

Variant eotaxin: Its effects on the asthma phenotype

Hidetoshi Nakamura, MD,^a Andrew D. Luster, MD, PhD,^b Toshiko Nakamura, MD,^a
Kwang Ho In, MD,^a Larry A. Sonna, MD, PhD,^c Aaron Deykin, MD,^a
Elliot Israel, MD,^a Jeffrey M. Drazen, MD,^a and Craig M. Lilly, MD^a *Boston, Natick, and
Charlestown, Mass*

Background: Eotaxin, a CC chemokine expressed in the asthmatic lung, has been associated with impaired lung function. The role of its variant form is unknown.

Objective: The purpose of this study was to detect the population frequency and effects of a known single-nucleotide polymorphism in the eotaxin gene in which a threonine residue (THR₂₃) is substituted for the wild-type alanine (ALA₂₃) at the 23rd amino acid at the terminus of the peptide leader sequence.

Methods: We measured eotaxin protein secretion in 293 cells transfected with expression vectors and in PBMCs obtained from individuals bearing the alternative forms of the gene. A case-control study of plasma eotaxin levels and eosinophil counts, a comparison of baseline lung function by genotype in a population of 806 subjects with asthma, and a comparison of the allele frequency with a nonasthmatic population were performed.

Results: Human 293 cells and PBMCs with THR₂₃ variant eotaxin secreted significantly less eotaxin protein than did ALA₂₃-bearing cells. In the case-control study, THR₂₃-THR₂₃ individuals had lower plasma levels of eotaxin (310 [240-350] vs 420 [270-700] pg/mL; $P < .05$) and eosinophil counts (120 [5-220] vs 190 [110-470] cells/ μ L; $P < .05$) than ALA₂₃-ALA₂₃ subjects; heterozygous subjects had intermediate levels. Higher levels of lung function were associated with THR₂₃ eotaxin (percent of predicted FEV₁, 65% $\pm$ 3.5% [THR₂₃-THR₂₃] vs 58% $\pm$ 0.9% [THR₂₃-ALA₂₃] and 56% $\pm$ 0.5% [ALA₂₃-ALA₂₃]; $P < .05$).

Conclusion: The THR₂₃ variant is associated with both decreased eosinophil counts and higher levels of lung function in subjects with asthma. (*J Allergy Clin Immunol* 2001;108:946-53.)

Key words: Eotaxin, single-nucleotide polymorphism, chemokines, human genetics, asthma

Abbreviations used

ALA₂₃: Wild-type form of eotaxin with an alanine residue at position 23
ARMS: Amplification refractory mutation system
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
SSCP: Single-stranded conformational polymorphism
THR₂₃: Variant form of eotaxin with a threonine substitution at position 23

Eotaxin is a chemotactic cytokine or chemokine with potent effects on eosinophils.¹ The effects of eotaxin on the CCR3 receptor and the expression of this receptor on cell types associated with allergic diseases, including eosinophils,^{2,3} T_H2 lymphocytes,⁴ and basophils,⁵ have led to studies associating eotaxin with asthma.⁶⁻⁹ Asthma is known to have a significant but complex genetic component; genetic mapping studies in families have established linkage between asthma diagnosis and markers near the eotaxin gene in 2 populations.^{10,11} Expression of eotaxin protein and mRNA is excessive in the asthmatic lung at baseline^{6,7} and increases after allergen challenge at airway loci where eosinophils accumulate in asthma.⁸ Eotaxin expression is strongly and directly associated with pulmonary eosinophil recruitment early after pulmonary allergen challenge,⁸ and its increased level of expression in the asthmatic lung correlates with increased sensitivity to contractile agonists, the physiological hallmark of asthma.⁷

Studies in larger populations have demonstrated that plasma eotaxin levels are directly related to asthma diagnosis and inversely related to lung function.⁹ In addition, subjects with acute asthma symptoms and airflow obstruction have higher plasma eotaxin levels than subjects with stable asthma, and higher levels are associated with less airflow reversibility after emergent asthma treatment.¹² Although these studies clearly associate eotaxin expression with compromised lung function, they do not allow us to determine whether eotaxin causes impaired lung function or is expressed by asthmatic lungs in response to damage by other mediators.

We reasoned that if eotaxin expression were causing loss of lung function, then a genetic variant that limited its expression would be associated with better lung function. If, on the other hand, eotaxin were expressed in response to lung damage caused by other mediators, then lung function would not be affected by an eotaxin-limiting variant. We characterized the eotaxin-limiting effects of a known

From ^athe Combined Program in Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston; ^bthe Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Boston; and ^cthe US Army Research Institute of Environmental Medicine, Natick, and Harvard Medical School, Charlestown.

Supported by National Heart, Lung, and Blood Institute grant HL/AI-64104 and National Institute of Allergy and Infectious Diseases grant AI 40618.

A. D. Luster is a Culpeper Medical Scholar and the recipient of a Cancer Research Institute/Benjamin Jacobson Family Investigator Award.

Received for publication June 5, 2001; revised September 11, 2001; accepted for publication September 12, 2001.

Reprint requests: Craig M. Lilly, MD, Respiratory Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/81/120135

doi:10.1067/mai.2001.120135

eotaxin genetic variant in vitro and ex vivo as well as its effects on plasma eotaxin levels, peripheral eosinophil counts, and lung function in subjects with asthma.

