**RAPID PUBLICATION**

**Eotaxin protein and gene expression in guinea-pig lungs: constitutive expression and upregulation after allergen challenge**

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**ABSTRACT:** Eotaxin is an eosinophil-specific chemoattractant originally identified in bronchoalveolar lavage fluid after allergen challenge of sensitized guinea-pigs. We have determined and quantified for the first time the cellular sources of guinea-pig lung eotaxin and localized gene expression in structural cells of large and small airways and in alveolar macrophages. We used anti-guinea-pig eotaxin monoclonal and polyclonal antibodies and a complementary ribonucleic acid (cRNA) probe to detect eotaxin protein and cytoplasmic messenger ribonucleic acid (mRNA) transcripts by the techniques of immunohistochemistry and in situ hybridization in: 1) naive; 2) ovalbumin-sensitized/saline-exposed; and 3) ovalbumin-sensitized/ovalbumin-challenged animals (n=5 for each group).

Compared with the naive animals, there was a fivefold increase of eotaxin protein and a 25 fold upregulation of eotaxin gene expression in the airway epithelium 3 h after ovalbumin challenge of sensitized animals (p<0.001). The average percentages of alveolar macrophages staining for eotaxin protein and mRNA in the naive animals were approximately 30 and 10% respectively: both increased significantly in the sensitized/ovalbumin-challenged animals to 78 and 57%, respectively (p<0.0001). Compared with the naive animals, the procedure of sensitization significantly increased eotaxin gene expression in both bronchial epithelium and alveolar macrophages (p<0.0001). The results indicate that there are multiple cellular sources of guinea-pig lung-derived eotaxin, including bronchial and bronchiolar epithelial cells, airway smooth muscle, bronchial vascular endothelium, and chondrocytes and alveolar macrophages, and that there are relatively rapid and marked increases of eotaxin protein and gene expression in airway epithelium and alveolar macrophages following allergen challenge.


Airway wall eosinophilia is a key characteristic of asthma and of the allergic response in a variety of tissues [1–6]. Whilst no animal model demonstrates the associated thickening of the reticular basement membrane and remodelling process seen in mild and severe asthma respectively [7, 8], the guinea-pig model of acute and late phase reactions to allergen challenge provides a useful experimental means of investigating the inflammatory mediators of importance in allergic reactions [9]. Following their recruitment to tissues, activated eosinophils release preformed toxic cationic granule proteins, such as major basic protein, eosinophil cationic protein and eosinophil peroxidase, which are thought to damage the airway mucosa and lead to the airways hyperresponsiveness of asthma [10–12]. It is probable that understanding and targeting the process of eosinophil tissue recruitment and activation will assist the development of new and specific anti-inflammatory and anti-allergic drugs [6].

A guinea-pig CC chemokine named eotaxin has been demonstrated with the capacity to recruit eosinophils, but not neutrophils or mononuclear cells, when instilled into the airways or injected into the guinea-pig skin [13–15]. Guinea-pig eotaxin complexed deoxyribonucleic acid (cDNA) and its mouse and human cDNA homologues have been cloned [14, 16–20]. Eotaxin messenger ribonucleic acid (mRNA) increases in the lungs 3 h after allergen challenge of sensitized guinea-pigs [14, 20] and in skin following injection of interleukin (IL)-4-producing tumours in the mouse [16]. Human eotaxin also appears to be an early response gene in cytokine-stimulated epithelial [21] and endothelial cells, and can be induced in peripheral blood eosinophils by IL-3 [19]. In guinea-pig and mouse models of allergic inflammation in vivo, eotaxin mRNA and eotaxin protein levels parallel the kinetics of eosinophil accumulation in bronchoalveolar lavage fluid (BALF) and the lung [22, 23]. The effect of eotaxin on

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eosinophil recruitment appears to be a direct one since, in purified form, eotaxin attracts guinea-pig eosinophils both in vitro [13] and in guinea-pig skin in vivo where the effect is completely inhibited by an anti-eotaxin antibody [23].

