSA was lowered to 1.25 mg to assess more effectively changes in activation of NF-κB. Immediately after intratracheal instillation of anti-BSA, 10 mg BSA in 0.5 ml PBS was injected i.v. Negative control rats received PBS, pH 7.4, intratracheally. For analysis of pulmonary vascular permeability, trace amounts of 125I-labeled albumin were injected i.v. Unless otherwise indicated, 4 h after initiation of IgG-IC deposition rats were exsanguinated, the pulmonary circulation flushed via the pulmonary artery with 10 ml PBS, and the lungs surgically dissected. The 4-h time point is when neutrophil accumulation and lung injury are peaking (31). The extent of lung injury was quantified by calculating the lung permeability index (dividing the amount of radioactivity (125I-labeled albumin) in the perfused lungs by the amount of radioactivity in 1.0 ml of blood obtained from the inferior vena cava at the time of exsanguination), and then multiplying the result by 100. For analysis of NF-κB, lungs were immediately frozen in liquid nitrogen after vascular perfusion with PBS. Congo-red staining of BAL cells demonstrated the absence of any eosinophils in BAL fluids obtained from normal and eotaxin-treated rats 4 h after IgG-IC deposition (data not shown).

Identification of eotaxin by Western blot analysis and ELISA

BAL fluids were collected by instilling and withdrawing 5 ml sterile saline three times from the lungs via an intratracheal cannula. BAL fluids were concentrated 10 times with an Ultrafree-4 Centrifugal Filter Unit (Millipore, Bedford, MA). Interfering anti-BSA IgG in BAL fluids was removed with GammABind G Sepharose (Amersham-Pharmacia Biotech, Piscataway, NJ). Samples containing 100 µg protein were electrophoresed in a denaturing 12.5% polyacrylamide gel and then transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with TBST (40 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20). Membranes containing 5% nonfat dry milk for 12 h at 4°C. Membranes were incubated in a 1:1000 dilution of rabbit polyclonal anti-murine eotaxin. After three washes in TBST, membranes were incubated in a 1:50,000 dilution of HRP-conjugated donkey anti-rabbit polyclonal anti-murine eotaxin. After three washes in TBST, membranes were incubated in a 1:50,000 dilution of HRP-conjugated donkey anti-rabbit polyclonal anti-murine eotaxin (Amersham-Pharmacia Biotech). The membrane was developed by an enhanced chemiluminescence technique according to the manufacturer’s protocol (Amersham-Pharmacia Biotech). Eotaxin levels in BAL fluids were also measured using standard mouse ELISA kits according to the manufacturer’s instructions (R&D Systems).

Northern blot analysis

RNA was extracted from alveolar macrophages and lung homogenates using TRRizol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. Ten micrograms of total RNA was fractionated electrophoretically in a 1% formaldehyde gel and transferred to a nylon blot (MSI, Westboro, MA). The rat eotaxin, MIP-2, CINC, MIP-1α, and MIP-1β cDNAs were radioisotopically labeled with 32PdCTP using a Redivue labeling kit (Amersham, Little Chalfont, U.K.). The blots were prehybridized at 42°C for 2 h in 50% formamide, 5% saline sodium phosphate-EDTA (SSPE), 2% SDS, 10 × Denhardt’s solution, and 100 µg/ml salmon sperm DNA solution. Hybridization was performed in the same solution at 42°C overnight with 1.5 × 106 cpm 32P-labeled probes. After hybridization, the blot was washed twice at 50°C for 20 min in 0.1X SSC 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 32PdCTP-radio labeled rat GAPDH or β-actin cDNA. Northern blots were quantitated by laser densitometry using ImageQuant software and Phosphorimager 445 SI (both obtained from Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry

Lungs from control and IgG-IC-injured rats were frozen in OCT compound (Miles, Elkhart, IN). Sections (4–5 µm) were prepared from the embedded tissue discs. The samples were fixed in methanol at −20°C for 10 min and then stained with biotinylated anti-eotaxin mouse polyclonal Ab in PBS containing 0.1% BSA for 1 h in a humidified chamber. Slides were then washed three times in PBS and incubated for 1 h with HRP-Streptavidin (Bio-Rad, Hercules, CA). Eotaxin was visualized using diaminobenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the tissues were counterstained with hematoxylin. BAL cells were also stained for eotaxin by using similar immunostaining methods after cyotoxin preparations.