METHODS

Subject characteristics

We studied 1492 subjects, of whom 873 met the American Thoracic Society's criteria for the diagnosis of asthma^{13,14} and 619 did not. The asthmatic subjects had mild to moderate asthma by spirometric classification and at the time of measurement were using only inhaled β -agonists for asthma treatment and loratadine for nasal symptoms; none of our subjects was taking oral or inhaled steroids. Normal subjects were defined as those without a physician's diagnosis of any disease who answered negatively to a screening questionnaire for respiratory symptoms. All subjects were free of symptoms of respiratory infection for the 4-week interval preceding the study.

In our case-control study, 17 subjects homozygous for the variant form of the eotaxin gene (THR₂₃-THR₂₃) were matched for age, race, sex, and asthma diagnosis with 51 heterozygous subjects (ALA₂₃-THR₂₃) and 51 subjects homozygous for the wild-type (ALA₂₃-ALA₂₃) form of the gene. In studies of cytokine expression in PBMCs, 6 normal subjects homozygous for the THR₂₃ variant were matched for race, sex, age, IgE level, and peripheral blood eosinophil count with 6 normal subjects homozygous for the ALA₂₃ form of the gene. All subjects gave written informed consent using documents approved in advance by the appropriate institutional review boards.

Mutation identification

SSCP. The source of DNA for single-stranded conformational polymorphism (SSCP) analysis was a panel of immortalized cells from normal and asthmatic subjects.¹⁵ Genomic DNA corresponding to the entire coding region of the eotaxin gene of each of these individuals was subjected to SSCP screening for mutations by the method of Beier.¹⁶ The following oligonucleotide primers (5' to 3') were synthesized and used for PCRs: AGAAACCACCACCTCTCACC and GAGAATTTGCAGTGAGTCTGT for exon 1, GTCATGCTTGGAATGTCTATG and AGTCTATGACTGCCACAGTTC for exon 2, and CAATCCTTCCAATTGCATCCT and GGACTGATAATGAGGTTACCT for exon 3. For kinase labeling of the primers, a mixture of 3.0 μ L [γ -³²P]ATP (3000 Ci/mmol, 10 mCi/mL), 1.7 μ L 20- μ mol/L forward primer, 37.1 μ L dH₂O, 5.0 μ L 10x T4 polynucleotide kinase buffer, and 1.5 μ L T4 polynucleotide kinase (10 μ mol/L) was prepared; the solution was subsequently incubated, first at 37°C for 45 minutes and then at 70°C for 10 minutes. The PCR solution (12.5 μ L) contained 0.1 μ g of genomic DNA in the presence of 1 μ L each of ³²P-labeled forward primer and unlabeled reverse primer (0.13 μ mol/L), dNTPs (0.4 mmol/L each), MgCl₂ (1.6 mmol/L), and Taq DNA polymerase (0.5 units, Perkin-Elmer/Cetus, Foster City, Calif). Before addition of the enzyme, the mixture was incubated for 5 minutes at 94°C. PCR was performed for 35 cycles, a 1-minute 55°C annealing segment being followed by a 1-minute 72°C extension segment and a 1-minute 94°C denaturation segment. A 5-minute 72°C extension segment followed the final cycle. The PCR solution was diluted 3:20 in a stop solution of 100% formamide containing xylene cyanol and bromophenol blue, denatured for 5 minutes at 94°C, and placed on ice. A 4- μ L sample was loaded onto an MDE gel (AT Biochem, Malvern, Pa) and subjected to electrophoresis for 5 to 6 hours at 4°C in 0.6 $\times$ TBE at 60W

constant power. The PCR products were transferred from the gel to filter paper, dried, and subjected to autoradiography overnight with an intensifying screen.

ARMS. DNA was harvested with a commercially available kit (Qiagen, La Jolla, Calif), used according to the manufacturer's instructions, from the whole blood of 873 asthmatic subjects and 619 normal subjects; it was then subjected to amplification refractory mutation system (ARMS) analysis. In this procedure, the presence of a 530-base pair (bp) band corresponding to the THR₂₃ form of the gene or the absence of a 530-bp band corresponding to the ALA₂₃ form of the gene was determined for paired PCR reactions under the conditions described in this paragraph. The sequences of the reverse primers were GGGGCTTACCTGGCCCAAC (wild-type primer) and GGGGCTTACCTGGCCCAAT (mutant primer), and the sequence of the common forward primer was TCAAGGAAGGTTCTTAGATCG. Each 20- μ L PCR solution contained 0.1 μ g of DNA, 2.0 mmol/L MgCl₂, a 0.2-mmol/L dNTP mixture, and 0.5 μ mol/L primers. Each reaction was subjected to the following amplification procedure: denaturation at 94°C for 5 minutes, addition of 2.0 units of AmpliTaq DNA polymerase, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute, and denaturation at 94°C for 40 seconds. Thirty amplification cycles were followed by a 5-minute 72°C extension period. The electrophoretic mobility of the reaction products was determined on a 2% agarose gel. All samples were run in quadruplicate. The ARMS band detection pattern agreed with the DNA sequence (Life Technologies, Gaithersburg, Md) and SSCP-determined genotype for each of the 40 samples studied.