Northern blot analyses have demonstrated that eotaxin mRNA is highly expressed in human small bowel and colon, at intermediate levels in heart, kidney and pancreas, and at low levels in lung, spleen, thymus, prostate, ovary, liver and skeletal muscle [17, 19]. For the purposes of eotaxin localization, it is most convenient to make use of human nasal mucosa and polyp tissue in which eotaxin immunostaining was localized to surface ciliated columnar epithelium and to a variety of subepithelial leukocytes including tissue eosinophils. In the present study, histological study of guinea-pig airways and lung, monoclonal and polyclonal anti-guinea-pig eotaxin antibodies have been used in order to localize the sources of eotaxin protein. In addition, guinea-pig eotaxin complementary ribonucleic acid (cRNA) probes and the technique of in situ hybridization have been applied to detect intracellular mRNA transcripts indicative of the sites of gene expression for guinea-pig eotaxin. We demonstrate for the first time that guinea-pig eotaxin and its gene expression are present in a variety of structural cells present in the airway mucosa and also in alveolar macrophages. Furthermore, eotaxin expression is markedly up-regulated following intratracheal challenge of previously sensitized animals.

Materials and methods

Animals

Fifteen male Dunkin Hartley guinea-pigs purchased from Charles River (Margate, Kent, UK) were used in this study. They were divided into: 1) naïve; 2) sensitized/saline-exposed; and 3) sensitized/ovalbumin-challenged groups (n=5 for each group). The procedures of sensitization of guinea-pigs with intraperitoneal ovalbumin and aerosol allergen challenge have been described previously [13–15]. All animals were killed with a barbiturate overdose 3 h after saline exposure or ovalbumin challenge.

Histology and immunohistochemistry

Immediately after killing, the lungs were expanded with 4% paraformaldehyde (Sigma, Dorset, UK) in phosphate-buffered saline (PBS) via the trachea until the pleural margins became sharp, and fixed by immersion for 4 h at 4°C, transferred into 15% sucrose in PBS and processed as paraffin blocks. Six micron thick sections were cut and mounted on poly-l-lysine (Sigma, Dorset, UK) coated slides. Haematoxylin and eosin-stained sections were used to assess the morphological changes associated with sensitization and allergen challenge. For the immunohistochemical localization of eotaxin both alkaline phosphatase anti-alkaline phosphatase (APAAP) [24] and peroxidase antiperoxidase (PAP) [24] methods were used. Briefly, in the APAAP method, the primary monoclonal anti-guinea-pig eotaxin antibody (mouse clone 72-B, immunoglobulin (Ig) G2a isotype screened against guinea-pig eotaxin and showing cross-reactivity by enzyme-linked immunosorbent assay (ELISA) with human eotaxin, (Geneva Biomedical Research Institute, Geneva, Switzerland)) was applied, at 1:25 dilution followed by the secondary rabbit antimosue (dilution 1:25, (Z0109) Dako, Bucks, UK) and tertiay APAAP complex (1:50, (D0651) Dako, UK). New fuchsin substrate (K596, Dako, UK) was added according to manufacturer’s recommendations and the sections were counterstained with haematoxylin. Negative control monoclonal antibody (mouse IgG2a antibody to Aspergillus niger glucose oxidase, dilution 1:25, Dako, UK) was used. For the PAP method, the slides were pretreated in 0.3% H2O2 in absolute ethanol to block endogenous staining and the primary polyclonal antibody (rabbit anti-guinea-pig eotaxin, dilution 1:100) was added and incubated for 1 h at room temperature. After washing in TRIS (hydroxymethyl) aminomethane-buffered saline (TBS), a second layer mouse anti-rabbit monoclonal antibody (IgG1, Dako, UK, dilution 1:50 (M0737)) was added, followed by a third layer rabbit anti-mouse antibody conjugated with horse radish peroxidase (dilution 1:50, (P0260) Dako, UK). Wherever the primary antibody detected eotaxin (AEC), 3-amino-9-ethylcarbazole substrate system ((K0697) Dako, UK) was used to produce a dark red precipitate. As negative control, normal rabbit IgG (reconstituted to give an IgG concentration equivalent to that of the primary antibody and also used at 1:100 dilution) was substituted for the primary antibody. The sections were counterstained with methyl green and mounted with glycergel ((C0563) Dako, UK).

