

Increased expression of IL-16 immunoreactivity in bronchial mucosa after segmental allergen challenge in patients with asthma

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Background: We have previously shown increased expression of the CD4⁺ cell chemoattractant IL-16 in bronchial mucosa of patients with asthma. We investigated the effects of allergen challenge on airway IL-16 expression.

Methods: We investigated the expression of IL-16 immunoreactivity in bronchial biopsy samples obtained from atopic asthmatic subjects (n = 19) and normal subjects (n = 6) 24 hours after segmental allergen challenge. Control biopsy samples were obtained either at baseline or after diluent challenge. IL-16 expression was correlated to numbers of CD4⁺ cells, CD25⁺ cells, and activated eosinophils. IL-16 bioactivity was assessed in bronchoalveolar fluid obtained from patients with asthma. **Results:** IL-16 expression was higher in control biopsy specimens obtained from subjects with asthma compared with normal subjects ($P < .05$). In patients with asthma, numbers of IL-16 immunoreactive cells were significantly higher in biopsy specimens obtained after allergen challenge compared with control biopsy specimens ($P < .001$). Allergen provocation was associated with release of IL-16 in bronchoalveolar fluid in patients with asthma. In normal subjects, there was no difference in the number of IL-16-immunoreactive cells in biopsy specimens obtained after allergen challenge compared with biopsy specimens obtained after diluent challenge. Allergen challenge was associated with an increase in the numbers of EG2⁺ eosinophils in patients with asthma but not in normal subjects. IL-16 expression correlated with the numbers of CD4⁺ cells and CD25⁺ cells after allergen challenge in asth-

matic subjects with a provocative concentration required to decrease the FEV₁ by 20% of its baseline value ($PC_{20}FEV_1$) < 4 mg/mL. IL-16-immunoreactive cells were identified mainly as T cells and eosinophils in asthmatic subjects after allergen challenge.

Conclusion: Endobronchial allergen provocation in atopic asthmatic patients resulted in increased airway expression of IL-16 and release of bioactive IL-16 in airways. IL-16 may contribute to the immunoregulation of the inflammatory infiltrate in the airways in response to antigen. (*J Allergy Clin Immunol* 2000;106:293-301.)

Key words: Asthma, IL-16, segmental challenge, lymphocyte, chemotaxis

Bronchial asthma is a chronic inflammatory disease of the airways that is characterized by tissue accumulation of activated CD4⁺ T lymphocytes and eosinophils.^{1,2} CD4⁺ T cells play an essential role in asthma both as part of the sensitization phase to antigen, providing help to B cells for IgE synthesis, and as part of the inflammatory cascade through the release of T_H2-type cytokines, which are critical for IgE synthesis and accumulation of eosinophils in inflamed airways.³ The role of CD4⁺ T cells in regulating allergic airway responses has been supported by numerous animal and human studies.^{1,4-7} Hence, adoptive transfer of antigen-primed specific CD4⁺ T cells in unsensitized animals results in antigen-induced bronchoconstriction, airway hyperresponsiveness, and airway eosinophilia in rats,⁸⁻¹⁰ whereas in vivo depletion of murine CD4⁺ T cells prevents the development of antigen-induced airway eosinophilia and development of airway hyperresponsiveness.^{11,12} Improved lung function is observed in patients with severe asthma after experimental therapy with a chimeric mAb to CD4.¹³ Because of the regulatory role of CD4⁺ T cells in the pathogenesis of atopic asthma, the study of the mechanisms responsible for the recruitment of these cells in the bronchial mucosa is of primordial importance. Tissue accumulation of T cells is a highly regulated process that likely depends on the selective expression of adhesion molecules, local release of specific chemotactic and growth factors, and suppression of cell surface receptors responsible for apoptosis.¹⁴

IL-16 has biological properties that are pertinent to the development of allergic airway inflammation. The bio-

