

Asthmatic airway biopsy specimens are more likely to express the IL-4 alternative splice variant IL-4 δ 2

Eric M. Glare, BSc,^a Maja Divjak, BAppSc,^a Jennifer M. Rolland, PhD,^b and E. Haydn Walters, MD, FRCP, FRACP^a Melbourne, Australia

Background: The human *IL4* gene, has been shown to express the alternatively spliced messenger (m)RNA IL-4 δ 2. IL-4 δ 2 is missing the entire sequence from exon 2 and has been identified as an IL-4 receptor antagonist.

Objective: We sought to distinguish IL-4 and IL-4 δ 2 mRNA in respiratory tract tissue for the first time.

Methods: A novel competitive PCR assay was established with primers designed on either side of the alternative splice junction of the *IL4* gene, allowing the simultaneous quantitation of both IL-4 and IL-4 δ 2 mRNA from one reaction.

Results: IL-4 and IL-4 δ 2 were differentially expressed in 4 nasal polyps. No difference was seen in endobronchial biopsy specimens for IL-4 mRNA expression between control subjects (median, 2.8×10^2 copies/ μ g RNA; range, $0.3.7 \times 10^3$ copies/ μ g RNA) and asthmatic subjects (median, 1.4×10^2 copies/ μ g RNA; range, $0.4.7 \times 10^2$ copies/ μ g RNA). However, significantly more asthmatic subjects (6 of 9) than control subjects (1 of 7) expressed IL-4 δ 2 ($P = .036$). Expression of IL-4 variants was unaffected by atopic status.

Conclusions: Given that IL-4 δ 2 is an IL-4 receptor antagonist, these results indicate that it is crucial to be able to distinguish IL-4 δ 2 from IL-4 when assessing *IL4* gene expression.

Increased expression of IL-4 δ 2 in stable asthmatic subjects suggests that the balance of IL-4 and IL-4 δ 2 may modulate asthmatic inflammation. (*J Allergy Clin Immunol* 1999;104:978-82.)

Key words: IL-4, IL-4 δ 2, splice variant, endobronchial biopsy, airway tissue, competitive PCR, nasal polyp, asthma, gene expression

IL-4, which is produced by CD4⁺ T cells and some CD8⁺ T cells, mast cells, B cells, and basophils, is a key cytokine that, along with IL-5, defines the T_H2 response.¹ The production of IL-4 and IL-5 by T cells is further promoted by IL-4 in a positive feedback system.

The human *IL4* gene contains 4 exons separated by 3 introns.² IL-4 messenger (m)RNA contains all 4 exons spliced together, whereas an alternatively spliced mRNA,

IL-4 δ 2, contains the coding sequences of exons 1, 3, and 4 spliced together in an open reading frame with the sequence of exon 2 omitted.³ Alternative splicing of human IL-4 mRNA was first described in PHA-stimulated PBMCs³ and subsequently in mitogenically stimulated CD3⁺ T cells⁴ and B cells.⁵

The deletion of exon 2 does not result in a frameshift, and IL-4 δ 2 mRNA encodes a truncated IL-4 protein. IL-4 δ 2 is a naturally occurring receptor antagonist of IL-4-induced T-cell stimulation, monocyte inhibition of PGE₂ secretion, and B cell-IgE synthesis and CD23 expression.^{6,7} Receptor-binding studies with lymphoma cell lines⁶ or purified monocytes⁷ showed that IL-4 δ 2 bound specifically and in competition with IL-4. The phenomenon of alternative splicing is shared by the *IL2* gene, which produces IL-2 δ 2 and IL-2 δ 3 mRNA, which are missing exons 2 and 3, respectively, as well as IL-2 mRNA.⁸ Studies with IL-2 δ 2 and IL-2 δ 3 indicate that they are also competitive inhibitors of IL-2.

We report a novel competitive RT-PCR assay for the simultaneous quantitation of both IL-4 and IL-4 δ 2 mRNA and its application to respiratory tract tissue. Previous studies have not attempted to differentiate these potentially competing IL-4 mRNA species and have only been able to report total *IL4* gene expression incorporating both mRNA forms.

METHODS

Samples

PHA-stimulated PBMCs were prepared as described previously.⁹ Aliquots of 3×10^6 cells were stimulated with 3 μ g/mL PHA (Sigma-Aldrich, Castle Hill, NSW, Australia) for 24 hours and then collected by centrifugation, snap frozen in liquid nitrogen, and stored at -80°C .