In situ hybridization (ISH)

Plasmid pEo3122 in BK-CMV, containing a 777 base pair (bp) guinea-pig eotaxin cDNA [14], was linearized with EcoRI (Boehringer Mannheim, East Sussex, UK) and XhoI (Boehringer Mannheim, UK), and ribonucleic acid (RNA) polymerase T3 and T7 (Boehringer Mannheim, UK) were used respectively to produce digoxigenin-labelled sense and antisense RNA probes. ISH was carried out according to the method of Martínez-Montero et al. [25] with minor modifications. Briefly, after deparaffinization, tissue sections were permeabilized with 1 µg·mL−1 proteinase K (Boehringer Mannheim, UK) for 10 min and postfixed in 2% paraformaldehyde for 5 min. Either sense or antisense 50 ng RNA probes were applied to each section. Hybridization was performed at 42°C overnight. Stringent washing was carried out in 0.1×standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) at 37°C followed by 20 µg·mL−1 RNase A (Sigma, Dorset, UK) digestion to remove all unhybridized RNA. Chromogen development was carried out after incubation with sheep antidigoxigenin Fab fragment conjugated with alkaline phosphatase (Boehringer Mannheim, UK, dilution 1:200), washing in TBS and addition of buffer solution containing 0.33 mg·mL−1 nitroblue tetrazolium and 0.16 mg·mL−1 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim, UK) substrate with 1 mM levamisole and kept in the dark overnight. The slides were counterstained with methyl green and mounted with aqueous mountant (BDH, Poole, UK).
Quantification and statistical analyses

Quantification of the immuno- and ISH-positivity of the epithelium and alveolar macrophages was carried out for the: 1) naive; 2) sensitized/saline-exposed; and 3) sensitized/ovalbumin-challenged animals. Assisted image analysis of microscopic slides was performed by application of Apple Macintosh computer with National Institutes of Health Image 1.5 software (National Institute of Health, Bethesda, MD, USA). The percentage area of epithelium immunopositive for eotaxin protein or that positive by ISH was calculated after measurement of the total area of surface epithelium in all available intrapulmonary cartilaginous airways in one tissue section and that which was immunopositive expressed as percentage of the total. Total macrophage numbers and those positive for eotaxin protein or those which were mRNA positive were counted in the alveolar spaces of 10 randomly selected consecutive 260×260 µm fields of lung tissue and expressed as mean cell number per microscopic field. The percentage of the total number of macrophages which were positive was also calculated. As the data were normally distributed, all values were expressed as the mean±SEM. The StatView (Abacus Concepts Inc., Berkeley, CA, USA) software programme was used to assess the statistical significance of group differences by unpaired t-test. Fisher's correlation (r<corr> was applied to test the concordance of upregulation for eotaxin protein in large airway epithelium and the number of immunopositive alveolar macrophages: the test incorporates an r to z transformation to ensure normalization of the data. A p-value of less than 0.05 was taken as statistically significant and for simplicity four levels of significance are reported: p<0.05; p<0.01; p<0.001; and p<0.0001. The counts of errors of repeat measurement by a single observer (DL) expressed as the percentage coefficient of variation were 4.2 and 4.6% for counts of macrophages and measurements of epithelial areas, respectively.