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Abbreviations used

APAAP:	Alkaline phosphatase–anti-alkaline phosphatase
BAL:	Bronchoalveolar lavage
EG2 ⁺ :	Eosinophil cationic protein
MBP:	Major basic protein
PC ₂₀ FEV ₁ :	Provocative concentration required to decrease FEV ₁ by 20% of baseline value
RAST:	Radioallergosorbent [assay]

logically active IL-16 has chemotactic activities for CD4⁺ cells, including CD4⁺ T cells and CD4-bearing eosinophils, with a median effective dose of recombinant protein in the range of 10⁻¹¹ M range.¹⁵⁻¹⁷ In addition to activation of cell migration, IL-16 induces functional high-affinity IL-2 receptors on CD4⁺ T cells and provides a synergistic signal for IL-2-induced proliferation of CD4⁺ T cells.^{18,19} These observations raise the hypothesis that IL-16 may play an important role in airway inflammation in subjects with allergic asthma. This hypothesis is supported by our previous findings that IL-16 expression is increased in bronchial biopsy specimens from atopic asthmatic subjects compared with normal control subjects.²⁰ Consistent with the view that IL-16 may, at least in part, be implicated in the recruitment of CD4⁺ cells in asthmatic airways, airway expression of IL-16 correlates with CD4⁺ cell infiltration. The response to antigen is an important factor in the development of airway inflammation. This study was conducted to further investigate the role of IL-16 in the development of allergen-driven airway inflammation. We investigated the effects of segmental allergen challenge on airway IL-16 expression in atopic asthmatic patients and normal control subjects and related IL-16 expression to the accumulation of inflammatory cells. In addition, cellular sources of IL-16 in response to allergen were determined.

METHODS**Subjects**

The study was approved by the Ethics Committee of The Royal Brompton and Harefield Hospitals NHS Trust, London, United Kingdom. The clinical characteristics of the study subjects are shown in Table I. Eighteen subjects with a clinical history of house dust mite-sensitive or grass pollen-sensitive asthma and 6 nonatopic normal control subjects took part in the study. Informed written consent was obtained from all subjects. All allergic asthmatic subjects had a clear clinical history of bronchospasm after allergen exposure. All asthmatic subjects were studied while in stable condition. Baseline FEV₁ was greater than 80% of predicted value. All asthmatic subjects showed increased bronchial reactivity to inhaled methacholine (ie, provocative concentration required to decrease the FEV₁ by 20% of its baseline value [PC₂₀FEV₁] < 16 mg/mL) or a reversibility of the airway obstruction of 12% of FEV₁ with bronchodilator. Their atopic status was also confirmed by a strong positive result on skin prick testing (>5-mm skin wheal) to either *Dermatophagoides pteronyssinus* or *Phleum pratense* (timothy grass) pollen extracts (Soluprick; ALK, Horsholm, Denmark), as well as positive results on radioallergosorbent (RAST) assay for the same antigen extracts (CAP system; Pharmacia Diagnostics,

Uppsala, Sweden). Inhaled glucocorticoid therapy was withheld for at least 2 weeks before bronchoscopy, and none of the subjects had received oral glucocorticoids for at least 6 months before the study or immunotherapy within the previous 5 years. Smokers or ex-smokers who had smoked more than 5 pack-years were excluded. The nonatopic healthy control subjects had a lifelong absence of any symptoms indicative of allergic disease, had normal bronchial reactivity to histamine (PC₂₀FEV₁ > 32 mg/mL), and had negative results to skin prick testing and RAST testing to common aeroallergens including *D pteronyssinus* and *P pratense*.