Freshly resected nasal polyps from 4 atopic patients were obtained. They were dissected and snap frozen in liquid nitrogen before storing at -80°C .

Endobronchial biopsy specimens were obtained from 9 asthmatic volunteers with well-documented illness (Table I). All had a bronchodilator response of 15% or greater increase in FEV₁. They used albuterol for relief of symptoms as needed, with (7 patients) or without (2 patients) regular inhaled corticosteroids. No patient was taking any other antiasthma medication. Seven control subjects had no history of asthma or other respiratory diseases, were using no medication, and had no bronchodilator response to albuterol and no measurable airway response to 3.2 mg of methacholine. Volunteers who had acute respiratory tract infection during the previous 4 weeks or who had any change in asthma medication or hospital admission caused by airway disease in the 4 weeks before the study

From the Departments of ^aRespiratory Medicine and ^bPathology and Immunology, Alfred Hospital and Monash University Medical School, Melbourne.

Supported by Glaxo Wellcome Australia and the National Health and Medical Research Council, Australia.

Received for publication Apr 26, 1999; revised July 1, 1999; accepted for publication July 1, 1999.

Reprint requests: E. Haydn Walters, MD, FRCP, FRACP, Department of Respiratory Medicine, Alfred Hospital, Prahran, Melbourne, Victoria, 3181, Australia.

Copyright © 1999 by Mosby, Inc.

0091-6749/99 \$8.00 + 0 1/1/101264

were excluded. All asthmatic and control subjects were nonsmokers and gave informed written consent before commencing the study, which was approved by The Alfred Hospital Ethics Committee.

Atopic status was assessed by skin prick testing with a panel of 7 common aeroallergens and was defined by a response of 3 mm or greater diameter to 1 or more of the aeroallergens. FEV₁ and bronchial responsiveness to methacholine were measured in the morning and after a bronchodilator-free period of at least 8 hours. A rolling-seal spirometer (SensorMedics Corporation, Yorba Linda, Calif) was used for measurement of FEV₁ according to American Thoracic Society guidelines. Bronchial responsiveness to methacholine was assessed by a previously established technique¹⁰ and expressed as PD₂₀ by using linear interpolation from a dose-response plot.

Bronchoscopy was performed shortly after recovery from methacholine challenge. Subjects were premedicated with intravenous administration of 0.4 mg of atropine and 5 to 15 mg of midazolam. Inhaled albuterol (200 µg) was given 15 minutes before bronchoscopy. Four percent lignocaine spray was applied to the pharynx and larynx, and 2% lignocaine was applied below the cords in 2-mL aliquots as required up to a maximum dose of 6 mL. Subjects were monitored by using pulse oximetry and administered oxygen at 4 L/min during the procedure. Endobronchial biopsy specimens were taken from the segmental subcarinae of the right lower lobe of each patient by using alligator forceps (Olympus code No. FB 15C). Biopsy specimens were transported on ice to the laboratory, snap frozen in liquid nitrogen within 15 minutes, and stored at -80°C.

Construction of competitor

A competitor template for IL-4 PCR was obtained by PCR-mediated mutagenesis of a commercially available construct. Human IL-4 DNA Mimic was obtained (Clontech, Palo Alto, Calif), and the primers were redesigned by using Oligo 5.0 Primer Analysis Software (National Biosciences, Plymouth, Minn) to improve their specificity and annealing profiles. IL-4 DNA Mimic (6 × 10⁷ copies/100 µL) was amplified in 4 × 100 µL reactions as for samples. The reactions were combined and purified by phenol/chloroform/isopropanol (25:24:1; Sigma-Aldrich) extraction and ethanol precipitation⁹ before resuspending in 100 µL of 10 mmol/L Tris-HCl, pH 7.5. The 499-bp competitor template was quantitated by using a PicoGreen fluorometric microplate assay (Molecular Probes, Eugene, Ore) detected in a laser-based fluorescence scanner (FluorImager 575, Molecular Dynamics, Sunnyvale, Calif). The IL-4 competitor was diluted to working concentrations with 50 µg/mL glycogen (Boehringer Mannheim Australia, Castle Hill, NSW, Australia) in 10 mmol/L Tris-HCl, pH 7.5.

