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EOTAXIN: CLONING OF AN EOSINOPHIL CHEMOATTRACTANT CYTOKINE AND INCREASED mRNA EXPRESSION IN ALLERGEN-CHALLENGED GUINEA-PIG LUNGS

P.J. Jose, I.M. Adcock†, D.A. Griffiths-Johnson, N. Berkman†, T.N.C. Wells*, T.J. Williams‡‡, and C.A. Power *

Departments of Applied Pharmacology and †Thoracic Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, U.K.

*Glaxo Institute for Molecular Biology, 14 Chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland

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Summary. Eotaxin was recently identified as the major eosinophil chemoattractant in bronchoalveolar lavage fluid obtained 3h after allergen challenge of sensitised guinea-pigs. We now report the cDNA cloning of this C-C chemokine. The 777 base-pair clone, pEo3122, consists of a 40 base 5' untranslated region, an open reading frame of 288 bases predicting a 73 amino acid mature protein plus a 23 amino acid signal peptide, and a 3' untranslated region of 449 bases containing a poly A tail. Northern blot analysis showed eotaxin mRNA in the lungs of naive and sensitised guinea-pigs, which was considerably increased after allergen challenge. Eotaxin may be an important mediator of eosinophil accumulation and activation in allergic reactions. As eotaxin stimulates human eosinophils, this chemokine and related molecules may be involved in human diseases such as asthma where eosinophil accumulation is a prominent feature.

Eotaxin was recently identified as a potent eosinophil chemoattractant detected in bronchoalveolar lavage (BAL) fluid obtained after allergen challenge of sensitised guinea-pigs (1,2). Low doses of eotaxin induce eosinophil accumulation in guinea-pig airways and skin *in vivo*, and the stimulation of guinea-pig and human eosinophils *in vitro* (1,2). Eosinophil accumulation and activation are characteristic features of IgE-mediated allergic reactions such as asthma (3), rhinitis (4), eczema (5) and parasitic infections (6). In asthma, eosinophil accumulation and activation are associated with hyperresponsiveness to constrictor mediators and damage to the bronchial epithelium (7-10). Experimental inhibition of eosinophil accumulation in sensitised monkeys suppresses the bronchial hyperresponsivness induced by allergen challenge (11).

‡‡ Corresponding author. Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, U.K. Fax:(44) 171 351 8270.

Abbreviations. BAL, bronchoalveolar lavage; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; OA, ovalbumin; RANTES, regulated on activation T-cell expressed and secreted; RT-PCR, reverse transcription-polymerase chain reaction.

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Eotaxin is a member of the C-C branch of the platelet factor 4 family of cytokines (reviewed in 12,13). Since many C-X-C and C-C cytokines are chemotactic for leukocytes, they are now often called "chemokines". Human C-C chemokines induce chemotaxis and activation of eosinophils, basophils and mononuclear cells via seven transmembrane spanning, G-protein coupled receptors. Different effects are seen in different target cells depending on the particular chemokine tested (14). Eotaxin appears to act selectively on eosinophils (1,2).

We report here the cDNA cloning of guinea-pig eotaxin and use of the cDNA probe to demonstrate significantly increased mRNA expression after allergen challenge of sensitised guinea-pig lungs.

Materials and Methods

Sensitisation and challenge of lungs *in vivo*. Male Dunkin Hartley guinea-pigs (350-400g) were sensitised by intraperitoneal injection of ovalbumin (1mg) on day 1, boosted by exposure to an aerosol of ovalbumin on day 8, and treated prophylactically with an antihistamine before challenge with an aerosol of the allergen, or saline as control, on day 18-20 as described previously (2). After 3h, atropine (0.06mg/kg, i.p.)was administered to prevent bronchoconstriction and the animals killed with a barbiturate overdose. Bronchoalveolar lavage (3x10ml saline) was performed, the heart and lungs removed, and the pulmonary vasculature flushed through the left ventricle (50ml saline/10mM EDTA). Lung tissue was snap frozen in liquid nitrogen and stored at -70°C for RNA extraction. The lavage fluid was centrifuged (300g, 10 min, 4°C). The pellet was resuspended in TRIzol (Gibco BRL, Basel, Switzerland, 10^7 cells per ml) and stored at -70°C for RNA extraction.

