Brief Definitive Report

Constitutive and Allergen-induced Expression of Eotaxin mRNA in the Guinea Pig Lung

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Summary

Eotaxin is a member of the C-C family of chemokines and is released during antigen challenge in a guinea pig model of allergic airway inflammation (asthma). Consistent with its putative role in eosinophilic inflammation, eotaxin induces the selective infiltration of eosinophils when injected into the lung and skin. Using a guinea pig lung cDNA library, we have cloned full-length eotaxin cDNA. The cDNA encodes a protein of 96 amino acids, including a putative 23–amino acid hydrophobic leader sequence, followed by 73 amino acids composing the mature active eotaxin protein. The protein-coding region of this cDNA is 73, 71, 50, and 48% identical in nucleic acid sequence to those of human macrophage chemoattractant protein (MCP) 3, MCP-1, macrophage inflammatory protein (MIP) 1α, and RANTES, respectively. Analysis of genomic DNA suggested that there is a single eotaxin gene in guinea pig which is apparently conserved in mice. High constitutive levels of eotaxin mRNA expression were observed in the lung, while the intestines, stomach, spleen, liver, heart, thymus, testes, and kidney expressed lower levels. To determine if eotaxin mRNA levels are elevated during allergen-induced eosinophilic airway inflammation, ovalbumin (OVA)-sensitized guinea pigs were challenged with aerosolized antigen. Compared with the lungs from saline-challenged animals, eotaxin mRNA levels increased sixfold within 3 h and returned to baseline by 6 h. Thus, eotaxin mRNA levels are increased in response to allergen challenge during the late phase response. The identification of constitutive eotaxin mRNA expression in multiple tissues suggests that in addition to regulating airway eosinophilia, eotaxin is likely to be involved in eosinophil recruitment into other tissues as well as in baseline tissue homing.

E osinophils are circulating leukocytes that are thought to dwell predominantly in tissues where they are believed to survive for at least 2 wk (1, 2). Here they can mediate pro-inflammatory changes by the release of preformed toxic cationic proteins and by the de novo synthesis of oxygen radicals and lipid mediators (3, 4). The mechanisms that regulate baseline eosinophil tissue homing and tissue recruitment in selected diseases (e.g., asthma, parasitic infections, and malignancy) are areas of active investigation (5). Eosinophils express several adhesion molecules (e.g., β1 and β2 integrins) that participate in their interaction with the endothelium. Furthermore, various chemoattractants active on eosinophils, including leukotriene B4, platelet activating factor, and several chemokines (e.g., macrophage chemoattractant protein [MCP]-3, RANTES, macrophage inflammatory protein [MIP]-1α, and IL-8), have been identified as mediators of eosinophil trafficking (5–10). In addition, IL-5, a cytokine released by Th2 lymphocytes, specifically regulates eosinophil growth and differentiation (11). However, while IL-5 overproduction in transgenic mice causes a marked rise in blood eosinophilia, it does not result in eosinophil tissue accumu-

lation and organ damage (12). None of these molecules are eosinophil specific and their relative importance in selected diseases and in experimental animal models of allergy remains unclear.

Allergic airway inflammation in the guinea pig provides a model for examining eosinophil accumulation in tissues and thus provides a system by which to assess the effectors of eosinophilic inflammation (13–15). As in patients with allergic asthma, exposure of sensitized guinea pigs to aerosolized antigen results in an immediate airway hypersensitivity characterized by mast cell degranulation. This is followed by a late phase of hypersensitivity that is associated with the accumulation of large numbers of eosinophils in the airways. Using an in vivo bioassay to measure eosinophil infiltration into guinea pig skin, a novel C-C chemokine, eotaxin, has been recently purified (16, 17). Eotaxin appears to be unique among the chemokines since it causes the selective infiltration of eosinophils into the lungs and skin of animals. To further study the role of this molecule in inflammation and in experimental models of airway hypersensitivity, we have cloned the full-length guinea pig eotaxin cDNA and studied the expression

of its mRNA as a function of both the normal and allergen-challenged state.