Analysis of specificity of PCR

The IL-4 primers produce 339- and 291-bp PCR products as predicted by Oligo software when used to amplify complementary (c)DNA from PHA-stimulated PBMCs. To confirm the identity of the PCR products as amplicons of IL-4 and IL-482, the individual bands were isolated from a polyacrylamide gel by using QIAquick PCR Purification Kits (Qiagen, Melbourne, Australia) and subjected to restriction enzyme analysis. Excised gel slices were crushed, and the DNA was eluted passively into 500 µL of PB buffer, incubating for 20 minutes at 94°C followed by 4°C overnight. The gel fragments were collected by centrifugation and washed with 500 µL of PB buffer. The aliquots of PB buffer were combined, and PCR products were purified before reamplification in 5 × 100 µL reactions and purification directly by the QIAquick kit.

Aliquots of 400 ng of putative IL-4 and IL-482 PCR products were restriction digested by 15 U *Bpu*AI (exon 1-2 splice junction), *Hind*II (exon 2), *Pst*II (exon 3) and *Pvu*II (exon 3; Boehringer

TABLE I. Airway biopsy subject profiles

	Control subjects	Asthmatic subjects
No.	7	9
Sex (M:F)	6:1	5:4
Mean age (range)	24 (20-38)	48 (24-65)
Atopy (positive/negative)	4/3	5/4
Mean PD ₂₀ methacholine, mg (range)	≥6.4	0.035 (0.001-0.625)
Median FEV ₁ , % predicted (range)	109 (95-125)	96 (58-103)
Median inhaled corticosteroid, µg/d (range)*	—	800 (0-1000)

*Dose per day of beclomethasone dipropionate: 2 taking nothing, 2 taking 500 µg/d, 2 taking 800 µg/d, and 3 taking 1000 µg/d.

Mannheim). Digests were analyzed by PAGE and fluorescence scanning. The specificity of the PCR products was further tested by sequencing with a Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS (Perkin-Elmer, Foster City, Calif) followed by analysis with the Basic Local Alignment Search Tool (National Centre for Biotechnology Information).

Dual-mRNA species-competitive PCR

To establish that dual-mRNA species-competitive PCR is a valid quantitative method, a *changing input ratio* series of reactions was set up. Aliquots of PHA-stimulated PBMC cDNA, previously shown to be positive for both IL-4 and IL-482, were combined in 100-µL PCRs with serial titration of IL-4 competitor DNA from 8 × 10⁵ to 3.9 × 10² copies. Reactions were split into duplicate 50-µL aliquots and amplified for 35 and 40 cycles.

RNA extraction

Nasal polyps and endobronchial biopsy specimens were extracted with RNeasy Total RNA Kit (Qiagen). Tissue was homogenized in 700 µL of lysis buffer RLT by using a Polytron rotor-stator (Kinematica, Luzern, Switzerland) followed by aspiration 10 times through a 21-gauge syringe needle to shear chromosomal DNA. RNA was eluted from the spin column with 40 µL of diethylpyrocarbonate-treated water. Aliquots of 4 µL of RNA were quantitated by capillary spectrophotometry by using an Absorbance Capillary Adaptor Cell (Helix, San Diego, Calif), background correction at 320 nm and a Cary 1 spectrophotometer (Varian, Melbourne, Australia). Samples with (A₂₆₀-A₃₂₀)/(A₂₈₀-A₃₂₀) ratios less than 1.7 and/or yields less than 0.5 µg of total RNA were excluded from subsequent analysis.

Reverse transcription and competitive PCR

cDNA was synthesized from up to 1 µg of RNA in a volume of 20 µL consisting of 1× GeneAmp PCR buffer, 5 mmol/L MgCl₂, 1 mmol/L deoxyribonucleoside triphosphate, 0.8 U of RNase inhibitor, 2 µmol/L Oligo d(T)₁₆, and 2 U of MuLV reverse transcriptase (all from Perkin-Elmer). Reactions were incubated for 1 minute at 15°C, and then the temperature was increased to 42°C over 9 minutes followed by 1 hour at 42°C, 5 minutes at 85°C, and 1 minute at 4°C.