RT-PCR, subcloning and sequencing. RNA was isolated from allergen-challenged guinea-pig lung by the method of Chomczynski and Sacchi (15). Single stranded cDNA was prepared from 1μg of total RNA using avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Lewes, Sussex, UK) according to the manufacturer's instructions. The reaction mixture was subjected to 35 cycles of PCR (1 min at each of 95°C, 50°C and 72°C) in 10mM Tris-HCl, pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM deoxynucleotide triphosphates and 2.5 units of AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT, USA) in a Techne PHC-3 thermal cycler (Techne Ltd, Cambridge,UK). The degenerate primers were based on the amino acid sequence of the mature protein (2) (sense 5' TGC TGT TTC CGI GTI ACI AAC AAA, antisense 5' CAT CTT GTC IGG CTT IAT TTC). PCR reaction products were visualised on 1% agarose gels containing 0.5μg/ml ethidium bromide. Reaction products with the predicted size were gel purified, treated sequentially with T4 polynucleotide kinase and *E. coli* DNA polymerase I (Klenow fragment) to render them blunt ended and subcloned into the *Eco*RV site of plasmid Bluescript II SK-. The recombinant cDNA inserts were sequenced on an ABI automated sequencer using T3 and T7 primers. One clone, pEo9, was subsequently digested with *Hind*III and *Eco*RI to release a 130 bp fragment which was labelled with [^{32}P] by random-priming and used to screen the guinea-pig lung cDNA library.

cDNA library screening. Total RNA was extracted from a sensitised guinea-pig lung, 3h after allergen challenge, using TRIzol according to the manufacturer's instructions. Poly A+ mRNA, prepared from total RNA using oligotex dT latex beads (Qiagen Inc, Chatsworth, CA, USA), was used to generate a cDNA library in the λZAP EXpress vector (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The [^{32}P]-labelled, 130 bp probe was used to screen 5 x 10^5 PFUs of the cDNA library by standard methods. Following hybridisation, duplicate filter lifts were washed at high stringency (0.1xSSC, 0.1% SDS, 55°C) and duplicating positives were rescreened with the same probe until pure positive phage plaques were obtained. Phagemid DNA was then screened with T3 primer and an antisense internal eotaxin primer (5' GAC AAT GGC CGT CTG GGG AC). Phagemid DNA was rescued from 3 independent plaques into plasmid BK-CMV, according to the manufacturer's instructions and sequenced using T3 and T7 primers. One clone, pEo3122, which contained the putative 5' end of eotaxin and a poly A tail at the 3' end was then resequenced with 2 internal primers (sense 5' CGT GTG ACC AAT AAG AAG ATC , antisense 5' GAG TTC ACA GAG AGC AAG G) based on the T3 and T7 sequence results, to give the full length cDNA sequence.

Northern blot analysis. Total RNA was extracted from guinea-pig lung, spleen and bone marrow using TRIzol. RNA samples (5μg) were separated on 1% agarose gels, containing 2.2% formaldehyde, transblotted onto nylon membranes (Boehringer-Mannheim) and fixed by UV irradiation. Membranes were hybridised with the random-primed, [^{32}P]-labelled, full-length eotaxin cDNA (*Xho*I/*Eco*RI fragment of pEo3122). Membranes were washed at a final stringency of 0.1xSSC, 0.1% SDS, 55°C and autoradiographed at -70°C. The membranes were then stripped, rehybridised using a [^{32}P]-labelled 1272 bp rat glyceraldehyde-3-phosphate (GAPDH) cDNA probe and washed at the same high stringency. Autoradiographs were assessed by laser densitometry (Protein & DNA ImageWare System, Discovery Series, USA) and specific RNA levels in lung tissues expressed as the ratio of eotaxin to GAPDH mRNA. The median value of five naive lungs was given an arbitrary value of one and other lung samples are expressed relative to this.

Total RNA from BAL cell pellets (limited to 2.7μg by availability) was analysed for eotaxin mRNA as described above, together with single representative samples (5μg) of spleen, bone marrow, naive and allergen-challenged lung.

Results

cDNA Cloning. Degenerate oligonucleotide primers based on the published sequence of eotaxin (2) were used to obtain the corresponding partial cDNA by PCR on reverse transcribed RNA isolated from the lung of a sensitised guinea-pig after exposure to an aerosol of allergen *in vivo*. The PCR products (120 bp) were subcloned into pBluescript II SK- and shown to contain coding sequence for eotaxin. A *Hind*III/*Eco*RI fragment of one of these clones was used to screen a guinea-pig lung cDNA library, prepared from an allergen-challenged animal, for the full length cDNA. Sequencing of three second-round positive clones revealed that one clone, pEo3122 encoding a 777 bp cDNA, was full length (Fig. 1).