Assessment of NF-κB activation by EMSA

Nuclear extracts of alveolar macrophages and whole-lung tissues were prepared as previously described (32) and analyzed by EMSA. Double-stranded consensus oligonucleotide (NF-κB; 5′-AGTTGAGGGGACTTTCG-3′, Promega, Madison, WI) was end-labeled with [32P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham-Pharmacia Biotech). Binding reactions containing 5 µg of protein for nuclear extracts and 35 fmol (~50,000 cpm, Cherenkov counting) of oligonucleotide were performed for 30 min in binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, pH 8.0, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris, pH 7.6, 50 µg/ml poly(dIdc); Amersham-Pharmacia Biotech). Reaction volumes were held constant to 15 µl. Reaction products were separated in a 4% polyacryl- amide gel and analyzed by autoradiography. NF-κB activation was quantitated from digitized autoradiography films using image analysis software (Adobe Systems, San Jose, CA).

In vitro stimulation of alveolar macrophages

Alveolar macrophages were isolated by repeatedly lavaging lungs of anesthetized Long-Evans rats (Charles River Breeding Laboratories, Portage, MI) with 10 ml of saline. Fresh fluid lavage fluids, cells were suspended in medium (DMEM; BioWhittaker, Walkersville, MD), plated in 48-well microtiter plates (Corning Glass, Corning, NY) at a concentration of 1 × 106 cells/well or in 100-mm cell culture dishes at a concentration of 1.5 × 107/dish, and allowed to settle for at least 1 h. Plates or dishes were then washed with medium to remove nonadherent cells. IgG-IC were formed by incubation of BSA with rabbit anti-BSA IgG in a 1:4 molar ratio. The centrifuged precipitate containing IgG-IC was resuspended in DMEM to a final concentration of 100 µg/ml. This concentration was established based on dose responses of alveolar macrophages using increasing amounts of IC and resulted in maximal chemokine stimulation of cell cultures (33). All studies involved at least quadruplicate replicates. Rabbit anti-beta IgG (negative control, 100 µg/ml), IC (100 µg/ml), or eotaxin (0, 25, 50, 100, and 200 ng/ml) resuspended in DMEM was added to cells. After an incubation period, supernatant fluids and cells were collected for next step analysis. All ELISA results were normalized to the cell number present.

Quantitation of chemokines and cytokines

BAL fluids were collected by instilling and withdrawing 5 ml sterile saline three times from the lungs via an intratracheal cannula. BAL fluids were measured using Ab-sandwich ELISA. Ab-sandwich ELISA was developed from digitized autoradiography films using image analysis software (Adobe Systems, San Jose, CA).

Statistical analysis

In groups with equal variances, data sets were analyzed using one-way ANOVA, and individual group means were then compared with the Student-Newman-Keuls multiple comparison test. In groups containing unequal variances, Kruskal-Wallis ANOVA was performed followed by Dunn’s method for multiple comparison. All values were expressed as mean ± SEM. Significance was assigned where p < 0.05. For percent change between groups, values obtained from negative controls were subtracted from each data point.
Results

Expression of eotaxin mRNA and protein in inflamed lungs

Lungs from rats undergoing IgG-IC deposition were evaluated over a 4-h time course for expression of eotaxin mRNA and protein. As shown in Fig. 1A, very low levels of constitutive eotaxin mRNA were present in whole lung extracts at time 0 and during the first 2 h. By 4 h, eotaxin mRNA was greatly increased in lung extracts. Loading conditions of the gels were documented by analysis of β-actin mRNA (Fig. 1A). Image analysis of eotaxin mRNA blots revealed the constitutive presence of eotaxin mRNA and up-regulation of eotaxin mRNA 4 h after intrapulmonary deposition of IgG-IC (Fig. 1B).

When 10^3 concentrated BAL fluids were evaluated by Western blot analysis for eotaxin protein, there was clear evidence for increased eotaxin content in BAL fluids 2 and 4 h after onset of the inflammatory reaction (Fig. 1C). There was a double-banding pattern for eotaxin, especially at the 4-h time point. This pattern was also observed in the eotaxin standard, suggesting that eotaxin may be present both in monomeric (~8 kDa) and dimeric (~16 kDa) forms. To confirm that the anti-mouse eotaxin Ab was reactive with rat eotaxin, Western blots were performed using a recombinant murine eotaxin standard (std) as well as the BAL fluids obtained 4 h after onset of lung injury. When the anti-eotaxin Ab (diluted 1:1000) was preabsorbed with murine eotaxin (1 mg/ml), the banding pattern for murine eotaxin as well as for the BAL fluids completely disappeared (Fig. 1D, right hand frame), indicating that anti-mouse eotaxin was reactive with rat eotaxin. Eotaxin levels in BAL fluids were also determined by ELISA (Fig. 1E). In agreement with Western blot results, eotaxin levels were barely detectable at time 0 h, increased to 24.50 ± 4.96 (p < 0.01, n = 5) at 2 h, and further increased to 72.97 ± 8.86 (p < 0.001, n = 5) at 4 h.