Bronchoscopy and endobronchial challenge

Study design. All volunteers taking part in this study (n = 18 atopic asthmatic patients and n = 6 nonatopic nonasthmatic control subjects) underwent segmental allergen challenge with endobronchial biopsy sampling from the challenged site 24 hours later. Control biopsies were performed either at baseline (n = 14 atopic asthmatic patients), or 24 hours after diluent challenge (n = 4 atopic asthmatic patients and n = 6 nonatopic nonasthmatic control subjects). Subjects were asked to undergo 2 fiberoptic bronchoscopies separated by 24 hours as previously described.²¹ All subjects were premedicated with nebulized albuterol (2.5 mg) and intravenous atropine (0.6 mg) and midazolam (5 to 10 mg) 10 minutes before the procedure. Oxygen was delivered throughout the procedure via nasal cannulas, and oxygen saturation was monitored by pulse oxymetry. Topical anesthesia of the upper airways was achieved with 2% to 4% lidocaine solution. For subjects who underwent dual diluent/allergen challenge, the bronchoscope (Olympus BFP20; Olympus Corp, London, UK) was wedged in a lateral subsegment of the right middle lobe to undertake a control challenge with 5 mL of sterile saline solution prewarmed to 37°C. The bronchoscope was then passed into the lateral subsegment of the left lingula and the allergen challenge was performed by instilling 5 mL of prewarmed saline solution containing 100 BU of either *D pteronyssinus* or *P pratense* (Aquagen, ALK; Abello, Denmark). The allergen challenge site was observed for 5 minutes, and in the absence of excessive local bronchoconstriction, an additional 400 BU of allergen was introduced in 5 mL of saline solution. The subjects were observed closely and peak flow and FEV₁ were recorded hourly. A second bronchoscopy was performed with the same premedications at 24 hours after challenge, and 2 bronchial biopsy specimens were taken from each challenge site. For the remaining subjects (n = 14), bronchoalveolar lavage (BAL) was first performed at baseline by instilling two 60-mL aliquots of sterile warmed saline solution and 2 baseline endobronchial biopsy specimens were taken from the carinae of the left lingular lobe or the right middle lobe. Allergen challenge was then performed in the right middle lobe or the lingula of the left lung, the opposite side from which baseline BAL and biopsy specimens were taken. A second bronchoscopy was performed 24 hours later, at which time a second BAL of the allergen-challenged segment was performed and 2 endobronchial biopsy specimens were taken from the same segments.

Bronchial biopsy preparation

Endobronchial biopsies were fixed in a 4% paraformaldehyde/PBS solution for 2 hours, washed in 15% sucrose/PBS 3 times, embedded in OCT compound (Tissue-Tek, Miles Inc, Elkhart, Ind), and snap-frozen in isopentane cooled in liquid nitrogen. Cryostat sections 10-μm thick were cut on 0.1% poly-L-lysine-coated slides, air-dried overnight at 37°C, and stored at -80°C.

Processing of BAL fluid

BAL fluid was passed through 2 layers of sterile gauze to remove debris and then centrifuged at 500g for 10 minutes at 4°C. The cells were separated from the supernatant and the supernatant was kept at -80°C. Supernatants were assessed for lymphocyte chemotaxis as described below.

Immunocytochemistry

We used a variety of monoclonal antibodies including an anti-human IL-16 (clone 14.1 raised against human rIL-16 molecule, AGMED Inc; Bedford, Mass), an anti-human CD4 (Dako Canada Inc; Mississauga, Ontario, Canada), anti-CD25 (Becton-Dickinson; Cowley, Oxford, UK), and anti-EG2 (cleaved form of eosinophil cationic protein, a marker of activated eosinophils; Pharmacia Diagnostics; Uppsala, Sweden). We used the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method as previously described.²⁰ Briefly, cryostat sections were first incubated with the primary antibodies at the appropriate dilution at 4°C overnight. Sections were then incubated with the unconjugated rabbit anti-mouse Ig secondary antibody followed by the APAAP complex. The reaction was visualized with Fast Red alkaline phosphatase substrate. In all immunocytochemical studies, the negative controls included omission of the primary antibody or the use of irrelevant isotype and species-matched primary antibodies.