Cloning and expression of THR₂₃ eotaxin

RNA was extracted from PBMCs of subjects homozygous for the THR₂₃ and ALA₂₃ forms of eotaxin by the guanidinium thiocyanate method, which has been described previously.¹⁷ This source RNA was reverse-transcribed with the SuperScript preamplification system (Life Technologies) according to the manufacturer's instructions. A forward oligonucleotide primer in exon 1 preceding the ATG start codon and a reverse primer at the end of exon 3 after the C-terminal proline residue were synthesized and had the sequences GCCTCCAACATGAAGGTCTC and GGCTCTGGTTTGTTTCAAAA, respectively. Each PCR solution (20 μ L) contained 0.1 μ g of cDNA, 2.0 mmol/L MgCl₂, a 0.2-mmol/L mixture of dNTPs, and 0.5 μ mol/L primers and was subjected to the following amplification procedure: denaturation at 95°C for 4 minutes, addition of 2.0 units of AmpliTaq DNA polymerase, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and denaturation at 95°C for 1 minute. Thirty-five amplification cycles were followed by a 10-minute 72°C extension period. The electrophoretic mobility of the product was determined on a 2% agarose gel. PCR amplification products were ligated into the pCR2.1 vector and cloned through use of the Original TA Cloning kit (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. The sequence of each insert was determined by means of the dsDNA Cycle Sequencing System (Life Technologies). Each insert was excised by enzymatic digestion with *Bam*HI and *Xba*I, ligated into the pCDNA 3.1 vector (Invitrogen), and cloned as described above, and the sequences were again confirmed.

Human embryonic kidney epithelial 293 cells, obtained from the American Type Culture Collection, were grown to confluence in Dulbecco modified Eagle medium (DMEM) with 10% FBS. These cells were stably transfected with an empty-vector construct and constructs corresponding to the THR₂₃ and ALA₂₃ forms of the gene through use of LipofectAMINE (Life Technologies), used according to the instructions of the manufacturer, and an identical

vector containing an insert for the expression of green fluorescent protein. Clones with robust and equivalent fluorescence were selected for further characterization. Distinct clones transfected with constructs containing the alternative genotypes with equivalent eotaxin mRNA expression were selected; mRNA expression was not detectable in untransfected or empty vector-transfected cells. Twenty-four hours after confluent cells had been cultured in serum-free medium, the cell supernatant was harvested and an aliquot was taken for protein determination (Micro BCA assay system, Pierce, Rockland, Ill). The remaining supernatant was applied to a prepared Sep-Pak C₁₈ column (Millipore, Bedford, Mass), eluted with 100% methanol, and dried under vacuum. The pellet was resuspended in ELISA buffer, and the eotaxin concentration was determined by ELISA and normalized to the concentration of protein in the corresponding sample.

Cell culture

PBMCs were isolated by density gradient centrifugation from 6 normal subjects homozygous for the THR₂₃ variant of eotaxin and 6 subjects homozygous for the ALA₂₃ (wild-type) form of the gene. PBMCs with purity greater than 98% were cultured on plastic or Teflon (DuPont, Wilmington, Del) plates in the presence or absence of IL-1 β (10 ng/mL) in RPMI-1640 with 10% FBS at 2 to 5 $\times$ 10⁶ cells/mL. The cells were harvested after 4 or 24 hours in culture. Human 293 cells were obtained from the American Type Culture Collection and cultured in DMEM modified to include 4.5 g/L glucose, 20 mmol/L L-glutamine, 0.1 mol/L HEPES, 10% FBS, 10,000 units/dL penicillin, and 10,000 μ g/dL streptomycin (Fisher Scientific, Pittsburgh, Pa). Twenty-four hours before harvesting, the FBS was removed from the medium. After centrifugation, the cell supernatant was collected, the cells were harvested by trypsin/EDTA digestion (Fisher), and cell-associated protein was extracted in NP-40 lysis buffer^{18,19} and immediately assayed for eotaxin or subjected to Western analysis.

RNA analysis

Total RNA was isolated from freshly harvested cells by guanidinium-thiocyanate-phenol-chloroform extraction (Stratagene Inc, La Jolla, Calif). Northern analysis was performed as previously described.¹⁸ In brief, 20 μ g of total RNA was subjected to gel electrophoresis on a formaldehyde-2% agarose gel and transferred to a nylon membrane (Schleicher and Schuell, Keene, NH). After UV crosslinking, the membrane was hybridized at 68°C in ExpressHyb Hybridization Solution (Clontech, Palo Alto, Calif) with a ³²P-labeled 0.35-kb cDNA probe containing the entire coding region of the human eotaxin gene or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech). The membranes were washed for 10 minutes at room temperature in 2 $\times$ SSC-0.05% SDS and then for 20 minutes at 50°C in 0.2 $\times$ SSC-0.1% SDS. The blots of eotaxin and GAPDH were exposed to BioMax films (Kodak, Rochester, NY) for 40 and 5 hours, respectively. In each case, the eotaxin signal was detected at the site expected for a 0.8-kb transcript and GAPDH was detected at the site expected for a 1.3-kb transcript. The optical density of each band was determined by densitometry of a scanned image (GelPro Analyzer Version 3.0, Media Cybernetics, Silver Springs, Md). To control for differences in RNA loading, the hybridization signal for eotaxin was normalized to that for GAPDH in each sample.

Eotaxin protein measurement

ELISA for human eotaxin was performed as previously described.^{12,18} The amount of eotaxin recovered from PBMC lysate and supernatant was calculated from the ELISA concentration and the cell numbers and was reported as the amount recovered per 10⁶ cells.