Materials and Methods

Animals. Airway inflammation was induced in male Hartley guinea pigs (300–500 g body weight) by OVA sensitization as previously reported (18). Briefly, guinea pigs were pretreated with pyrilamine malate by i.p. injection before aerosolized OVA (1% wt/vol in 0.9% sterile sodium chloride) or saline alone. Animals were exposed in an aerosol chamber on three occasions at 7-d intervals and the lungs were harvested at various points after the final exposure. Organs were frozen in liquid nitrogen and used for subsequent RNA isolation. A guinea pig lung epithelial cell line (JH4 clone 1) and a guinea pig colon adenocarcinoma cell line (GPC-16) were purchased from American Type Tissue Culture Collection (Rockville, MD).

Eotaxin Cloning. Based on the published amino acid sequence of guinea pig eotaxin (17), the following degenerate oligonucleotide primers containing EcoR1 and BamH1 restriction sites, respectively, were synthesized: CCGGAATTCCA(CT)CC(AGCT)GG(AGCT)-AT(ACT) (128-fold degeneracy) and CGCGGATCCGC(AG)CA-(AGT)ATCAT(CT)TT(AG)TC (32-fold degeneracy). First strand cDNA was synthesized from guinea pig lung RNA and PCR was performed with an initial five cycles at 37°C for 60 s, followed by 25 cycles at 50°C for 60 s (denaturation at 95°C for 30 s and extension at 72°C for 90 s) to amplify a 130-bp eotaxin cDNA fragment that was subsequently subcloned into Bluescript II KS (Stratagene Inc., La Jolla, CA). Construction of a cDNA library using poly(A)$^+$ RNA isolated from the lung of an OVA-sensitized guinea pig was performed using Stratagene ZAP Express Vector according to the directions of the manufacturer. 500,000 independent clones were subsequently amplified and an aliquot of this cDNA library containing 10^6 phage was screened with the 130-bp eotaxin cDNA that had been ^{32}P-labeled with Klenow enzyme. Two phagemids were isolated and subsequently subjected to automated sequencing on both strands using instrumentation (model 373a; Applied Biosystems Inc., Foster City, CA) and the dye-terminator protocol. Sequence analysis was performed using software developed by the University of Wisconsin genetics computer group (19).

DNA and RNA Analysis. RNA was isolated by CsCl centrifugation in guanidine isothiocyanate (20). DNA was isolated from these gradients as well. In some cases, RNA was isolated using RNAzol (Biotecx Lab, Inc., Houston, TX) according to the directions of the manufacturer. Poly(A)$^+$ RNA was enriched by elution through an oligo dT column (Pharmacia LKB Nuclear, Gaithersburg, MD). 10 mcg of total RNA, 2 mcg of poly(A)$^+$ RNA, or 10 mcg of restriction endonuclease cut DNA was electrophoresed in agarose and transferred to Gene Screen (Dupont-New England Nuclear, Boston, MA) membranes. Membranes were hybridized with ^{32}P-labeled full-length cDNA or a fragment encoding the translated protein (bp 57–356). High-stringency hybridization was performed in 50% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt's solution (0.0002% [wt/vol] polyvinylpyrrolidone, 0.0002% [wt/vol] BSA, 0.0002% [wt/vol] Ficoll 400), 1% (wt/vol) SDS, 100 mcg/ml denatured herring sperm DNA, and 20 mM Tris at 42°C and blots were washed with 0.2× SSC, 0.5% SDS at 65°C. Low stringency hybridization was performed in 0.6 M NaCl, 80 mM Tris–Cl, 4 mM EDTA, 0.1% (wt/vol) sodium pyrophosphate, 0.1% (wt/vol) SDS, 10× Denhardt's, 100 mcg/ml denatured herring sperm DNA at 50°C, and washed with 1× SSC, 0.05% SDS at 50°C. Quantitation of the intensity of band hybridization was determined using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis. The statistical significance of differences between means was determined by analysis of variance (ANOVA). $P < 0.05$ was considered significant. When ANOVA indicated a significant difference, the Newman-Keuls test was used to determine which groups were significantly different from each other.