Identification of phenotype of IL-16+ cells by double immunocytochemistry

To examine the phenotype of IL-16-immunoreactive cells, cryostat sections of bronchial biopsy specimens were studied using the technique of double immunocytochemistry as previously described.²² Briefly, endogenous peroxidase activity was blocked using 1% H₂O₂ in Tris-buffered saline solution for 30 minutes. A mixture of primary antibodies was applied to the sections consisting of the rabbit anti-human IL-16 polyclonal antibody (AGMED Inc) used to detect IL-16 immunoreactivity, and the appropriate mAbs to determine cellular phenotype: anti-CD3 (Becton-Dickinson; Mississauga, Ontario, Canada), anti-eosinophil major basic protein (MBP) (gift from Dr. R. Moqbel, University of Alberta, Edmonton, Canada), anti-CD68 (Dako Canada Inc), and anti-human mast cell tryptase (Chemicon International Inc, Mississauga, Temecula, Calif). After incubating with the appropriate secondary antibodies, a tertiary layer of streptavidin-peroxidase and murine APAAP conjugate was then applied. Sections were developed sequentially in Fast Red (the APAAP substrate) and diaminobenzidine (a peroxidase substrate). IL-16-immunoreactive cells stained brown and cells immunoreactive for CD3, CD68, MBP, or tryptase stained red. Double immunoreactive cells stained red-brown.

Lymphocyte chemotaxis assay

The presence of bioactive IL-16 in BAL fluid samples obtained from asthmatic subjects before and after challenge was assessed by chemotaxis assay. Human lymphocytes were isolated from heparinized venous blood samples of healthy normal volunteers by density centrifugation on Ficoll-Paque (Pharmacia; Uppsala, Sweden). Preparations were enriched for T lymphocytes by nylon wool adherence. The nonadherent mononuclear cells were >97% T cells as determined by fluorescent staining with anti-CD3 mAb (Becton-Dickinson, Mountain View, Calif). Migration of lymphocytes was assessed by a modification of a Boyden chemotaxis chamber technique as previously described.²³ Human T cells (10 × 10⁷/mL) were resuspended in Medium 199 enriched with 0.4% BSA and loaded into the upper wells of the chamber; 32 µL of cell-free unconcentrat-

TABLE I. Clinical characteristics of study population

Characteristic	Atopic asthmatic subjects	Normal control subjects
No.	18	6
Age (y)*	31.1 ± 7.5	22.4 ± 2.6
Sex (M/F)	8/10	6/0
Baseline FEV ₁ (L)*	3.68 ± 0.84	4.43 ± 0.51
PC ₂₀ FEV ₁ (mg/mL)*	3.59 ± 3.61	>32
Total IgE (IU/mL)*	319.1 ± 270.1†	85.0 ± 114.5
RAST to <i>Der p</i> or G	Positive	Negative
Skin prick tests to <i>Der p</i> or G	Positive	Negative
Inhaled steroids (yes/no)	6/12	0/6

Der p, *Dermatophagoides pteronyssinus*; G, Timothy grass pollen.

*Data presented as mean ± SD.

†P = .01 compared with normal control subjects.

ed BAL fluid or control buffer was placed in the lower wells. The upper and lower wells were separated by 8-µm micropore nitrocellulose filters (Neuroprobe, Cabin John, Md). Chambers were incubated for 2 hours, after which the filters were fixed in ethanol, stained with hematoxylin, and dehydrated. Cell migration was quantitated by light microscopy by counting the numbers of cells that had migrated beyond the depth of 50 µm. All migration data are expressed as percentage values of cell migration in control buffer (designated as 100%). A value >130% represents significantly different migration from control migration. All samples were tested in duplicate, and 4 high-power fields were examined in each duplicate. Data are expressed as mean values ± SD of all samples tested. To assess specificity for IL-16, neutralizing experiments were conducted incubating BAL fluid samples for 15 minutes with neutralizing concentrations of affinity-purified rabbit anti-IL-16 antisera (5 µg/mL, which neutralizes the chemotactic activity of 50 ng/mL recombinant human IL-16) before the addition of T cells in upper compartments.