pEo3122 consists of 40 bases of 5' untranslated sequence which contains a putative Kozak sequence (16) at nucleotides 35-40, an open reading frame of 288 bases encoding a protein of 96 amino acids and a 3' untranslated region of 449 bases which contains a poly A tail. The deduced protein sequence contains a putative 23 amino acid signal peptide which is cleaved to produce a mature protein of 73 amino acids. The latter corresponds to the sequence identified for eotaxin purified from BAL fluid except that position 65 is encoded as glycine, not aspartic acid as previously determined by sequencing two peptide fragments (2). The three previously unidentified amino acids in the mature protein are encoded as Lys^{54}, Lys^{55} and Thr^{70}. The molecular weight of the predicted 73 amino acid sequence of the mature protein is 8298.81.

mRNA analysis. Northern blot analysis showed eotaxin mRNA in the lung, but not spleen, of naive guinea-pigs (Fig. 2a). There was no difference in eotaxin mRNA levels between naive and saline-exposed sensitised animals after correction for RNA loading by reference to the constitutive GAPDH mRNA (Fig. 2b). A striking increase in eotaxin message was seen in the lungs 3 hours after allergen challenge of sensitised guinea-pigs (Fig. 2a,b). In contrast, no eotaxin message was detected in BAL cell pellets taken from the same sensitised animals, with or without allergen challenge, nor in bone marrow (data not shown).

Discussion

Eotaxin was identified as the major, stable eosinophil chemoattractant in bronchoalveolar lavage fluid 3h after allergen challenge of sensitised guinea-pigs (2). We now report the cDNA cloning of eotaxin.

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1   CGGCACGAGGTCACGCTGCAACCCATCTGCACACTGCACCATGAAAGTCTCCACAGCGTT   60
                                              M  K  V  S  T  A  F

61  TCTGTGCCTGCTGCTCACAGTCTCTGCTTTCAGCGCCCAGGTGCTCGCCCATCCAGGTAT  120
    L  C  L  L  L  T  V  S  A  F  S  A  Q  V  L  A  H  P  G  I

121 CCCAAGTGCCTGCTGCTTTCGTGTGACCAATAAGAAGATCTCCTTTCAGCGACTGAAGAG  180
    P  S  A  C  C  F  R  V  T  N  K  K  I  S  F  Q  R  L  K  S

181 CTACAAAATAATCACCAGCAGCAAATGTCCCCAGACAGCCATTGTCTTTGAGATCAAACC  240
    Y  K  I  I  T  S  S  K  C  P  Q  T  A  I  V  F  E  I  K  P

241 TGACAAAATGATATGTGCGGACCCCAAGAAGAAGTGGGTTCAGGATGCCAAGAAGTACCT  300
    D  K  M  I  C  A  D  P  K  K  K  W  V  Q  D  A  K  K  Y  L

301 GGGCCAAATATCCCAAACTACAAAGCCGTAATCATCGTGCTTGAGATGACAAACCAGAAA  360
    G  Q  I  S  Q  T  T  K  P

361 ATTGCTTGATTTATTTTTCCTTCCTAAAATGCATTCTGAAATAATATTATTATTCCCCAA  420

421 AGGGATGACTTTTATTTAATAATTTTAAAAAGCAAATTGCATTTATGTTATCAGTCTTTA  480

481 AACATATCTTTCATGTATATCACTCATTTTTAAAGGTTGCCTTGCTCTCTGTGAACTCCC  540

541 ATTCCGGTACCCTGCCATGTGTAGGAAATGTGACTCCAGGCTTGCTGGAGACTCTTTCTC  600

601 CTACCTCCCTGGACTCTTGTAAGGATCCAACAAAGACCATTGATGTGAAATTGCCTTTTA  660

661 ATTTTCAGAGGACATTGTTCTTGTGAAGCCAGGTTGTGATTCACAGTGATGAGTGGAAGT  720

721 AAATGTGTTTTCAGAATACATGAAATATATACATACTTTAAAAAAAAAAAAAAAAAAA  777
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Figure 1. The nucleotide sequence and deduced amino acid sequence of guinea-pig eotaxin. The predicted signal sequence, preceding the sequence of the mature protein isolated from BAL fluid (2), is underlined. The nucleotide sequence has been deposited in EMBL/GenBank/DDBJ data bases (accession number X77603).

The sequence of the predicted mature protein contains important information in addition to the sequence obtained previously. Firstly, the three previously unidentified amino acids are now identified as Lys^{54}, Lys^{55}, and Thr^{70}. The identification of threonine at position 70 is in accord with our previous conclusion of probable O-glycosylation of this residue, there being no potential N-glycosylation sites. Human MCP-1 and MCP-3 also contain lysine and threonine in positions equivalent to residues 55 and 70. Similarly, human RANTES contains lysine at positions equivalent to eotaxin residues 54 and 55. Secondly, the amino acid at position 65, which was previously identified as aspartic acid in each of two peptide fragments, is encoded as glycine. The peptide sequencing data has been rechecked and the signal is clearly aspartic acid. This suggests polymorphism in the C-terminus, which could help to explain the resolution of two closely migrating peaks of eosinophil chemoattractant activity with identical N-termini by reversed phase HPLC (2). Thirdly, the overlap of the C-terminal tryptic peptides, which were previously alligned by reference to the sequence of human MCP-1, is confirmed. Fourthly, the stop codon after Pro^{73} confirms the C-terminus of the protein which was not unequivocally identified in our previous report (2).