Results and Discussion

Analysis of Guinea Pig Eotaxin cDNA. Using degenerate oligonucleotide primers based upon the amino acid sequence of guinea pig eotaxin, a 130-bp cDNA was amplified by PCR from guinea pig lung single stranded cDNA. This PCR product encoded a peptide identical to eotaxin and was used to screen an amplified cDNA library made from the inflamed lung of an OVA-sensitized guinea pig. From 60 positive plaques, six plaques were subsequently purified and their excised phagemids had an insert size of ~700–800 bp. Sequence analysis of the longest two inserts revealed that the regions of overlap were identical. The cDNA was 818 bp long with an open reading frame that encoded 96 amino acids. The predicted protein sequence of the COOH-terminal 73 amino acids agreed exactly with the protein sequence of eotaxin isolated from guinea pig bronchoalveolar fluid (shown underlined in Fig. 1) except for three amino acids that were previously ambiguous (shown surrounded by a box in Fig. 1) (17).

The 5' region of the cDNA encoded a putative hydrophobic leader sequence whose cleavage site was predicted to occur at the NH$_2$-terminal site at which the active eotaxin protein sequence starts (shown with the arrow in Fig. 1) (21). This structure strongly suggested that eotaxin was unlikely to exist as a precursor protein requiring additional proteolytic cleavage for activation. This type of biochemical processing has been seen with the platelet basic proteins, members of the C-X-C chemokine family (22). A Kozak consensus sequence for translation initiation was identified 5' of the AUG (23). The 3' untranslated region encoded a mRNA with 59% AU nucleotides including two "AUUUA" domains (shown with the hatched lines) that have been reported to decrease the mRNA stability of other cytokine mRNAs (24).