By immunohistochemical analysis, alveolar macrophages obtained by BAL from normal rat lungs showed low constitutive levels of cytoplasmic eotaxin (Fig. 2A), whereas alveolar macrophages obtained 4 h after initiation of the lung inflammatory reactions showed intense cytoplasmic staining for eotaxin in virtually all cells (Fig. 2B). When frozen sections of lungs at time 0 were evaluated for eotaxin by immunostaining there was limited, if any, staining of lung cells (Fig. 2C), whereas in lungs obtained 4 h after IgG-IC deposition there was intense staining of cells, which appear to be type II epithelial cells and lung macrophages (Fig. 2D, arrows). In addition, there was staining along alveolar walls, suggesting that type I alveolar epithelial cells also express eotaxin during the inflammatory response.

Effects of in vivo blockade of eotaxin

Rats undergoing IgG-IC deposition were treated i.v. or intratra-}

![FIGURE 1. Expression of rat eotaxin mRNA and protein during IgG-IC-induced lung injury. A, Lung RNA extracts were analyzed by Northern blots for eotaxin and β-actin mRNA. B, Autoradiographs were digitized and eotaxin mRNA expression was quantitated by image analysis. Results are representative of two independent and separate experiments. C, Western blot analysis of eotaxin protein in BAL fluids obtained 0, 2, and 4 h after deposition of IgG-IC in lung. Recombinant mouse eotaxin was used as a reference standard (std). Rabbit polyclonal anti-mouse eotaxin IgG was used as the primary Ab. D, Western blot analysis of mouse eotaxin (std) and 4 h concentrated (10×) BAL fluids (BALF) from inflamed lungs was performed with nonabsorbed anti-mouse eotaxin (left) or with eotaxin-absorbed Ab (right). E, Quantitation of eotaxin protein in BAL fluids obtained 0, 2, and 4 h after deposition of IgG-IC in lung by ELISA. *, p < 0.01; **, p < 0.001 compared with the time 0 value (n = 5 for each vertical bar).}
μg anti-eotaxin rabbit IgG. In preliminary experiments, it was determined that these doses of anti-eotaxin would consistently affect the intensity of lung inflammatory injury, whereas lower doses (100–200 μg IgG) failed to show consistent effects (data not shown). Four hours after IgG-IC deposition, the lung permeability index and BAL content of neutrophils were determined. In animals

FIGURE 2. Immunohistochemical expression of eotaxin protein in BAL macrophages and frozen sections of lungs. BAL macrophages were obtained at time 0 (A) and 4 h (B) after initiation of the inflammatory response. Frozen sections of lung tissue were obtained at time 0 (C) and 4 h (D) after initiation of lung inflammatory reactions. Arrows indicate staining of what appears to be a type II cell and a macrophage. All specimens were counterstained with hematoxylin (A and B, ×150; C and D, ×100).

FIGURE 3. Effects of 400 μg preimmune IgG or anti-eotaxin IgG (administered i.v. or intratracheally) on the permeability index (A), BAL content of neutrophils (B), MIP-2 (C), CINC (D), and TNF-α (E), and IL-10 content in lung homogenates (F). All measurements were made 4 h after intratracheal administration of PBS or 2.5 mg anti-BSA (IgG-IC) followed by i.v. infusion of 10 mg BSA. Preimmune IgG or anti-eotaxin IgG (400 μg) was administered intratracheally with the anti-BSA or i.v. with BSA. In this and subsequent figures, as indicated, the dashed lines represent negative control values that were subtracted from the positive control groups to calculate percent change. Values are mean ± SEM for each vertical bar; A (n = 8); B–F (n = 5). Results are representative of three separate experiments.
treated with preimmune IgG, the lung permeability index increased nearly 3-fold compared with negative controls \( (p < 0.001; \text{Fig. 3A}) \). Intravenous infusion of anti-eotaxin IgG caused a significant additional increase in the mean permeability index, increasing by 96% when compared with values obtained in rats treated with preimmune IgG \( (p < 0.001) \). When 400 \( \mu \)g anti-eotaxin IgG was instilled intratracheally, the mean permeability index in the positive control groups increased by 95% compared with the group receiving preimmune IgG \( (p = 0.002; \text{Fig. 3A}) \).

Parallel changes were found in the BAL content of neutrophils. Four hours after IgG-IC deposition, rats treated either i.v. or intratracheally with 400 \( \mu \)g preimmune IgG showed a >30-fold increase in neutrophil content in BAL fluids compared with negative controls \( (p < 0.001; \text{Fig. 3B}) \). In rats treated with anti-eotaxin IgG, i.v. or intratracheal administration caused substantial increases \( (45\% \, p = 0.045; \text{and} \, 49\% \, p = 0.028, \text{respectively}) \) in the numbers of neutrophils compared with positive control groups treated with preimmune IgG. In a separate experiment, normal rats treated i.v. with 400 \( \mu \)g anti-eotaxin IgG displayed no difference in numbers of circulating blood neutrophils (data not shown). Thus, treatment of rats with anti-eotaxin, whether by the i.v. or intratracheal route, resulted in significantly increased numbers of neutrophils recruited into lung following intrapulmonary deposition of IgG-IC as well as the related pulmonary vascular leak of albumin.