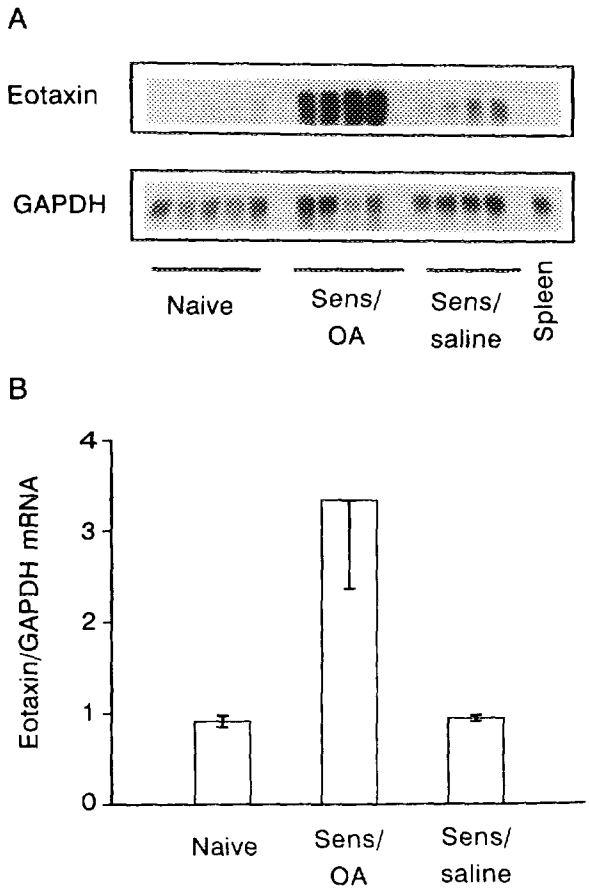

Figure 2. Northern blot analysis of eotaxin mRNA expression in guinea-pig lungs and spleen. A) RNA samples from the lungs of 5 naive, 4 sensitised/ovalbumin(OA)-challenged, 4 sensitised/saline control-challenged animals and, for comparison, the spleen of 1 naive guinea-pig were hybridised with a cDNA probe prepared from the eotaxin clone pEo3122. After stripping, the membranes were probed for the constitutive GAPDH message. B) Eotaxin/GAPDH ratios for the 3 groups of lung samples. Allergen-challenge of sensitised guinea-pigs induced a significant increase in eotaxin message, compared with saline-exposed sensitised animals, $p<0.05$, Mann-Whitney U-test (2-tailed).

The eotaxin clone was used to probe Northern blots prepared from guinea-pig tissues. Eotaxin mRNA (approximately 800 bp) was detected in lung and was up-regulated after allergen challenge, but was not detected in spleen, bone marrow or BAL cell pellets. These results suggest that the source of eotaxin may be lung cells, eg. epithelial or connective tissue cells, and/or infiltrating inflammatory cells which have not reached the airway lumen within 3 hours of aerosolised allergen challenge. *In situ* hybridisation should provide a clearer understanding of the cell source(s) eotaxin. The up-regulation of eotaxin message as well as protein after allergen challenge shows that the response is, at least to a large extent, at the level of transcription rather than translation of the existing message. Since eosinophils are present in guinea-pig lung and are increased after challenge with allergen (17) or eotaxin (1), the results of mRNA analysis support our conclusions, based on the generation and activity of the protein, that eotaxin is an important mediator of eosinophil accumulation in guinea-pig lung particularly in response to allergen challenge.

The predicted eotaxin protein sequence of clone pEo3122 permits a re-evaluation of homology with human C-C chemokines. The highest homology is with the MCP-3 (54%), MCP-1 (53%) and MCP-2 (52%); these are all chemotactic for monocytes but MCP-3 is also a potent eosinophil activator (18). MIP-1β shows 39% homology with eotaxin whilst MIP-1α and RANTES, both eosinophil chemoattractants (19-21), show lower homology (32 and 30%, respectively). The results presented here provide the basis to examine further the role of eotaxin in inducing eosinophil accumulation and activation in the guinea-pig lung and to look for a human equivalent since eotaxin may be involved in these events in the asthmatic lung.

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