The nucleotide sequence showed significant homology to other members of the C-C chemokine family, in particular to members of the MCP family (25). The full-length cDNA was 61, 58, 42, 38, and 52% identical in nucleic acid to human MCP-3, MCP-1, MIP-1a, RANTES, and guinea pig MCP-1, respectively. Even greater homology was seen when only the region of the cDNA that encoded protein was compared (Table 1). Comparison of the leader sequence with other sequences in the gene data bank revealed that the eotaxin leader sequence was homologous only to other leader sequences of the MCP family (81 and 74% nucleotide identity and 78 and 70% amino acid identity to human MCP-3 and MCP-1, respectively). This level of homology suggested that these leader sequences may have an additional biological role, perhaps in cellular targeting (e.g., localization to a granule sub-compartment). Comparison of the homology to other C-C chemokines revealed that the nucleotide identity was almost always greater than the amino acid identity and similarity (Table 1). Although the MCPs were initially characterized by their ability to activate and
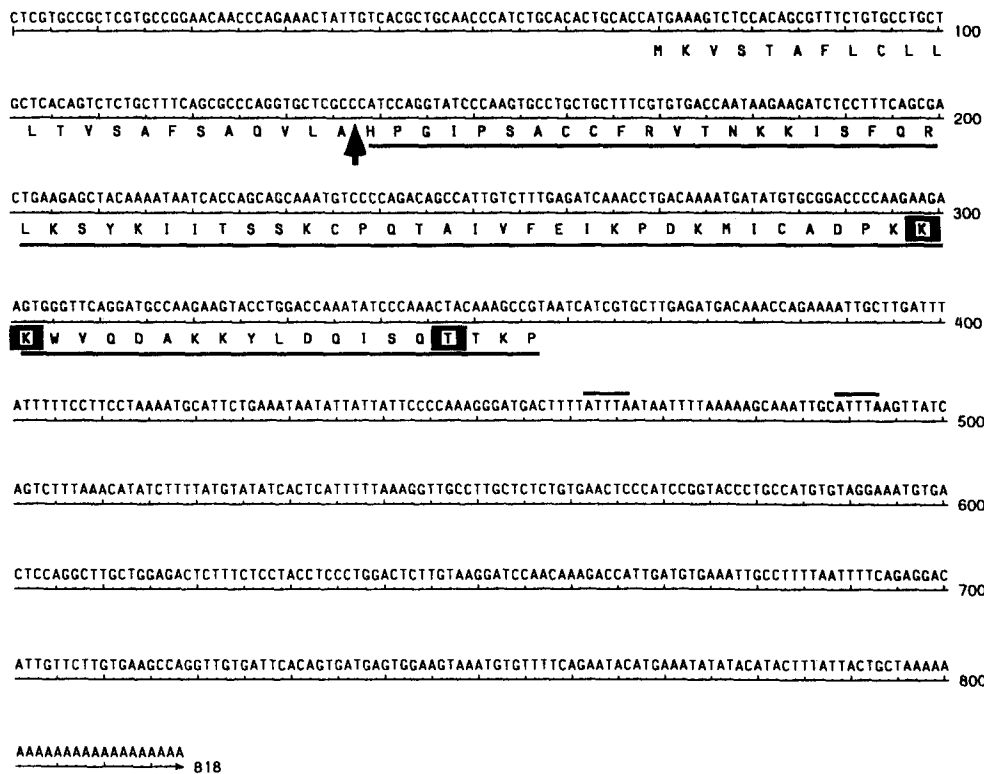
CTCGTGCCGCTCGTGCCGGAACAACCCAGAAACTATTGTCACGCTGCAACCCATCTGCACACTGCACCATGAAAGTCTCCACAGCCGTTTCTGTGCCTGCT
⎣_____⎦ 100
                                                                 M   K   V   S   T   A   F   L   C   L   L

GCTCACAGTCTCTGCTTTCAGCGCCCAGGTGCTCGCCCATCCAGGTATCCCAAGTGCCTGCTGCTTTCGTGTGACCAATAAGAAGATCTCCTTTCAGCGA
⎣_____⎦ 200
  L   T   V   S   A   F   S   A   Q   V   L   A▲H   P   G   I   P   S   A   C   C   F   R   V   T   N   K   K   I   S   F   Q   R

CTGAAGAGCTACAAAATAATCACCAGCAGCAAATGTCCCCAGACAGCCATTGTCTTTGAGATCAAACCTGACAAAATGATATGTGCGGACCCCAAGAAGA
⎣_____⎦ 300
  L   K   S   Y   K   I   I   T   S   S   K   C   P   Q   T   A   I   V   F   E   I   K   P   D   K   M   I   C   A   D   P   K   ▣

AGTGGGTTCAGGATGCCAAGAAGTACCTGGACCAAATATCCCAAACTACAAAGCCGTAATCATCGTGCTTGAGATGACAAACCAGAAAATTGCTTGATTT
⎣_____⎦ 400
  ▣   W   V   Q   D   A   K   K   Y   L   D   Q   I   S   Q   ▣   T   K   P