Western analysis

Cell lysate (1 μ g) or recombinant eotaxin derived from COS cells was subjected to gel electrophoresis on a 10% to 20% tricine gel (Novex, San Diego, Calif) and transferred to a Protran nitrocellulose membrane (Schleicher and Schuell). The membrane was incubated for 12 hours at 4°C in an incubation solution composed of PBS (pH 7.4) containing 1 g/L Tween 20 and 10 g/L powdered milk and then for 1 hour at room temperature with 5 μ g of a goat antibody to eotaxin (R&D Systems, Minneapolis, Minn) diluted in 10 mL incubation buffer. The membrane was washed 3 times for 5 minutes in incubation buffer containing milk and incubated for 1 hour at room temperature in 10 mL incubation buffer containing 4 μ g horseradish peroxidase-conjugated anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, Calif). The membrane was washed 3 times for 20 minutes in incubation buffer containing milk and then 3 times in incubation buffer not containing milk. The membrane was developed with the Renaissance Western blot chemiluminescence reagent (NEN Life Science Products, Boston, Mass) according to the instructions of the manufacturer. The membrane was exposed for 5 to 20 minutes to Blue XB-1 film (Kodak).

Statistical analysis

Values were expressed as means $\pm$ SEMs or as medians and 25th and 75th percentiles after being tested for normality and equal variance. Mean values were compared through use of the Student *t* test or ANOVA for parametrically distributed data; this was followed by the Student-Newman-Keuls test. Nonparametric data were compared through use of ANOVA on the basis of ranks; this was followed by Dunn's test. The case-control study was analyzed through use of ANOVA on the basis of ranks and confirmed by 2-factor ANOVA, genotype and case match being used as factors on rank-transformed eotaxin data. Differences in allele frequency among groups were calculated by χ^2 analysis, and significance was determined with 1 degree of freedom. A *P* value of less than .05 was considered significant.

RESULTS

Determination of eotaxin genotype

SSCP analysis of DNA harvested from the immortalized lymphocytes of 40 asthmatic subjects demonstrated 3 distinct electrophoretic patterns (Fig 1, A). A single distinct band was noted in lanes corresponding to 26 subjects with the ALA₂₃ wild-type form of eotaxin, and 2 bands were present in lanes corresponding to 12 heterozygous subjects. In lanes corresponding to 2 subjects, a single band with distinct electrophoretic mobility was present. This was identified by sequence analysis to represent the THR₂₃ variant form of eotaxin. The frequency of the THR₂₃ allele was 0.2 in the screening population. Sequencing of the DNA from each of these 40 subjects demonstrated that the 3 distinct sequence patterns of SSCP conformers corresponded exactly to the alternative genotypes (Fig 1). A single-base pair transition mutation was detected 67 bp in the 3' direction from the ATG initiation codon of the eotaxin gene (Fig 1, B). SSCP screening of the entire eotaxin coding sequence, 700 bp of the core promoter region, and 1000 bp of the 3' untranslated region for these 40 individuals detected no other coding region sequence alterations that changed the eotaxin amino acid sequence and no other variants that could explain our findings.

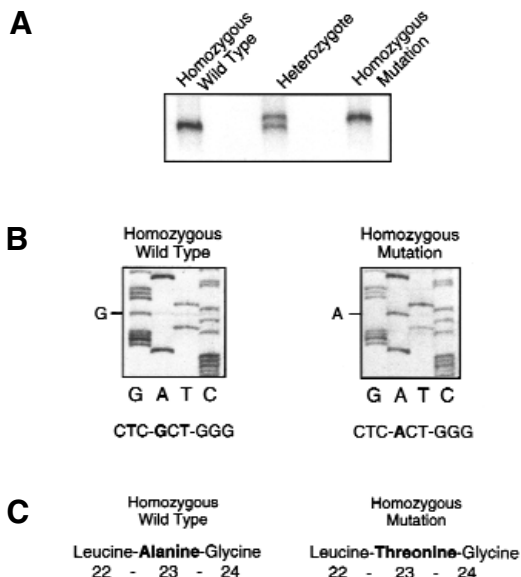


FIG 1. Analysis of the eotaxin coding sequence polymorphism by SSCP and direct cycle sequencing. **A**, SSCP demonstrates 3 distinct patterns; 2 bands are present in heterozygotes, one with the electrophoretic mobility of the wild-type conformer and the other with that of the mutant conformer. **B**, Direct cycle sequencing indicated a single band for G in the homozygous wild-type and a single band for A in the homozygous mutation. **C**, Predicted amino acid sequences for the alternative forms of the eotaxin gene.

Amino acid substitution at the signal peptidase cleavage site

The single-base pair substitution of an adenosine for a guanine residue 67 bp after the ATG initiation codon altered the predicted protein sequence of eotaxin by substituting a threonine for the alanine at amino acid residue 23 (Fig 1, C). This alteration occurred at the terminus of the predicted hydrophobic leader sequence. Signal peptidase hydrolysis analysis²⁰ was performed on the alternative forms of the human eotaxin leader sequence. The most efficient cleavage site was on the carboxyl-terminal side of the 23rd amino acid for the ALA₂₃ form of eotaxin ("Nielsen y" value of 0.877). The predicted site of cleavage for THR₂₃ eotaxin is at the 24th amino acid (glycine) and is less efficient ("Nielsen y" value of 0.659).