For competitive PCR, 20 µL of cDNA was made up to a 100-µL reaction, consisting of 2 U AmpliTaq Gold, 1× GeneAmp PCR buffer, 2.5 mmol/L MgCl₂, deoxyribonucleoside triphosphate (all from Perkin-Elmer), 0.2 µmol/L upper primer CCG GCA ACT TTG TCC AC from exon 1 and 0.2 µmol/L lower primer TCT GGT TGG CTT CCT TCA C from exon 4 (synthesized by Bresatec, Ade-

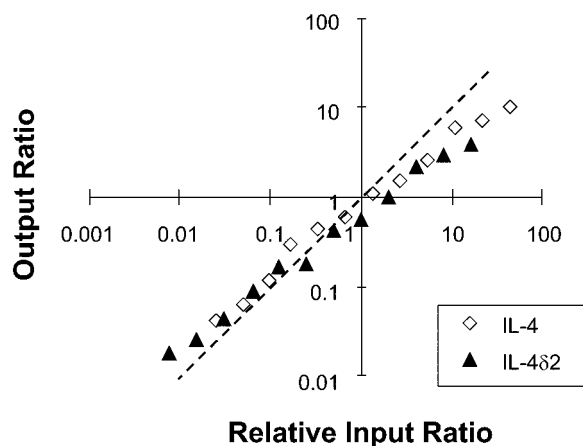


FIG 1. Test of dual-mRNA species-competitive PCR by titration of competitor. IL-4 competitor was titrated against a constant aliquot of PHA-stimulated PBMC cDNA, and PCR was conducted for 40 cycles. Quantitation of the native/competitor ratios for IL-4 and IL-4δ2 is shown. The line of prediction is indicated by a dashed line with a slope of 1. Similar data was obtained with 35 cycles of amplification.

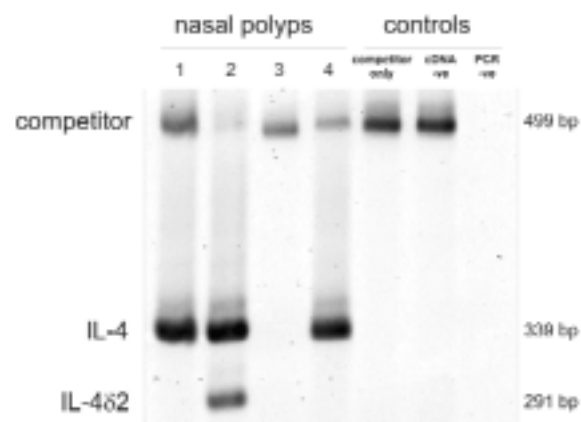


FIG 2. IL-4 and IL-4δ2 mRNA are differentially expressed in nasal polyps. The sizes of the PCR products are indicated.

laide, SA, Australia) and 40 copies of IL-4 competitor. Thermal cycling was conducted in an MJ Research PTC-200 (Watertown, Mass) with an initial denaturation of 8 minutes at 94°C, followed by 50 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minute and concluding with 7 minutes at 72°C and 1 minute at 4°C. The PCR products representing IL-4 and IL-4δ2 were resolved by electrophoresis through 1.5 mm 8% 29:1 polyacrylamide gels (acrylamide/bisacrylamide; Bio-Rad Laboratories, Hercules, Calif) in 1× Tris-acetate-EDTA buffer⁹ by using active cooling to maintain a temperature of 16°C to 18°C. PAGE gels were stained by overlay with 1:10,000 SYBR Green I (Molecular Probes) in Tris-acetate-EDTA buffer for 45 minutes and then scanned directly in the FluorImager. PCR product band volumes were quantitated with Image-QuaNT software (Molecular Dynamics). Native-competitor product ratios were calculated, and the levels of gene expression were reported as copies of mRNA per micrograms of total RNA.

Statistical analysis

For comparison between normal and asthmatic endobronchial biopsy mRNA levels, the 2-way Mann-Whitney *U* test was used.

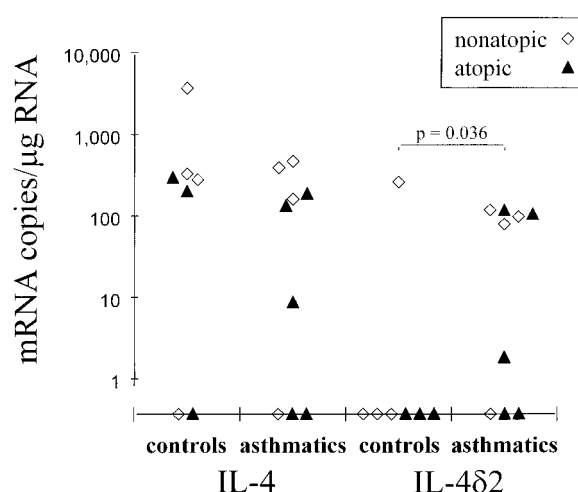


FIG 3. IL-4 and IL-4δ2 expression in airway biopsy specimens.