To determine whether the increases in BAL neutrophils and intensified lung injury in rats treated with 400 \( \mu \)g anti-eotaxin IgG were associated with altered levels of inflammatory mediators in lung, BAL fluids were examined for content of TNF-\( \alpha \), MIP-2, and CINC, whereas lung homogenates served as the source for IL-10 because its levels in BAL fluids were quite low (data not shown). In this model of lung injury, the first three factors are known to be an important proinflammatory mediator, whereas IL-10 is known to have powerful anti-inflammatory functions in this model \( (17, 25, 32, 34, 35) \). Rats undergoing IgG-IC deposition and treated intratracheally with preimmune IgG showed the expected increased levels of MIP-2, CINC, and TNF-\( \alpha \) in BAL fluids \( (\text{Fig. 3, C, D, and E}) \) and increased IL-10 content in lung homogenates \( (\text{Fig. 3F}) \) compared with negative controls \( (p < 0.010) \). In rats treated with 400 \( \mu \)g anti-eotaxin IgG, BAL levels of MIP-2 increased by 41% \( (p = 0.047; \text{Fig. 3C}) \), and CINC levels increased by 84% \( (p = 0.029; \text{Fig. 3D}) \), but BAL levels of TNF-\( \alpha \) were statistically indistinguishable \( (p > 0.05) \) from rats treated with preimmune IgG \( (\text{Fig. 3E}) \). In a companion group of animals, treatment with anti-eotaxin caused a significant reduction in IL-10 content in lung homogenates by 73% \( (p = 0.013) \) when compared with rats treated with preimmune IgG \( (\text{Fig. 3F}) \). As will be discussed below, reduced levels of lung IL-10 would be expected to cause enhanced NF-\( \kappa \)B activation.

**Effects of anti-eotaxin on NF-\( \kappa \)B activation in lung**

In this model of lung injury, activation of NF-\( \kappa \)B is known to be required for production of relevant inflammatory mediators \( (24, 32, 34, 36) \). Because the data in Fig. 3 indicated that intratracheal administration of 400 \( \mu \)g anti-eotaxin IgG \( (\text{with anti-BSA}) \) would lead to intensified inflammatory injury, suggesting enhanced activation of NF-\( \kappa \)B, rats undergoing intrapulmonary deposition of IgG-IC were treated intratracheally with either 400 \( \mu \)g anti-eotaxin or preimmune IgG, and whole lung nuclear extracts were obtained at 4 h for evaluation of NF-\( \kappa \)B. For these experiments a lower dose of anti-BSA \( (1.25 \text{mg}) \) was used to optimize the ability to detect changes in NF-\( \kappa \)B activation. Very little NF-\( \kappa \)B was found in lung nuclear extracts obtained from rats receiving PBS and 400 \( \mu \)g preimmune IgG intratracheally \( (\text{Fig. 4, left two lanes}) \). In rats undergoing IgG-IC deposition treated intratracheally with preimmune IgG, there was clear evidence of increased nuclear translocation of NF-\( \kappa \)B \( (\text{Fig. 4, middle two lanes}) \). In rats undergoing IgG-IC deposition and treated with 400 \( \mu \)g anti-eotaxin, there was intensified activation of NF-\( \kappa \)B \( (\text{Fig. 4, right two lanes}) \). By densitometry analysis of EMSAs obtained from nuclear extracts from lungs of 12 rats \( (n = 4 \text{ for each of the groups displayed in the upper frame}) \) the amount of NF-\( \kappa \)B activation increased by 92% in lungs of animals treated with anti-eotaxin. Quantitation of autoradiographs by image analysis indicated that the increase in NF-\( \kappa \)B induced by anti-eotaxin was statistically significant \( (p = 0.048) \) compared with rats treated with preimmune IgG. These data suggest that in vivo blockade of eotaxin leads to augmented activation of NF-\( \kappa \)B, which are consistent with increases in BAL levels of MIP-2 and CINC \( (\text{Fig. 3}) \).

**Effects of exogenously administered eotaxin**

To investigate the effects of exogenously administered eotaxin in the IgG-IC model of lung injury, recombinant murine eotaxin \( (1–10 \mu \text{g}) \) was infused i.v. \( (\text{at time 0}) \), and the pulmonary vascular leak of \( ^{125}\text{I}-\text{albumin} \) (expressed as permeability index) was assessed 4 h after initiation of lung injury. Although i.v. infusion of 1 or 5 \( \mu \)g eotaxin did not statistically affect the permeability index in rat lungs 4 h after deposition of IgG-IC, i.v. infusion of 10 \( \mu \)g eotaxin caused a 34% decrease \( (p = 0.043) \) in the permeability index \( (\text{Fig. 5A}) \). In the IgG-IC model, the effects of i.v.-infused eotaxin \( (10 \mu \text{g}) \) on BAL neutrophils and on cytokine/chemokine
content of BAL fluids were also assessed. Intravenous treatment with eotaxin (10 μg) resulted in a 36% decrease (p < 0.001) in the numbers of BAL neutrophils induced by IgG-IC deposition (Fig. 5B). Under the same experimental conditions, eotaxin treatment resulted in a 24% decrease in BAL MIP-2 levels (p < 0.044; Fig. 5C), but no effect on the levels of CINC (Fig. 5D) and TNF-α (Fig. 5E).