ATTTTTCCTTCCTAAAATGCATTCTGAAATAATATTATTATTCCCCAAAGGGATGACTTTTATTTAATAATTTTAAAAAGCAAATTGCATTTAAGTTATC
⎣_____⎦ 500

AGTCTTTAAACATATCTTTTATGTATATCACTCATTTTTAAAGGTTGCCTTGCTCTCTGTGAACTCCCATCCGGTACCCTGCCATGTGTAGGAAATGTGA
⎣_____⎦ 600

CTCCAGGCTTGCTGGAGACTCTTTCTCCTACCTCCCTGGACTCTTGTAAGGATCCAACAAAGACCATTGATGTGAAATTGCCTTTTAATTTTCAGAGGAC
⎣_____⎦ 700

ATTGTTCTTGTGAAGCCAGGTTGTGATTCACAGTGATGAGTGGAAGTAAATGTGTTTTCAGAATACATGAAATATATACATACTTTATTACTGCTAAAAA
⎣_____⎦ 800

AAAAAAAAAAAAAAAAAA
⎣_____⟶ 818

Figure 1. Nucleotide sequence and predicted amino acid translation of full-length guinea pig eotaxin cDNA. The underlined protein sequence corresponds to the sequence of mature active eotaxin isolated from the bronchoalveolar fluid except for the boxed amino acids which were previously ambiguous (17). The arrow indicates the predicted site for signal peptide cleavage. The hatched bars overlie the ATTTA sequences that has been reported to decrease mRNA stability. These sequence data are available from EMBL/GenBank/DDBJ under accession number U18941.

attract macrophages, MCP-3 has been reported to cause eosinophil chemotaxis (10). It remains possible that guinea pig eotaxin is the homologue of human MCP-3, but this seems unlikely since eotaxin does not display any effect on macrophages in vivo. Furthermore, the proposed murine homologue

Table 1. C-C Chemokine Comparison with Guinea Pig Eotaxin*

	% Amino acid		% Nucleotide Identity
Chemokine	Identity	Similarity	
Human MCP-1	56	66	71
Human MCP-2	54	66	ND
Human MCP-3	57	65	73
Guinea pig MCP-1	45	61	60
Murine MARC	46	64	64
Human MIP-1α	35	49	50
Human MIP-1β	40	56	53
Human MIP-2α	23	42	42
Human MIP-2β	23	38	42
Human I-309	31	51	47
Human RANTES	28	47	48

* Comparison over entire 96-amino acid protein and cDNA encoding entire translated protein (bp 69–359). Chemokines in bold have been shown to be active on eosinophils.

of human MCP-3, MARC, is even less homologous to eotaxin than to human MCP-3 (Table 1) (26, 27).

Eotaxin Gene in the Guinea Pig and Mouse Genome. Restriction endonuclease treatment of guinea pig DNA (Fig. 2, lanes a and b) with analysis by Southern blotting under conditions of low stringency, revealed a single hybridizing band. Mouse genomic DNA cut with EcoRV (Fig. 2, lane c) also revealed a single hybridizing band. This data suggests that a single gene encodes guinea pig eotaxin and suggests the existence of a closely related gene in the mouse.

Eotaxin mRNA Expression in Different Organs. Northern blot analyses of total RNA isolated from different guinea pig tissue samples revealed easily detectable constitutive expression of eotaxin in the lung (Fig. 3). The predominant hybridizing band had a size of ∼0.8 kb. Other hybridizing bands were not detectable using poly A–selected RNA (data not shown). Lower levels were detectable in the intestines, stomach, heart, thymus, spleen, liver, testes, and kidney. In these latter tissues, eotaxin mRNA was more easily detected on poly A blots (data not shown). The intestine showed some variability in expression between two different animals (Fig. 3). In addition, no RNA was detectable in the brain, bone marrow, or skin. Likewise, macrophages isolated and cultured from the spleen, a lung epithelial cell line, and a colon adenocarcinoma cell line were not found to express eotaxin mRNA (data not shown). The finding of constitutive eotaxin mRNA in mucosal tissues wherein eosinophils predominantly reside (lung and intestines) suggests that eotaxin may play a role in the normal tissue homing and turnover of eosinophils.
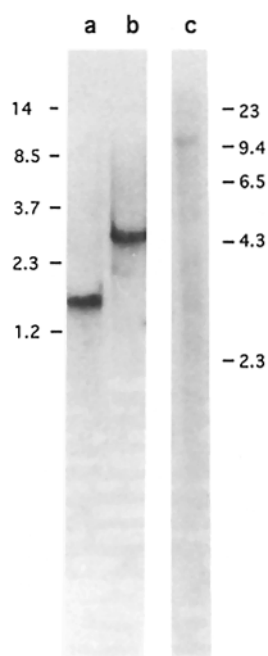
Figure 2. Guinea pig and mouse genomic analysis. Guinea pig genomic DNA was digested with EcoR1 (lane *a*) and PvuII (lane *b*) and mouse genomic DNA was digested with EcoRV (lane *c*). After electrophoresis and transfer to nylon membranes, a guinea pig eotaxin probe limited to the coding region of the cDNA was hybridized and washed under low stringency conditions. X-ray film was exposed for 2 wk. Molecular weight markers (in kb) are shown to the left for lanes *a* and *b* and to the right for lane *c*.