The χ^2 test was used to compare the frequencies of positive samples.

RESULTS

IL-4 PCR specificity

RT-PCR of PHA-stimulated PBMC RNA yielded PCR products of 339 and 291 bp, which are consistent with being derived from IL-4 and IL-4δ2 mRNA, respectively. Restriction enzyme analysis was conducted on PAGE-purified and reamplified PCR products (data not shown). As predicted, putative IL-4δ2 PCR products were cleaved by *Pst*I (exon 3) and *Pvu*II (exon 3) but not by *Bpu*AI (exon 1-2 splice junction) or *Hind*III (exon 2). IL-4 PCR products were cleaved by all 4 restriction enzymes, producing fragments of the expected sizes.

Dual-mRNA species-competitive PCR

Titration of IL-4 competitor against a constant aliquot of cDNA in a changing ratio experiment showed that the ratios closely followed the predicted line (Fig 1), indicating that both IL-4 and IL-4δ2 products and the IL-4 competitor compete equally in the PCR. Thus dual-mRNA species-competitive PCR allows both IL-4 and IL-4δ2 PCR products to be quantitated from 1 reaction.

IL-4 and IL-4δ2 in nasal polyps

IL-4-competitive RT-PCR was conducted on 4 nasal polyp samples in preliminary experiments conducted to optimize the assay system (Fig 2). Two samples expressed only IL-4, but one expressed both IL-4 and IL-4δ2, whereas the other was negative for both types of mRNA.

IL-4 and IL-4δ2 in airway biopsy specimens

No difference was seen in endobronchial biopsy specimens for IL-4 mRNA expression (Fig 3) between control subjects (median, 2.8×10^2 copies/μg RNA; range, $0-3.7 \times 10^3$ copies/μg RNA) and asthmatic subjects (median, 1.4×10^2 copies/μg RNA; range, $0-4.7 \times 10^2$ copies/μg RNA).

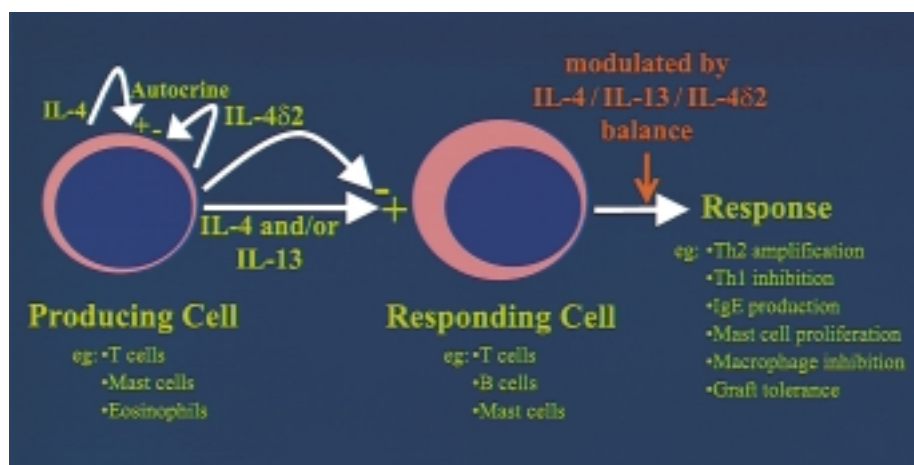


FIG 4. Potential role of IL-4δ2.

copies/ μ g RNA). However, significantly more asthmatic than control subjects expressed IL-4δ2 ($P = .036$). The single control subject expressing IL-4δ2 was negative for IL-4 mRNA. The detection of IL-4 or IL-4δ2 was not obviously affected by age, steroid use, or atopic status.