**Effect of eotaxin on mRNA expression of CINC, MIP-2, IL-1β, TNF-α, MIP-1α, and MIP-1β in alveolar macrophages stimulated by IgG-IC**

CINC, MIP-2, IL-1β, TNF-α, MIP-1α, and MIP-1β mRNA levels in alveolar macrophages were analyzed by Northern blots at 0, 1, 2, and 4 h following IgG-IC addition. Alveolar macrophages were incubated with a fixed amount of IgG-IC (100 μg/ml) in the presence or absence of eotaxin (100 ng/ml). In nontreated cells at 1 h, mRNA expression for CINC, MIP-2, TNF-α, MIP-1α, and MIP-1β was barely, if at all, detectable (Fig. 6). The presence of eotaxin did not change mRNA expression of these chemokines and cytokines in unstimulated cells (Fig. 6, second lanes). CINC and MIP-2 mRNA expression showed a similar pattern over the 2-h incubation time. One hour after stimulation, a new splicing form of CINC mRNA (more quickly migrating in the gel; ~1.3 kb) and a new splicing form of MIP-2 mRNA (~1.4 kb) were identified (Fig. 6, lane 3 of MIP-2 frame), and the induction was markedly suppressed in the presence of eotaxin (Fig. 6, lane 4 of MIP-2 frame). At 2 h, the suppressive effects of eotaxin on mRNA expression of CINC and MIP-2 were not observed (Fig. 6, lanes 5 and 6 of CINC and MIP-2 frames). At 1 h, IL-1β, TNF-α, MIP-1α, and MIP-1β mRNA expression was barely perceptible, if at all, when compared with mRNA expression in untreated cells. However, all mRNAs were greatly increased at 2 h, and at this time point eotaxin did not show suppressive effects. At 4 h, all genes remained up-regulated and showed exactly the same expression pattern as those at 2 h, suggesting that these genes were fully expressed 2 h after IC stimulation. The presence of eotaxin showed no impact on the gene expression at 4 h (data not shown). Equal loading of RNA for all measurements was confirmed by probing the same blot to determine GAPDH levels. Thus, the in vitro presence of eotaxin may transiently suppress mRNA expression of MIP-2 and CINC stimulated by IgG-IC in alveolar macrophages.

**Effect of eotaxin on cytokine and chemokine protein production in alveolar macrophages stimulated by IgG-IC**

To evaluate the effects of eotaxin on the production of cytokine and chemokine protein in alveolar macrophages stimulated by IgG-IC, alveolar macrophages were incubated with a fixed amount of IgG-IC (100 μg/ml) in the presence or absence of different concentrations of eotaxin (0, 25, 50, 100, and 250 ng/ml). After an incubation period of 4 h, the chemokine and cytokine content in supernatant fluids was determined by ELISA. Very low levels of
CINC, MIP-2, IL-1β, and TNF-α were found in supernatant fluids from alveolar macrophages treated only with anti-BSA (negative control, dose 0 of eotaxin) (Fig. 7, A–D), whereas MIP-1α and MIP-1β were below a detectable level in supernatant fluids from the same cells (Fig. 7, E and F). In the presence of eotaxin alone (25, 50, 100, and 250 ng/ml), there were no significant changes in CINC, MIP-2, IL-1β, and TNF-α levels. When the cells were stimulated with IgG-IC, in all cases cytokine production was robust (Fig. 7). Eotaxin at concentrations of 25–200 ng/ml did not cause statistically significant changes in IL-1β, TNF-α, MIP-1α, and MIP-1β production induced by IgG-IC. However, at a concentration of 100 ng/ml, there was a modest decrease of CINC production from 1705 ± 136 to 1343 ± 37 ng/ml, but this did not reach statistical significance (Fig. 7A). However, in the case of MIP-2, the presence of 100 or 200 ng/ml eotaxin resulted in significant decreases of IgG-IC-induced MIP-2 production from 17,225 ± 727 to 12,818 ± 197 and 14,315 ± 701 pg/ml, respectively (p < 0.05; Fig. 7B). Anti-eotaxin coculture completely abolished the ability of eotaxin to inhibit IC-induced MIP-2 release from macrophages (data not shown). MIP-2 production in macrophages in the presence or absence of eotaxin (100 ng/ml) was measured 0, 2, 4, and 8 h after IC stimulation (Table I). Significant suppressive effects of eotaxin on MIP-2 production were found at 2 and 4 h (p < 0.05, n = 8), but not at 8 h.