Cloning and expression of THR₂₃ and ALA₂₃ eotaxin

The sequence of the PCR product derived from RNA harvested from a subject homozygous for the ALA₂₃ form of eotaxin was identical to that reported by others²¹⁻²³ and included the entire coding sequence of eotaxin. The sequence of the product derived from RNA harvested from a subject homozygous for the THR₂₃ form of eotaxin differed from this sequence by a single-base pair transition from a guanine to an adenosine residue 67 bp after the ATG initiation codon (Fig 1, C). Unstimulated human 293 cells stably transfected with a CMV-based eukaryotic expression vector containing the ALA₂₃ form

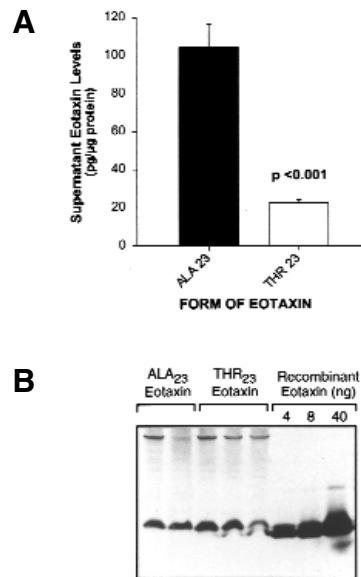


FIG 2. **A**, Human 293 cells stably transfected with expression vectors bearing the entire ALA₂₃ coding region (n = 4) of eotaxin secreted significantly more eotaxin than cells transfected with an identical vector bearing the entire THR₂₃ coding sequence (n = 5; $P < .001$). **B**, Western analysis of 293 cell lysate (1 μg/lane) and increasing amounts of recombinant (nonglycosylated) eotaxin standard.

of the eotaxin coding sequence secreted 110 ± 12 pg of eotaxin/μg of protein (n = 5); paired cells transfected with an identical vector containing the THR₂₃ construct secreted significantly less eotaxin (23 ± 2 pg of eotaxin/μg of protein; n = 4; $P < .001$; Fig 2, A). Eotaxin secretion was not detectable in untransfected or empty vector-transfected 293 cells. Western analysis confirmed that each transfected cell line produced a substance that had the molecular mass of glycosylated human eotaxin (Fig 2, B).

Population genotype screening by ARMS analysis

Three distinct genotypes were identified by ARMS analysis. Using wild-type primer sets, we detected a 530-bp band with DNA from subjects with the ALA₂₃ wild-type form of eotaxin and from heterozygotes, whereas amplification of DNA from subjects homozygous for the THR₂₃ variant form of eotaxin yielded a less distinct band with greater electrophoretic mobility. When the variant primer set was used, a 530-bp band was detected with DNA from subjects homozygous for the THR₂₃-THR₂₃ variant form of eotaxin and from heterozygotes but was not detected with DNA from subjects with the ALA₂₃-ALA₂₃ wild-type form of the gene.

Eotaxin expression in PBMCs

To compare the efficiency of eotaxin production and release from PBMCs between subjects homozygous for the THR₂₃-THR₂₃ variant and those homozygous for the ALA₂₃-ALA₂₃ wild-type form of the eotaxin gene, we

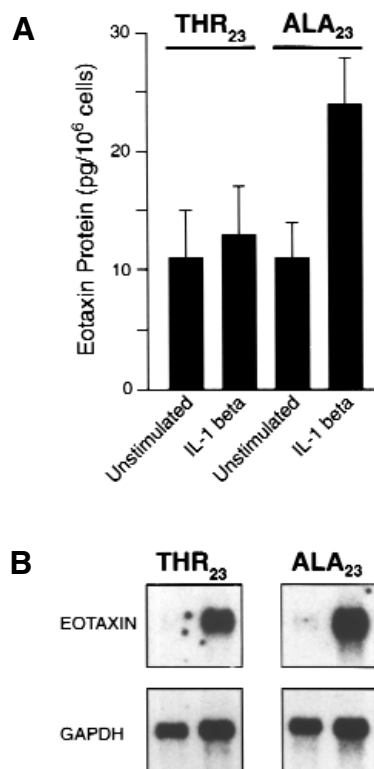


FIG 3. Effects of genotype on eotaxin expression. **A**, Supernatant eotaxin protein levels were significantly higher after 24 hours of stimulation with 10 ng/mL IL-1 β for ALA₂₃-ALA₂₃ PBMCs than for THR₂₃-THR₂₃-bearing cells ($P < .05$). **B**, Eotaxin mRNA expression before (left lane) and 4 hours after (right lane) stimulation with 10 ng/mL IL-1 β in PBMCs from individuals homozygous for the THR₂₃ and ALA₂₃ alleles. A representative blot from 5 sets of observations.

isolated PBMCs from 6 normal subjects in each group who were matched for age, race, sex, IgE level, and plasma level of eotaxin. PBMCs from subjects homozygous for the THR₂₃ variant secreted substantially less eotaxin protein (Fig 3, A; Table I) after stimulation with IL-1 β than did cells from subjects with the ALA₂₃ wild-type genotype. Stimulated THR₂₃ cells also expressed slightly lower levels of eotaxin mRNA than did ALA₂₃ cells ($P < .05$; Fig 3, B; Table I). Although cell-associated eotaxin levels did not differ between cells from THR₂₃ and ALA₂₃ homozygotes (11 ± 3 vs 11 ± 1 pg/10⁶ cells), IL-1 β -induced levels of eotaxin were lower in cells from subjects homozygous for THR₂₃ eotaxin than in cells from subjects homozygous for the ALA₂₃ form of the gene (13 ± 4 vs 24 ± 4 pg/10⁶ cells; $P < .05$; Table II).

Case-control and population studies

The demographic characteristics of the subjects in our case-control study are presented in Table II. There were no significant differences in the demographic data between the case groups and the control groups. The demographic characteristics of our populations are described in Table III. The proportion of women and the fraction of atopic subjects (defined by IgE levels of >100 IU/mL)²⁴ are similar to those reported by other investigators for adult populations.