Results

Morphological changes

In comparison with the naive animals, both the ovalbumin-sensitized/saline-exposed and sensitized/ovalbumin-challenged guinea-pig lungs showed eosinophil infiltration of airway epithelium and surrounding interstitial tissues by 3 h postchallenge. In comparison with the sensitized group, there was a more florid response in the allergen-challenged group. The eosinophilic infiltrate was associated with a lymphoedema localized to peribronchial and periarterial tissues in which neutrophils and mononuclear cells were also present. The airway mucosa was also infiltrated by inflammatory cells, most of which were eosinophils. There was no significant shedding of airway surface epithelium in any animal. Goblet cell hyperplasia was evident in large intrapulmonary airways. In small airways (i.e. bronchioli), there was evidence of epithelial hyperplasia and apparent contraction of the airways associated with obstruction of the airway lumina. The parenchyma of the lung was congested. Alveolar macrophages were scarce in the naive animals, the mean number (±SEM) being 1.8±0.3 per microscopic field. There was a significant, but small, increase in their number in sensitized/saline-exposed animals to 4.1±0.5·field<sup>-1</sup> (p<0.05). However, in comparison with the sensitized/saline-exposed group, there was an approximately fivefold increase in their number to 19.0±1.6·field<sup>-1</sup> in the sensitized/ovalbumin-challenged animals (p<0.0001).

Fig. 1. – Large (cartilaginous) intrapulmonary airways immunostained for guinea-pig eotaxin using a polyclonal antibody and the peroxidase antiperoxidase technique. There is eotaxin protein localized to epithelial cells (arrows), bronchial smooth muscle (M) and the endothelium lining bronchial vessels (V). (Internal scale bar = 100 µM).
Eotaxin localization

Immunohistochemical staining with both the mouse monoclonal and rabbit polyclonal anti-guinea-pig eotaxin antibodies showed positivity with identical localization, but the polyclonal antibody provided much stronger signals. The results for the polyclonal antibody were quantified and are presented herein.

In the large airways of the ovalbumin-challenged group, eotaxin positivity was present regularly in airway epithelium and appeared to be confined to ciliated cells and the basal aspects of the remaining epithelial cells (fig. 1). There was constitutive staining of the airways of the naive as well as the sensitized/saline-exposed animals: this included staining of airway epithelium, bronchial smooth muscle and chondrocytes. Approximately 5% of the epithelial area stained for eotaxin: this was similar in both the naive and sensitized/saline-exposed groups. In contrast, there was an approximately fivefold increase of eotaxin protein to 23.9±2.0% in the sensitized/ovalbumin-challenged group (p<0.0001 between sensitized/ovalbumin-challenged and sensitized/saline-exposed groups) (fig. 2). In contrast to the naive group, bronchial smooth muscle of both saline-exposed and allergen-challenged groups immunostained strongly whilst pulmonary vascular smooth muscle did not stain in any group. Bronchial vessels located within the airway wall were, however, positive. In bronchioli, positive staining was localized to nonciliated epithelial (Clara) cells but, in contrast to the large airway, there was a patchy distribution which made its quantification difficult (fig. 3). Figure 4 demonstrates the numbers of eotaxin-positive alveolar macrophages (expressed per microscopic field) in the three experimental groups. Compared with the naive group, there was a small but significant increase
in the mean number of eotaxin positive macrophages in the sensitized/saline-exposed group (p<0.05). In contrast, there was an approximately 30 fold increase in the ovalbumin-challenged group, which was significant when compared to either the sensitized/saline or naive groups (p<0.0001). As a percentage, 30–40% of the relatively few alveolar macrophages present in the naive and sensitized/saline-exposed animals stained for eotaxin. However, in the ovalbumin-challenged animals in which the alveolar macrophage number had increased, about 78% of the macrophages were immunostained for eotaxin (p<0.0001 compared with the sensitized/saline-exposed group) (fig. 3).

Comparison by correlation, of the up-regulation of eotaxin in large airway epithelium (determined by point counting) with that of the total numbers of alveolar macrophages staining for eotaxin resulted in the highly significant rcorr value of 0.95 (p<0.0001) (fig. 5). Occasionally, mononuclear cells present among the interstitial infiltrate stained for eotaxin. The negative control sections showed very weak diffuse staining not localized to any tissue structure.