Counting and data analysis

IL-16-immunoreactive cells in the epithelium were counted blindly by optical analysis and expressed as a semiquantitative score from 0 to 4 based on the percentage of the epithelium showing positive signal/total epithelium (0, no staining; 0.5, <12.5%; 1, 12.5% to 25%; 1.5, 25% to 37.5%; 2, 37.5% to 50%; 2.5, 50% to 62.5%; 3, 62.5% to 75%; 3.5, 75% to 87.5%; 4, 87.5% to 100%) as previously published.²⁰ In the subepithelial tissue, IL-16-immunoreactive cells, as well as CD4⁺ cells, CD25⁺ cells, and EG2⁺ cells, were counted with an eyepiece graticule (0.202 mm²) at ×200 magnification²⁰ and cell counts were presented as the numbers of cells per square millimeter. The numbers of cells that were positive for both IL-16 and cell markers (CD3, MBP, CD68, tryptase) were counted together with the number of cells individually staining for IL-16. Results are presented as the mean percentage of IL-16-immunoreactive cells that coexpressed immunoreactivity for CD3, MBP, CD68, or tryptase. The within-observer coefficient of variation for repeated measures was less than 5%.

Data were analyzed using a statistical package (Systat, version 5.1; Systat Inc, Evanston, Ill). The Mann-Whitney U test was used to compare data between the control and asthmatic groups, and the Wilcoxon matched-pairs signed rank test was applied for within-group comparisons. Significance was accepted at the 5% level of confidence. Within the atopic asthmatic group, there was no difference between the epithelial IL-16 immunoreactivity score in baseline biopsies (0.58 ± 0.19) and biopsies obtained from the diluent-challenged sites (0.56 ± 0.12, P = .63); similarly, epithelial IL-16 immunoreactivity score at 24 hours after allergen challenge did not differ in biopsy specimens taken from subjects who underwent

either single allergen challenge (1.4 ± 0.76) or dual saline solution/allergen challenge (1.50 ± 0.81 , $P = .89$). In addition, there was no difference in the numbers of subepithelial IL-16-immunoreactive cells in biopsy specimens from controls taken either at baseline (23.58 ± 19.28 cells/mm²) or after diluent challenge (18.50 ± 14.58 cells/mm², $P = .85$) in asthmatic patients. Therefore IL-16 immunoreactivity data in asthmatic patients have been grouped for further analysis regardless of the allergen provocation protocol. Correlations were done using the Pearson correlation coefficient.

RESULTS

IL-16 immunoreactivity

At baseline, IL-16-immunoreactive cells were identified both in the epithelial and subepithelial areas. In normal subjects, the epithelial IL-16 immunoreactivity scores were low and score values did not differ in biopsy specimens obtained from the diluent-challenged and the allergen-challenged sites (Fig 1). Within the atopic asthmatic group, the epithelial IL-16 immunoreactivity scores in biopsy specimens obtained 24 hours after allergen challenge were significantly higher compared with scores observed in control biopsy specimens obtained either at baseline or from the diluent-challenged sites ($P < .001$) (Fig 2).

Within the subepithelial area, the numbers of IL-16-immunoreactive cells in control biopsy specimens obtained either at baseline or from the diluent-challenged sites were significantly higher in asthmatic subjects compared with normal control subjects ($P = .03$) (Fig 3). In normal control subjects, there was no significant difference in the numbers of IL-16-immunoreactive cells in biopsy specimens obtained from the diluent sites compared with the allergen sites. Within the asthmatic group, the numbers of subepithelial IL-16-immunoreactive cells in biopsy specimens obtained 24 hours after allergen challenge were significantly higher than those from control biopsy specimens obtained either at baseline or from the diluent-challenged sites ($P < .001$).