Figure 4. Northern analysis of poly(A)+ RNA (2 mcg/lane) isolated from the lungs of guinea pigs challenged 3 h earlier with saline (lanes *a–f*) or OVA (lanes *g–l*). Each lane is RNA from a separate animal. Hybridization was performed with a guinea pig eotaxin probe (*top*) or a guinea pig β-actin probe (*bottom*). X-ray films were exposed for 24 h.

The Induction of Eotaxin mRNA in Allergic Airway Inflammation. Since eotaxin mRNA was found to be expressed at relatively high levels in the lung of healthy guinea pigs, it was important to determine if this mRNA could account for all the protein released after allergen challenge, or whether eotaxin mRNA levels also increased. Therefore guinea pigs were sensitized to either aerosolized OVA or exposed to saline twice at a 7-d interval. After a third dose of aerosolized antigen, the lungs of sensitized, but not saline exposed, guinea pigs develop a mucosal and submucosal eosinophil infiltra-

Figure 3. Northern analysis of total RNA from various guinea pig organs. 10 mcg of total RNA was resolved in 1.5% agarose gels and transferred to nylon membranes. Tissue source is indicated. Splenocytes were treated with 2.5 mcg/ml Con A for 48 h. RNA from intestine and lung were isolated from two different animals. Hybridization and washing were performed under conditions of high stringency using a guinea pig eotaxin probe that was limited to the coding region of the cDNA. X-ray film was exposed for 1 wk.
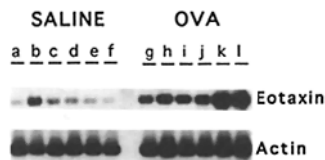
tion. The inflammation is most prominent at 17 h and persists for at least 3 d (13, 18). At various times after antigen challenge, poly A–selected RNA were isolated from replicate lungs and equal amounts were examined by Northern blot analysis for eotaxin mRNA expression. Eotaxin mRNA levels increased approximately sixfold ($p < 0.01$) by 3 h compared with the lungs of saline-treated guinea pigs (Fig. 4). All other time points were not significantly different (Fig. 5).

The cloning of guinea pig eotaxin cDNA has allowed us to make several observations relevant to the biology of eotaxin. The eotaxin gene is expressed at relatively high levels in the lungs of healthy guinea pigs without airway inflammation. In contrast, the chemotactic activity ascribed to eotaxin has been reported to be undetectable in the bronchoalveolar fluid of non-antigen–challenged guinea pigs (17). Thus, eotaxin mRNA is expressed at easily detectable constitutive levels in the lung when eotaxin activity is undetectable. This presents several alternative possibilities regarding the eotaxin protein: (*a*) it is rapidly degraded; (*b*) it is expressed at a low level which previously was not detectable; (*c*) it remains in an inaccessible location (e.g., a mast cell granule) and/or is biologically inhibited until after antigen challenge; or (*d*) it requires additional biochemical processing for activation or some combination of the above. It is unlikely that eotaxin requires additional processing for activation since the cDNA structure predicts that active eotaxin is generated directly after removal of the leader sequence. It is interesting that the lungs of healthy guinea pigs without eosinophilic inflammation have detectable eosinophils in the bronchoalveolar fluid at baseline (13). Low levels of eotaxin protein may regulate basal eosinophil tissue homing.