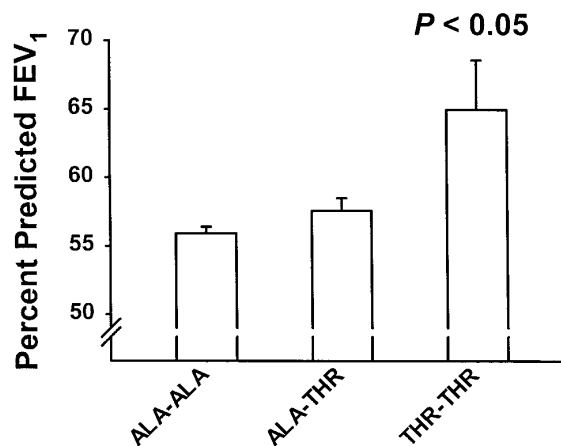


FIG 4. Lung function, as assessed by baseline percent of predicted FEV₁, was significantly higher in asthmatic individuals with the THR₂₃-THR₂₃ eotaxin genotype than in asthmatic individuals with the ALA₂₃-THR₂₃ or the ALA₂₃-ALA₂₃ genotype ($n = 806$; $P < .05$).

TABLE I. Effects of genotype on eotaxin expression in PBMCs

	Homozygous THR ₂₃ variant	Homozygous wild type
mRNA density ratio (eotaxin/GAPDH)		
US	0.048 $\pm$ 0.02	0.11 $\pm$ 0.07
IL-1	0.59 $\pm$ 0.06*	0.81 $\pm$ 0.05
Cell-associated protein (pg/10 ⁶ cells)		
US	11 $\pm$ 3	11 $\pm$ 1
IL-1	13 $\pm$ 3	12 $\pm$ 1
Secreted protein (pg/10 ⁶ cells)		
US	11 $\pm$ 4	11 $\pm$ 3
IL-1	13 $\pm$ 4*	24 $\pm$ 4

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; US, unstimulated; IL-1, treatment with 10 ng/mL IL-1 β .

* $P < .05$ in comparison with wild type.

Case-control study

Plasma eotaxin levels were significantly lower in subjects homozygous for the THR₂₃ variant form of eotaxin than in those with the homozygous ALA₂₃ genotype (310 [240-350] pg/mL; $n = 17$; vs 420 [270-700] pg/mL; $n = 51$; median and interquartile range, $P = .03$). Heterozygous subjects had intermediate levels (360 [250-500] pg/mL; $n = 51$). The genotype-associated differences in plasma eotaxin levels are directly related to differences in the number of eosinophils in peripheral blood. Subjects homozygous for the THR₂₃ variant form of eotaxin had significantly fewer peripheral eosinophils than did heterozygotes or subjects homozygous for the wild-type form of the gene (120 [5-220] cells/ μ L, 160 [140-370] cells/ μ L, and 190 [110-470] cells/ μ L, respectively; both $P < .05$). Differences in serum IgE levels among patients harboring alternative genotypes were not statistically significant.

TABLE II. A case-control study of the effects of genotype on plasma eotaxin and eosinophil levels

Genotype	Sex (M/F)	Age (y)	Disease (asthma/normal)	Plasma eotaxin (pg/mL)†	Eosinophil (cells/μL)†
THR ₂₃ -THR ₂₃	7/10	41 ± 3.5	6/11	310 [240-350]	120 [5-220]
ALA ₂₃ -THR ₂₃	21/30	41 ± 1.9	18/33	360 [250-500]	160 [110-470]*
ALA ₂₃ -ALA ₂₃	21/30	41 ± 2.0	18/33	420 [270-700]*	190 [110-470]*

M, Male; F, female.

**P* < .05 vs THR₂₃-THR₂₃.

†Median [interquartile range].

Population study

We detected the variant THR₂₃ eotaxin allele with a frequency of 0.16 in both the normal population and the asthmatic population: 28.2% of our normal subjects and 28.7% of the subjects in our asthmatic population carried at least 1 copy of this allele, and 3.2% of our normal subjects and 3.1% of our asthmatic population were THR₂₃ homozygotes (Table III). The frequency of THR₂₃, determined by the gene-counting method, was 0.156 in normal subjects and 0.159 in asthmatic subjects, and the population distribution of genotypes was consistent with the Hardy-Weinberg equilibrium (*P* = .5 and *P* = .2). Plasma IgE levels were not significantly different among subjects with the alternative genotypes. Lung function, measured as percent of predicted FEV₁, was significantly higher among THR₂₃ homozygotes than in the other groups (65% ± 3.5% [THR₂₃-THR₂₃; *n* = 25] vs 58% ± 0.9% [ALA₂₃-THR₂₃; *n* = 206] and 56% ± 0.5% [ALA₂₃-ALA₂₃; *n* = 575] of predicted FEV₁; *P* < .05; Fig 4).

DISCUSSION

We report the functional implications of a known mutation of the human eotaxin gene, which we have found to be present in 28% of individuals in 2 geographically dispersed and ethnically diverse US populations. This variant, termed THR₂₃, was detected by SSCP analysis of PCR products derived from the first exon of the eotaxin gene, which demonstrated a previously described variant of the eotaxin coding sequence.²³ The THR₂₃ form of eotaxin is common in US populations; at least 1 copy was present in 28% of an asthmatic population and a population of healthy adults (Table III).