Effects of eotaxin on NF-κB activation in alveolar macrophages

Because the NF-κB activation in alveolar macrophages is an early event (as soon as 0.5 h after initiation of IgG-IC deposition) during lung inflammation (32), macrophages were stimulated in vitro with IgG-IC in the presence or absence of eotaxin, and nuclear extracts were obtained at 0.5 and 1 h for NF-κB analysis. NF-κB activation was quantitated by densitometry analysis of EMSA blots (Fig. 8A, lower frame). In the presence or absence of eotaxin, there was little NF-κB presence in nuclei of alveolar macrophages (Fig. 8A, lanes 1 and 2). However, 30 min after IgG-IC deposition NF-κB activation significantly increased, but in the presence of eotaxin nuclear translocation of NF-κB was reduced by 46% (n = 3, p < 0.01) (Fig. 8A, lanes 3 and 4). Sustained NF-κB activation was observed at 1 h, and this was unchanged in the presence of eotaxin. These data imply that eotaxin may temporarily attenuate NF-κB activation in alveolar macrophages induced by IgG-IC.

In vivo effect of anti-eotaxin on induction of CINC and MIP-2 mRNA in alveolar macrophages

For in vivo evaluation of CINC and MIP-2 mRNA expression in alveolar macrophages during IgG-IC deposition, the dose of anti-BSA was lowered to 1.25 mg to assess more effectively changes in mRNA levels. CINC and MIP-2 mRNA expression was assessed in BAL alveolar macrophages, which were obtained 2 h after initiation of IgG-IC deposition. The animals were treated intratracheally with 400 μg anti-eotaxin or preimmune IgG. Very little, if any, CINC mRNA (~1.3 kb) and MIP-2 mRNA (~1.4 kb) could be detected in alveolar macrophages from normal rats (Fig. 8B, lane 1). Administration of 400 μg anti-eotaxin IgG did not cause any increases in CINC mRNA and MIP-2 mRNA expression in alveolar macrophages obtained from normal rats (Fig. 8B, lane 2). However, faint up-regulation of CINC and MIP-2 mRNA was observed in macrophages from rats undergoing IgG-IC deposition (Fig. 8B, lane 3). In contrast, intratracheal treatment of 400 μg anti-eotaxin significantly increased in vivo expression of IgG-IC-induced CINC and MIP-2 mRNA (Fig. 8B, lane 4). Equal loading of RNA was confirmed by probing the same blot to determine GAPDH levels. These data suggest that endogenous eotaxin negatively regulates CINC and MIP-2 gene expression in alveolar macrophages during IgG-IC-induced lung injury.
Discussion

The IgG-IC model of lung injury in rats serves as a useful model for analysis of the acute inflammatory response. In this model, IC deposition triggers complement activation and stimulation of resident lung macrophages via engagement of FcR. Lung macrophages generate and secrete a number of mediators that initiate a proinflammatory cascade that leads to up-regulation of vascular adhesion molecules followed by an intense recruitment of neutrophils into the intra-alveolar and interstitial compartments. Few, if any, eosinophils have been found in the inflammatory cell infiltrates in this model at the 4-h interval (data not shown). Lung neutrophil recruitment and the subsequent lung injury is dependent upon the expression of the cytokines TNF-α and IL-1 (17, 18) as well as the CXC chemokines MIP-2 and CINC (19) and the CC chemokines MIP-1α and MIP-1β (22, 23). Pulmonary expression of these mediators, especially TNF-α and IL-1, has been linked to activation (nuclear translocation) of the transcription factor NF-κB (37). This inflammatory response is endogenously regulated by IL-10 and IL-13 (25, 26) as well as by secretory leukocyte protease inhibitor (SLPI) (38), each of which interferes with lung NF-κB activation by preventing breakdown of IκB proteins (24, 36).

Eotaxin is a primary mediator of IgE-mediated allergic reactions in lung. IgE-mediated allergic inflammatory reactions in lung are characteristically associated with an early, transient accumulation of neutrophils, which gives way to their replacement by and sustained presence of eosinophils (39). Whether this biphasic response is controlled by two different mediator cascades or whether there is some type of regulation that eventually causes eosinophil accumulation to predominate is unclear. The data in the current report suggest that expression of eotaxin negatively regulates neutrophil accumulation in the lung inflammatory response occurring after intrapulmonary IgG-IC deposition. Constitutive eotaxin is known to exist on or in lung macrophages and in bronchial epithelial cells (6, 40). In this report we found up-regulation of eotaxin mRNA and protein (Figs. 1 and 2) in lung during IgG-IC deposition, which represents a model of neutrophil-dependent acute lung inflammatory injury. Although there seems to be some discrepancy in the timing of induction of eotaxin mRNA vs