In situ hybridization

ISH demonstrated constitutive eotaxin gene expression in both the naive and sensitized/saline-exposed animals, present in airway epithelial cells. Compared with the intensity of immunohistochemical staining, gene expression was relatively weak in airway smooth muscle (fig. 6). Approximately 1% (i.e. 1.4±0.5%) of the epithelial area contained mRNA transcripts in the naive group. The sensitization/saline-exposure procedure significantly increased the epithelial area containing eotaxin mRNA transcripts about sixfold to 8.9±1.9% (p<0.01 compared with the naive group). Ovalbumin challenge of sensitized animals further significantly increased eotaxin gene expression to approximately four times that of the sensitized saline-exposed animals to 34.7±5.2% (p<0.01 and p<0.001 compared with the sensitized/saline-exposed and naive animals, respectively) (fig. 2). The mRNA was localized particularly to cells present at the base of epithelium (fig. 6). In bronchioli, Clara cells also showed strongly positive signals for eotaxin gene expression in the ovalbumin-challenged animals but, as with the immunoreactivity, its distribution was patchy (fig. 7).

Figure 4 demonstrates the numbers of alveolar macrophages positive for eotaxin mRNA. The ISH procedure showed a sixfold upregulation of eotaxin mRNA in response to sensitization (p<0.0001): ovalbumin challenge was associated with a further fourfold increase
As a percentage of the total, about 10% (9.7±1.0%) of the relatively few alveolar macrophages present in the lungs of naive animals showed gene expression for eotaxin: this increased significantly to 36.6±1.0% (p<0.0001) in the sensitized/saline-exposed animals. Ovalbumin challenge was associated with a further increase to 57.2±2.7%, representing more than a fivefold increase over the naive animals (p<0.0001 for both saline-exposed and allergen-challenged groups compared with the naive animals and compared with each other).

In the oedematous interstitium, lymphocytes and mononuclear cells occasionally showed eotaxin gene expression. However, we did not observe ISH positivity in the eosinophils of either the ovalbumin-challenged or the saline-exposed animals. The sense ISH probe in both saline-exposed and allergen-challenged, and naive animals provided a "clean" negative control (figs. 8 and 9).
Discussion

Eotaxin is a CC chemokine acting selectively on eosinophils [14, 15, 17, 19, 20, 22]. In contrast, C5a, platelet-activating factor (PAF), monocyte chemotactic peptide-3 (MCP-3) and regulated on activation, normal T-cell expressed and secreted (RANTES) are also chemoattractants for eosinophils [26–30], but are less selective as they act additionally on other leucocytes. Eotaxin was originally purified from BALF of allergen-challenged guinea-pigs [13]. By Northern blot analysis, its gene expression has been shown to be up-regulated in ovalbumin-challenged guinea-pig lung [14, 20]. However the origins and the cellular localization of constitutive and allergen-induced upregulation of eotaxin in the guinea-pig model of allergic inflammation are not known. We found a multicellular source of constitutive eotaxin protein and mRNA, co-localized in large and small airway epithelial cells, airway smooth muscle, bronchial capillaries, chondrocytes and occasionally in alveolar macrophages. Its constitutive uniform expression in the guinea-pig large airways and patchy distribution in small airways and lung is perhaps not surprising as this species is unusual in its tendency to large airway tissue eosinophilia. In addition, constitutive mRNA [14, 20] and protein have been reported previously in guinea-pig airways and lung by Northern blot and radioimmunoassay, respectively.

Ponath et al. [17] have recently reported eotaxin immunolocalization to human ciliated columnar nasal and polyp epithelium and also to underlying eosinophils, lymphocytes and macrophages. Our demonstration of constitutive eotaxin protein in guinea-pig ciliated bronchial epithelium was therefore expected and is largely not surprising as this species is unusual in its tendency to large airway tissue eosinophilia. In addition, constitutive mRNA [14, 20] and protein have been reported previously in guinea-pig airways and lung by Northern blot and radioimmunoassay, respectively.