This single nucleotide polymorphism in exon 1 of the eotaxin gene alters the predicted eotaxin amino acid sequence by substituting a polar threonine residue for the hydrophobic alanine residue at position 23. As a secreted protein, eotaxin contains an amino-terminal sequence of hydrophobic amino acids that are a substrate for signal peptidases, which process proteins for transport to the cell surface and secretion.²⁰ The hydrolytic activity of these ubiquitous signal peptidases is highly dependent on the specific amino acids in the leader sequence of the substrate protein.²⁵ The efficiency of signal peptidase hydrolysis of secreted proteins is determined by the specific sequence of peptides at the amino-terminus.^{20,26} The substitution of threonine for alanine substantially reduces the predicted signal peptidase activity for the THR₂₃ variant of eotaxin. We therefore predicted that

TABLE III. A population study of variant eotaxin genotype frequency

Characteristic	Normal	Asthmatic
No. of subjects	505	806
Age (y): mean ± SEM	31.6 ± 0.5	35.5 ± 0.5
Sex (male/female)	237/268 (47%/53%)	395/411 (49%/51%)
Race/ethnicity		
Caucasian	66%	87%
African American	20%	11%
Hispanic	9%	1%
Other	4%	1%
Atopic/nonatopic	55/217 (20%/80%)	469/287 (62%/38%)
Genotype (no. observed)		
Homozygous THR ₂₃	16 (3.2%)	25 (3.1%)
Heterozygous ALA ₂₃ /THR ₂₃	126 (25.0%)	206 (25.6%)
Homozygous ALA ₂₃	363 (71.9%)	575 (71.3%)
THR ₂₃ allele frequency	0.156	0.159

this mutation would impair eotaxin secretion. We tested this hypothesis by creating expression vectors that did not contain core promoter elements but that included the entire coding region of the alternative forms of eotaxin. We found that human 293 cells stably transfected with eukaryotic expression vectors bearing the THR₂₃ variant of eotaxin secreted significantly less eotaxin than did paired cells transfected with vectors bearing the ALA₂₃ (wild-type) form.

This finding suggested that PBMCs from individuals with the THR₂₃ variant of eotaxin would have an impaired ability to secrete eotaxin. We therefore studied eotaxin-producing circulating cells from individuals with the alternative genotypes to determine whether genotype affected eotaxin secretion and mRNA expression. Because baseline eotaxin secretion was near the limits of detection in our assay system, we measured eotaxin secretion after stimulation with IL-1β under conditions that we had previously established as optimal for stimulation of PBMCs. To reduce the potential confounding effects of sensitization, eosinophil hematopoietic factors, autocrine behavior of eotaxin, and disease activity, we performed these studies on nonasthmatic subjects matched for these variables. Despite the statistically significantly lower eotaxin levels in THR₂₃-variant individuals, the variance in levels allowed us to select individuals of the alternative genotypes who had similar levels. We found that cytokine-stimulated PBMCs from subjects homozygous for the THR₂₃ variant secreted less protein

and had slightly impaired eotaxin mRNA expression; the decreased expression of eotaxin mRNA implies either that the effects of this mutation are not limited to secretory mechanisms or that it is associated with a common genetic variant that was not detected by our methods.

The reduced ability of PBMCs from THR₂₃-THR₂₃-bearing individuals to express eotaxin suggested that individuals with variant eotaxin genes would have lower plasma eotaxin levels. We tested this hypothesis in a case-control study of subjects with the various forms of the eotaxin gene. We found significantly lower levels of eotaxin in the plasma of subjects homozygous for the THR₂₃ variant of eotaxin than in subjects homozygous for the ALA₂₃ wild-type allele; patients with one wild-type and one mutant allele had intermediate levels of eotaxin. The presence of a naturally occurring genetic variant that limited eotaxin expression allowed us to explore the nature of the direct association between plasma eotaxin and eosinophil levels and its indirect relationship with lung function.

The relationships of eotaxin, IL-5, other growth factors, and peripheral eosinophilia are complex. In the mouse, eotaxin is reported to serve as a GM-CSF; specific antibodies against it prevent antigen-induced increases in bone marrow myeloid progenitor cells.²⁷ The ability of the THR₂₃ variant form to limit eotaxin expression allowed us to determine whether eotaxin expression was related to eosinophil counts in human beings. We found that the THR₂₃ variant eotaxin was associated with lower levels of peripheral eosinophils. Although these findings indicate that eotaxin has predictive value for eosinophil counts, the amount of variance in eosinophil counts that is explained by eotaxin levels is small. This association supports the notion that eotaxin contributes to other, more dominant factors that determine the number of circulating eosinophils.

The ability of the THR₂₃ variant form to limit eotaxin expression allows us to explore the nature of the known indirect relationship between plasma eotaxin levels and lung function.¹¹ This association of eotaxin with impaired lung function could occur either because eotaxin causes lung damage or because lungs damaged by other mediators secrete eotaxin. If eotaxin were expressed by lungs that have been damaged by other mediators, a genetic variant that limited eotaxin expression would have no effect on lung function. If, on the other hand, eotaxin were impairing function, a genetic variant that limited its expression would be associated with better lung function. We found that asthmatic individuals who were homozygous for the THR₂₃ form of eotaxin had significantly better lung function than asthmatic individuals with the alternative genotypes. Moreover, the difference in lung function was of clinically relevant magnitude. Our finding that asthmatic individuals who harbor the THR₂₃ eotaxin allele have higher levels of lung function implies that eotaxin is causally related to impaired lung function in people with asthma. In the broader sense, our findings implicate the CCR3 receptor as important for impaired lung function in asthma and in

this context might underrepresent the magnitude of benefit from complete CCR3 abrogation. We observed significant protection with modest reductions in plasma eotaxin levels in a setting in which only one of the several known ligands for the CCR3 receptor is limited.