Table 1. Time course of MIP-2 protein production in vitro in macrophages induced with IC in the presence or absence of eotaxin (100 ng/ml)

<table>
<thead>
<tr>
<th>MIP-2 Production (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>0 h         2 h            4 h            8 h</td>
</tr>
<tr>
<td>Control  &lt;10             147 ± 82       3,118 ± 655   5,607 ± 490</td>
</tr>
<tr>
<td>IC         &lt;10             1,688 ± 397   20,492 ± 1,985 31,821 ± 1,946</td>
</tr>
<tr>
<td>IC + eotaxin&lt;10            993 ± 269*   15,105 ± 627* 33,856 ± 2,623</td>
</tr>
</tbody>
</table>

* p < 0.05 when compared to IC control (n = 8).
Anti-eotaxin IgG or preimmune IgG and the i.v. infusion of 10 mg BSA. Administration of PBS or 1.25 mg anti-BSA serum induced in vitro with IgG-IC in the presence or absence of 100 ng/ml eotaxin 0.5 or 1 h after incubation, NF-κB activation was evaluated by EMSA. NF-κB activation was quantitated from digitzed autoradiography films using image analysis software. Lower frame, Values represent mean ± SEM, with n = 3 for each group. B, In vivo induction of CINC and MIP-2 mRNA in alveolar macrophages by anti-eotaxin instillation. Alveolar macrophages were harvested 2 h after intratracheal administration of PBS or 1.25 mg anti-BSA (400 μg) was instilled intratracheally at time 0. CINC and MIP-2 mRNA levels were evaluated by Northern blots. Equal loading of RNA was confirmed by probing the same blot with GAPDH. Results are representative of three separate experiments.

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>0.5</th>
<th>0.5</th>
<th>0.5</th>
<th>1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-IC</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**FIGURE 8.** A, Effects of eotaxin on NF-κB activation induced by IgG-IC. Rat alveolar macrophages were stimulated in vitro with IgG-IC in the presence or absence of 100 ng/ml eotaxin 0.5 or 1 h after incubation, NF-κB activation was evaluated by EMSA. NF-κB activation was quantitated from digitzed autoradiography films using image analysis software. Lower frame, Values represent mean ± SEM, with n = 3 for each group. B, In vivo induction of CINC and MIP-2 mRNA in alveolar macrophages by anti-eotaxin instillation. Alveolar macrophages were harvested 2 h after intratracheal administration of PBS or 1.25 mg anti-BSA (400 μg) was instilled intratracheally at time 0. CINC and MIP-2 mRNA levels were evaluated by Northern blots. Equal loading of RNA was confirmed by probing the same blot with GAPDH. Results are representative of three separate experiments.

It is curious as to why TNF-α was not similarly affected. In the case of exogenously administered eotaxin, the outcome was the converse of that described above, namely, diminished accumulation of neutrophils in lung and reduced lung vascular permeability.