DISCUSSION

In both nasal polyps and airway biopsy specimens we found that IL-4 and IL-4δ2 were variably expressed, with IL-4δ2 levels usually somewhat lower than those for IL-4. If we had used an assay that did not distinguish between IL-4 and IL-4δ2 for these samples, we would have concluded incorrectly that asthmatic subjects expressed more IL-4 compared with control subjects. Given that IL-4δ2 is an IL-4 receptor antagonist,^{6,7} these results indicate that it is crucial to be able to distinguish IL-4δ2 from IL-4 when assessing *IL4* gene expression.

In the absence of validated protein detection methods to discriminate between IL-4 variants, it is not possible to determine whether the transcribed mRNA is actually translated to protein. IL-4 immunoreactivity has been found in storage granules of eosinophils and mast cells.¹¹ If IL-4δ2 is indeed an *in vivo* physiologic antagonist of IL-4 activity, then conceivably, IL-4δ2 could be coexpressed with IL-4 and stored to rapidly moderate an IL-4-mediated response (Fig 4).

There is preliminary evidence that IL-4δ2 has differing affinities compared with IL-4 for at least 2 receptors.^{6,7} Thus IL-4δ2 may be expressed as a differential receptor antagonist to channel IL-4 from binding to the high-affinity IL-4 receptor to binding to the low-affinity receptor. Given the coexpression of IL-4 and IL-13 in T cells¹² and the sharing of receptors and/or receptor chains between IL-4 and IL-13,¹³ IL-4δ2 expression might also function to direct IL-13 from one receptor to another.

Increased expression of IL-4 and IL-5 in asthmatic airway biopsy specimens and lavage fluid has been reported.¹⁴⁻²⁰ These studies have used varying combinations of

immunochemistry, *in situ* hybridization, and semiquantitative PCR methods, but all the methods were applied in the traditional manner and would have failed to differentiate between the 2 IL-4 mRNA species. Although molecular modeling of IL-4δ2 structure indicates some changes in folding compared with IL-4, most mAbs are likely to detect both IL-4 and IL-4δ2.²¹ Our results indicate that both IL-4 and IL-4δ2 are commonly expressed in airway samples, and therefore previous studies would have reported additive results.

We have demonstrated that dual-mRNA species-competitive PCR is a valid quantitative method for assaying IL-4 and IL-4δ2 in airway biopsy specimens. This method is analogous to that previously reported for HIV viral load quantitation in which multiple competitors, differing by size, are added in known amounts to patient samples, allowing categorical reporting of HIV copy number.²² We believe this to be the first report of a quantitative assay with more than 2 simultaneously competing PCR products, as well as the first description of the IL-4δ2 variant in human respiratory tract tissue.

Perhaps the strongest evidence for IL-4 being important in the asthmatic lung comes from studies of allergen challenge in atopic patients.²³⁻²⁷ Although these studies have the same detection problems described above, they have shown upregulation of total *IL4* gene transcription at 18 to 24 hours after challenge compared with that found immediately after challenge or compared with vehicle only-challenged sites. However, not all lung allergen challenge studies have found increased expression of IL-4.^{28,29} We did not find IL-4 mRNA increased in a group of stable nonchallenged asthmatic subjects, but they were more likely to express IL-4δ2 mRNA compared with normal control subjects. Although numbers were small, there was no suggestion of trends for IL-4δ2 to vary with steroid use or atopy.

IL-4 has been correlated with asthma symptoms³⁰ and is possibly more important in subjects with acute asthma than in those with stable asthma. Increased IL-4δ2 in our relatively stable asthmatic subjects may reflect a resolv-

ing IL-4 response or active asthma being kept under critical control (Fig 4). These hypotheses and speculations obviously need further detailed work, but the potential importance of an IL-4/IL-482 balance modulating asthmatic inflammation is considerable.

We thank Ros Bish, RN; Dr Frank Thien; and Dr Xun Li for their clinical assistance.