In summary, we report the identification of a coding region polymorphism in the leader peptide sequence of the eotaxin gene that is associated with impaired eotaxin mobilization, reduced numbers of eosinophils in the peripheral blood, and higher levels of lung function in asthmatic subjects. The effects of this common mutation explain some of the population variance in peripheral eosinophil counts and identify a subgroup of subjects who express the asthma phenotype despite reduced numbers of peripheral eosinophils and a limited ability to mobilize eotaxin. THR₂₃ variant eotaxin does not appear to affect susceptibility to the development of asthma but appears to play a protective role with regard to lung function in asthmatic individuals by limiting eotaxin expression.

We acknowledge Amy Bastille and Murad Dikeidek for their technical assistance.

REFERENCES

1. Rothenberg ME. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *Am J Respir Cell Mol Biol* 1999;21:291-5.
2. Daugherty BL, Siciliano SJ, DeMartino JA, Malkowitz L, Sirotina A, Springer MS. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J Exp Med* 1996;183:2349-54.
3. Kitauro M, Nakajima T, Imai T, Harada S, Combadiere C, Tiffany HL, et al. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. *J Biol Chem* 1996;271:7725-30.
4. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 1997;277:2005-7.
5. Uguccioni M, Mackay CR, Ochensberger B, Loetscher P, Rhee S, LaRosa GJ, et al. High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. *J Clin Invest* 1997;100:1137-43.
6. Lamkhouiou B, Renzi PM, Abi-Younes S, Garcia-Zepeda EA, Allakhverdi O, Ghaffar O, et al. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol* 1997;159:4593-601.
7. Ying S, Robinson DS, Meng Q, Rotman J, Kennedy J, Ringler CR, et al. Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur J Immunol* 1997;27:3507-16.
8. Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin Exp Immunol* 1998;114:137-46.
9. Nakamura H, Weiss ST, Israel E, Luster AD, Drazen JM, Lilly CM. Eotaxin and impaired lung function in asthma. *Am J Respir Crit Care Med* 1999;160:1952-6.
10. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). *Nat Genet* 1997;15:389-92.
11. Kauffmann F, Dizier MH, Annesi-Maesano I, Bousquet J, Charpin F, Demenais D, et al. EGEA (Epidemiological Study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness and Atopy)—descriptive characteristics. *Clin Exp Allergy* 1999;29 Suppl 4:17-21.
12. Lilly CM, Woodruff PG, Camargo CA Jr, Nakamura H, Drazen JM, Nadel ES, et al. Elevated plasma eotaxin levels in acute asthma. *J Allergy Clin Immunol* 1999;104:786-90.
13. American Thoracic Society. Lung function testing: selection of reference values and interpretive strategies. *Am Rev Respir Dis* 1991;144:1202-18.

14. Guidelines for the diagnosis and management of asthma. 97-4051, 1-86. Bethesda (MD): National Institutes of Health/National Heart, Lung, Blood, Institute; 1997.
15. In KH, Asano K, Beier D, Grobholz J, Finn PW, Silverman EK, et al. Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription. *J Clin Invest* 1997;99:1130-7.
16. Beier DR. Single-strand conformation polymorphism (SSCP) analysis as a tool for genetic mapping. *Mamm Genome* 1993;4:627-31.
17. Rothenberg ME, Luster AD, Lilly CM, Drazen JM, Leder P. Constitutive and allergen-induced expression of eotaxin mRNA in the guinea pig lung. *J Exp Med* 1995;181:1211-6.
18. Lilly CM, Nakamura H, Kesselman H, Nagler-Anderson K, Garcia-Zepeda EA, Rothenberg MA, et al. Expression of eotaxin by human lung epithelial cells. Induction by cytokines and inhibition by glucocorticoids. *J Clin Invest* 1997;99:1767-73.
19. Nakamura H, Haley KJ, Nakamura T, Luster AD, Lilly CM. Differential regulation of eotaxin expression by TNF alpha and PMA in human monocytic U937 cells. *Am J Physiol Lung Cell Mol Physiol* 1998;275:L601-L610.
20. Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 1997;10:1-6.
21. Garcia-Zepeda EA, Rothenberg ME, Weremowicz S, Sarafi MN, Morton CC, Luster AD. Genomic organization, complete sequence, and chromosomal location of the gene for human eotaxin (SCYA11), an eosinophil-specific CC chemokine. *Genomics* 1997;41:471-6.
22. Ponath PD, Qin S, Ringler DJ, Clark-Lewis I, Wang J, Kassam N, et al. Cloning of the human eosinophil chemoattractant, eotaxin. *J Clin Invest* 1996;97:604-12.
23. Bartels J, Schluter C, Richter E, Noso N, Kulke R, Christophers E, et al. Human dermal fibroblasts express eotaxin: molecular cloning, mRNA expression, and identification of eotaxin sequence variants. *Biochem Biophys Res Commun* 1996;225:1045-51.
24. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989;320:271-7.
25. Dalbey RE, Lively MO, Bron S, van Dijk JM. The chemistry and enzymology of the type I signal peptidases. *Protein Sci* 1997;6:1129-38.
26. Folz RJ, Nothwehr SF, Gordon JI. Substrate specificity of eukaryotic signal peptidase. Site-saturation mutagenesis at position-1 regulates cleavage between multiple sites in human pre (delta pro) apolipoprotein A-II. *J Biol Chem* 1988;263:2070-8.
27. Peled A, Gonzalo JA, Lloyd C, Gutierrez-Ramos JC. The chemotactic cytokine eotaxin acts as a granulocyte-macrophage colony-stimulating factor during lung inflammation. *Blood* 1998;91:1909-16.