After antigen challenge, eotaxin gene expression in the lung is further increased during the early part of the late phase response. This change in mRNA parallels the peak changes in eotaxin protein release into the bronchoalveolar fluid which also peaks at three hours (17). Only antigen exposure is associated with eosinophilic airway inflammation and bronchial hyper-responsiveness (13–15). Thus, upregulation of gene expression, and not constitutive gene expression, is associated with the pathogenesis of airway disease. Eotaxin is likely to work in parallel with other cytokines generated during the late phase response. For example, IL-5, a cytokine produced during the late phase response, can prime eosinophils to respond to another C-C chemokine, RANTES, and can promote eosinophil tissue survival and activation (28, 29).
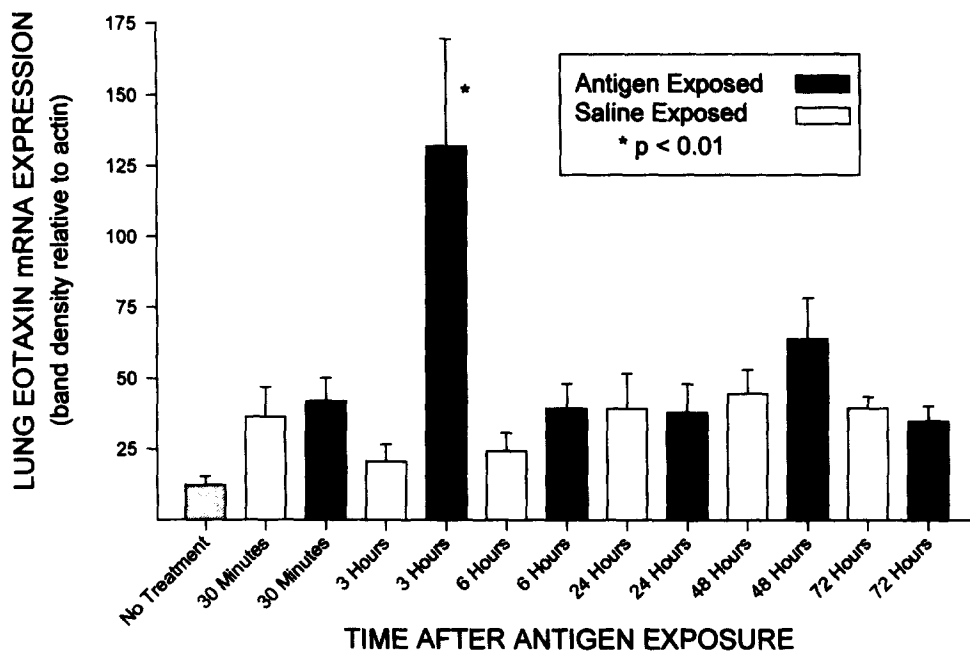
Examination of RNA samples from multiple tissues for

Figure 5. Eotaxin mRNA levels after OVA challenge. OVA sensitized or saline exposed guinea pigs were challenged with aerosolized OVA or saline, respectively, and eotaxin mRNA levels in the lungs were examined. The intensity of the band hybridization was determined by a Phosphor-Imager. The eotaxin mRNA expression is normalized to guinea pig β-actin mRNA expression in each sample. Data are represented in arbitrary units and the results are expressed as mean ± SEM (n = 5 or 6 animals for each group).

the expression of eotaxin mRNA reveals that in addition to the lung, lower levels are seen in a variety of other tissues. This suggests a more widespread function for this molecule. With the development of immunological reagents to detect the eotaxin protein, it will be important to compare protein and mRNA expression in these tissues. Finally, this cDNA will enable the identification of analogous genes in other species and the development of molecular and immunological tools to examine the role of this molecule in allergic models and human disease.

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