It is known that certain chemokines can regulate chemokine and cytokine expression. Monocyte chemotactic protein (MCP)-1 has been found to have marked anti-inflammatory properties in a model of endotoxemia (41). The administration of anti-MCP-1 Abs to endotoxin-challenged mice resulted in increased levels of TNF-α and IL-12 and decreased IL-10 levels. The converse outcome was observed when recombinant MCP-1 was administered exogenously. In vitro treatment of primary astrocyte cultures with MCP-2 or KC markedly up-regulated expression of MCP-1, inflammatory protein-10 (IP-10), and RANTES (42). MCP-1,-2,-3, and -4 inhibited IL-12 production by human monocytes in response to stimulation with Schistosoma mansoni egg Ag-sensitized mice, it was found that MCP-1 significantly increased IL-4 mRNA expression, and protein production MIP-1α treatment decreased IL-4 production (44). The expression of eotaxin by epithelial cells, endothelial cells, and fibroblasts was induced by proinflammatory cytokines, including IL-1, TNF-α, and IFN-γ (43). Using Th2-type lymphocyte populations from schistosomal egg Ag-sensitized mice, it was found that MCP-1 significantly increased IL-4 mRNA expression, and protein production MIP-1α treatment decreased IL-4 production (44). The expression of eotaxin by epithelial cells, endothelial cells, and fibroblasts was induced by proinflammatory cytokines, including IL-1, TNF-α, and IFN-γ (45). TNF-α also induced eotaxin expression in the human cell line, U-937 (46). Both IL-4 and IL-13 synergistically enhanced TNF-α-induced eotaxin production and down-regulated TNF-α-induced IL-8 production from bronchial epithelium (47). Additionally, eotaxin increased allergen-induced expression of IL-4 in basophils (48). These data suggest that eotaxin may play a role similar to that of IL-4 and IL-13 and
function in an opposing manner to IL-8. In IgG-IC-induced lung injury, exogenously administered IL-4 or IL-13 exhibited powerful anti-inflammatory properties (35), whereas Ab to IL-8 exerted an anti-inflammatory effect (49). IL-10 and IL-13 have been found to be up-regulated in the IgG-IC-injured lung, and in vivo blockade of IL-10 and IL-13 increased the lung injury caused by deposition of IgG-IC (25, 26). As described in this report, in vivo blockade of eotaxin resulted in decreased IL-10 content in the lung and increased lung injury. It seems possible that increased levels of IL-10 or IL-13 may contribute to the up-regulation of eotaxin production, synergistically leading to protective effects during IgG-IC-induced lung injury. In this study, intratracheal treatment with anti-eotaxin significantly increased IC-induced MIP-2 and CINC production in BAL fluids. Increased levels of MIP-2 and CINC correlated with increased numbers of BAL neutrophils. Conversely, exogenous administration of eotaxin resulted in a decrease in BAL MIP-2 levels, correlating with reduced neutrophil accumulation. Additionally, the studies have shown that eotaxin transiently suppresses MIP-2 mRNA and protein production induced by IgG-IC in alveolar macrophages (Figs. 6 and 7). However, there seem to be some discrepancies in CINC data. Anti-eotaxin treatment significantly increases CINC protein levels in BAL fluids and CINC mRNA in macrophages in vivo (Figs. 3D and 8B), and eotaxin temporarily suppresses CINC mRNA expression induced by IgG-IC in alveolar macrophages in vitro. In contrast, CINC protein production in BAL fluid was not changed by exogenously administered eotaxin (Fig. 5D), and eotaxin did not show significant suppressive effects of CINC protein production in alveolar macrophages in vitro (Fig. 7A). It should be pointed out that CINC protein is generated in vivo in much higher amounts when compared with other CXC chemokines, such as MIP-2 (Fig. 3). Similar differences were noted with in vitro IC challenge (Fig. 7). Thus, it is possible that the CINC expression system is more resistant to regulation (suppression). It has been reported that eotaxin chemokatracts neutrophils stimulated with IFN-γ; however, in this study recombinant murine eotaxin did not contain chemotactic activity for BAL neutrophils obtained from IC-injured rats (data not shown). These data suggest that, in IgG-IC-injured lung, eotaxin may not exhibit its chemotactic ability for neutrophils but instead alters the cytokine and chemokine balance to hinder the neutrophil infiltration and decrease lung injury. Pretreatment of blood neutrophils with eotaxin failed to affect rat blood neutrophil chemotactic responses to MIP-2 (data not shown), suggesting that eotaxin may not directly affect neutrophil response to chemotactic chemokines.

CCR3 is considered to be the principal receptor of eotaxin. Besides eotaxin, MCP-2, -3, -4, and RANTES are functional ligands for CCR3 (45). CCR3 is expressed on eosinophils, basophils, Th2-type lymphocytes, and human neutrophils stimulated with IFN-γ (8–12). CCR3 is found on alveolar macrophages isolated from healthy individuals (13). It is known that eotaxin is not able to chemotactically attract macrophages, implying that eotaxin may exert a nonchemotactic effect via CCR3 on macrophages. CCR3 may not only mediate chemotaxis of immune cells to the site of inflammation but also affect cell signal transduction. Binding of eotaxin to CCR3 induced an increase of the intracellular Ca²⁺ concentration and activated the mitogen-activated protein kinases in eosinophils (50). In this study, eotaxin had the ability to suppress the NF-κB translocation in alveolar macrophages stimulated by IC (Fig. 8A). The lack of a reliable blocking Ab to rat CCR3 has not allowed a definitive assessment of the role of CCR3 in NF-κB translocation. The linkage between CCR3 and NF-κB activation should be explored. NF-κB transcriptionally regulates MIP-2, CINC, MIP-1α, MIP-1β, TNF-α, and IL-1β gene expression (51, 52). It seems likely that NF-κB suppression by eotaxin was linked to down-regulation of MIP-2 and CINC mRNA in alveolar macrophages, but the mechanism by which eotaxin selectively suppresses MIP-2 and CINC gene expression remains to be determined.

Our data suggest that eotaxin may affect several cell systems in the lung in a manner that leads to suppression of neutrophil recruitment and resulting in attenuated lung injury. Accordingly, it may be that eotaxin, while being an important mediator in eosinophil-mediated inflammatory reactions, plays additional and different roles in other types of inflammatory responses in the lung.

Acknowledgments

We greatly appreciate the gift of preimmune IgG and anti-mouse eotaxin IgG from Dr. S. L. Kunkel (Department of Pathology, University of Michigan Medical School).

References