Fig. 9. – *In situ* hybridization sense (negative) control for the small airway and alveolus. (Internal scale bar = 200 µM).
is in keeping with reports of RANTES protein release [33] and our evidence for eotaxin expression demonstrates how bronchial smooth muscle may also play a proinflammatory effector role in the recruitment and activation of eosinophils. The number of activated eosinophils in hyperresponsive subjects is associated with the degree of airway responsiveness in asthma [2] and our findings demonstrate a way in which bronchial smooth muscle itself may influence airways responsiveness.

Our demonstration of the localization of eotaxin protein and mRNA to alveolar macrophages which not only increase in number in response to allergen but show a marked up-regulation of their gene expression and eotaxin protein is also novel. In respect of the eotaxin protein identified in the alveolar macrophages, the combination of the tenfold increase of macrophages after allergen challenge and the threefold increase in the percentage of eotaxin-positive cells results in a 30 fold increase of eotaxin-positive macrophages in the alveolar region compared with the naive animals. Macrophages as well as epithelial cells are ideally placed to sample allergen in inspired air and both are capable of expressing cell surface human leucocyte antigen-DR (HLA-DR) [34–36] and of responding in an antigen-specific fashion. Interestingly, the increase of eotaxin in macrophages showed a strong association with its up-regulation in large airway epithelium (r<corr>0.95; p<0.0001) indicating a co-ordinated allergic response throughout the respiratory tract. The relative expression and production of eotaxin by lumen-associated cells would determine the tissue gradient in airway or alveolus for the driving force for their migration into the airway lumen. The production of eotaxin by both bronchial and alveolar macrophages may be of relevance to the reported observations of an alveolar involvement in the inflammation of asthma [37, 38].

However, the tissue eosinophilia of the guinea-pig response does not depend upon eotaxin alone. HUMBLE S et al. [39] have recently shown in the guinea-pig that anti-IL-5 and dexamethasone can inhibit tissue eosinophilia, yet eosinophil production remains uninhibited by these molecules. Up-regulation of guinea-pig eotaxin has recently been shown to be associated with an early increase of lung tissue eosinophils, measured by concentration of eosinophil peroxidase [23], and also to recruit more eosinophils in vivo in IL-5 pretreated animals [40]. Recent experiments using targeted gene disruption to deplete mice of eotaxin have demonstrated that eotaxin enhances the magnitude of the early, but not the late, phase recruitment of eosinophils and that it may contribute both to the generation of peripheral blood eosinophilia and the recruitment of eosinophils to tissue [41]. In addition, TNF-α appears to be required to enhance vascular retention of the eotaxin-stimulated eosinophils via expression of their surface very late activation antigen-4 (VLA-4) ligand [42] and both IL-1-α and TNF-α appear to be important early response molecules which up-regulate expression of eotaxin itself [18, 19]. Mucosal mast cells may be the cell of origin for these proinflammatory molecules [19] and in this way may act as an important cell to trigger eotaxin expression and initiate eosinophil recruitment. Once produced, eotaxin may activate eosinophils to produce a variety of cytokines including IL-5 [43], inducing a proinflammatory positive cycle. The observation that human peripheral blood eosinophils may be induced by IL-3 to express significant amounts of eotaxin mRNA [19], and that tissue eosinophils may express the protein [17], is of interest. We did not, however, localize either eotaxin protein or mRNA to the allergen-induced recruitment of eosinophils in our guinea-pig model of acute allergic inflammation.

In conclusion, whilst the T helper-2 cell response involves interleukin-4 and interleukin-5, the production of tissue eosinophils from a variety of cell sources may be of fundamental importance to the early tissue recruitment and activation of eosinophils [40] which characterizes the allergic inflammation of asthma and other eosinophilic conditions. The localization of eotaxin production is important not only to our understanding of the basic mechanisms of tissue eosinophilia and tissue damage but also to the design of drug delivery which can now target the specific cells that generate the signal responsible for the selective recruitment of eosinophils. We have shown that the cellular sources are multiple and, following challenge, both airway epithelial cells and alveolar macrophages exhibit marked increases in their eotaxin gene expression and synthesis of this novel protein: these should now be included as candidate target cells for the prevention or treatment of airway and lung eosinophilia.

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References