REFERENCES

1. Borish L, Rosenwasser LJ. Update on cytokines. *J Allergy Clin Immunol* 1996;97:719-34.
2. Arai N, Nomura D, Villaret D, et al. Complete nucleotide sequence of the chromosomal gene for human IL-4 and its expression. *J Immunol* 1989;142:274-82.
3. Sorg RV, Enczmann J, Sorg UR, Schneider EM, Wernet P. Identification of an alternatively spliced transcript of human interleukin-4 lacking the sequence encoded by exon 2. *Exp Hematol* 1993;21:560-3.
4. Alms WJ, Atamas SP, Yurovsky VV, White B. Generation of a variant of human interleukin-4 by alternative splicing. *Mol Immunol* 1996;33:361-70.
5. Klein SC, Golverdingen JG, van Wichen DF, et al. Expression of two interleukin 4 mRNA isoforms in B lymphoid cells. *Cell Immunol* 1996;167:259-68.
6. Atamas SP, Choi J, Yurovsky VV, White B. An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL-4-stimulated T cell proliferation. *J Immunol* 1996;156:435-41.
7. Arinobu Y, Atamas SP, Otsuka T, et al. Antagonistic effects of an alternative splice variant of human IL-4, IL-482, on IL-4 activities in human monocytes and B cells. *Cell Immunol* 1999;191:161-7.
8. Tsytisikov VN, Yurovsky VV, Atamas SP, Alms WJ, White B. Identification and characterization of two alternative splice variants of human interleukin-2. *J Biol Chem* 1996;271:23055-60.
9. Ausubel FA, Brent R, Kingston RE, et al. *Current Protocols in Molecular Biology*. New York: John Wiley & Sons; 1999.
10. Beach JR, Young CL, Avery AJ, et al. Measurement of airway responsiveness to methacholine: relative importance of the precision of drug delivery and the method of assessing response. *Thorax* 1993;48:239-43.
11. Moqbel R, Ying S, Barkans J, et al. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. *J Immunol* 1995;155:4939-47.
12. Dolganov G, Bort S, Lovett M, et al. Coexpression of the interleukin-13 and interleukin-4 genes correlates with their physical linkage in the cytokine gene cluster on human chromosome 5q23-31. *Blood* 1996;87:3316-26.
13. de Vries JE. The role of IL-13 and its receptor in allergy and inflammatory responses. *J Allergy Clin Immunol* 1998;102:165-9.
14. Humbert M, Durham SR, Ying S, et al. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 1996;154:1497-504.
15. Robinson DS, Hamid Q, Ying S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
16. Ying S, Durham SR, Corrigan CJ, Hamid Q, Kay AB. Phenotype of cells expressing mRNA for TH2-type (interleukin 4 and interleukin 5) and TH1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am J Respir Cell Mol Biol* 1995;12:477-87.
17. Gelder CM, Thomas PS, Yates DH, Adcock IM, Morrison JF, Barnes PJ. Cytokine expression in normal, atopic, and asthmatic subjects using the combination of sputum induction and the polymerase chain reaction. *Thorax* 1995;50:1033-7.
18. Marini M, Avoni E, Hollemborg J, Mattoli S. Cytokine mRNA profile and cell activation in bronchoalveolar lavage fluid from nonatopic patients with symptomatic asthma. *Chest* 1992;102:661-9.
19. Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow JC Jr. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992;146:109-15.
20. Bradding P, Roberts JA, Britten KM, et al. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994;10:471-80.
21. Zav'yalov VP, Denesyuk AI, White B, Yurovsky VV, Atamas SP, Korpela T. Molecular model of an alternative splice variant of human IL-4, IL-4 delta 2, a naturally occurring inhibitor of IL-4-stimulated T cell proliferation. *Immunol Lett* 1997;58:149-52.
22. Vener T, Nygren M, Andersson A, Uhlen M, Albert J, Lundeberg J. Use of multiple competitors for quantification of human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol* 1998;36:1864-70.
23. Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR. Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993;92:313-24.
24. Kelly EA, Rodriguez RR, Busse WW, Jarjour NN. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. *Am J Respir Crit Care Med* 1997;156:1421-8.
25. Durham SR, Ying S, Varney VA, et al. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. *J Immunol* 1992;148:2390-4.
26. Ying S, Durham SR, Jacobson MR, et al. T lymphocytes and mast cells express messenger RNA for interleukin-4 in the nasal mucosa in allergen-induced rhinitis. *Immunology* 1994;82:200-6.
27. Barata LT, Ying S, Meng Q, et al. IL-4- and IL-5-positive T lymphocytes, eosinophils, and mast cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Allergy Clin Immunol* 1998;101:222-30.
28. Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 1993;8:35-42.
29. Virchow JC Jr, Walker C, Hafner D, et al. T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am J Respir Crit Care Med* 1995;151:960-8.
30. Robinson DS, Ying S, Bentley AM, et al. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J Allergy Clin Immunol* 1993;92:397-403.