Effects of allergen provocation on airway IL-16 expression were also assessed in asthmatic subjects according to their PC₂₀FEV₁ values. Subjects were divided into asthmatic subjects with a PC₂₀FEV₁ value < 4 mg/mL and asymptomatic seasonal asthmatic subjects with a PC₂₀FEV₁ value ≥ 4 mg/mL. Significant increases in the numbers of IL-16-immunoreactive cells after antigen challenge were observed in all asthmatic subjects regardless of their PC₂₀FEV₁ values and time of challenge (PC₂₀FEV₁ < 4 mg/mL, $P = .002$; PC₂₀FEV₁ ≥ 4 mg/mL, $P = .01$).

Phenotype of IL-16-immunoreactive cells

To determine whether IL-16 immunoreactivity was localized to T cells, eosinophils, macrophages, and mast cells, double immunocytochemistry was performed in bronchial biopsy samples obtained from 5 asthmatic individuals 24 hours after allergen challenge. Colocalization studies demonstrated that the majority of IL-16-immunoreactive cells were CD3⁺ T cells and MBP⁺ eosinophils (Table II). IL-16 immunoreactivity also

coexpressed, to a lesser extent, with CD68 (macrophages) and tryptase (mast cells).

Correlation between IL-16 expression and numbers of EG2⁺ cells, CD4⁺ cells, and CD25⁺ cells

Within the asthmatic group, the numbers of EG2⁺ eosinophils were similar in control biopsy specimens taken either at baseline (26.6 ± 24.7 cells/mm²) or from the diluent-challenged sites (38.6 ± 23.1 cells/mm²). In atopic asthmatic subjects, the number of EG2⁺ cells in the bronchial mucosa was significantly higher in biopsy specimens from the allergen-challenged sites compared with the control sites ($P < .01$) (Table III). In normal control subjects, the numbers of EG2⁺ cells did not differ in bronchial biopsy specimens obtained from the diluent-challenged and the allergen-challenged sites. Within the asthmatic group, allergen challenge was not associated with significant change in the numbers of CD4⁺ cells. In normal control subjects, the numbers of CD4⁺ cells did not differ in biopsy specimens obtained from the diluent-challenged and the allergen-challenged sites. Both in normal subjects and in patients with atopic asthma, allergen challenge was not associated with significant changes in the numbers of CD25⁺ cells in the bronchial mucosa.

In biopsy specimens from the asthmatic group taken as a whole, no correlation was found between the numbers of IL-16-immunoreactive cells and the numbers of EG2⁺ eosinophils, CD4⁺ cells, or CD25⁺ cells 24 hours after allergen challenge. In asthmatic subjects with PC₂₀FEV₁ < 4 mg/mL, the numbers of subepithelial IL-16-immunoreactive cells correlated with the numbers of CD4⁺ cells ($r = 0.77$, $P = .008$) and CD25⁺ cells ($r = 0.77$, $P = .01$).

Chemotactic activity of BAL fluid

No T-cell chemoattractant activity was detected in BAL fluid samples obtained at baseline from subjects with asthma with the exception of 2 subjects. Significant T-cell chemoattractant activity was detectable in all BAL fluid samples obtained 24 hours after allergen provocation. Table IV shows the migration of T cells induced by unconcentrated BAL fluid samples obtained from asthmatic subjects at baseline and 24 hours after allergen provocation. T-cell migration induced by BAL fluid obtained after allergen challenge was significantly higher than that induced by BAL fluid obtained before allergen challenge ($P < .01$). Neutralization of IL-16 in the BAL fluid caused a significant decrease in T-cell migration ($P < .05$).

DISCUSSION

The interaction of IL-16 and CD4⁺ cells may be of particular importance to asthma because IL-16 has the ability to selectively attract CD4⁺ cells in vitro through interaction with CD4. In this study, we used the tech-

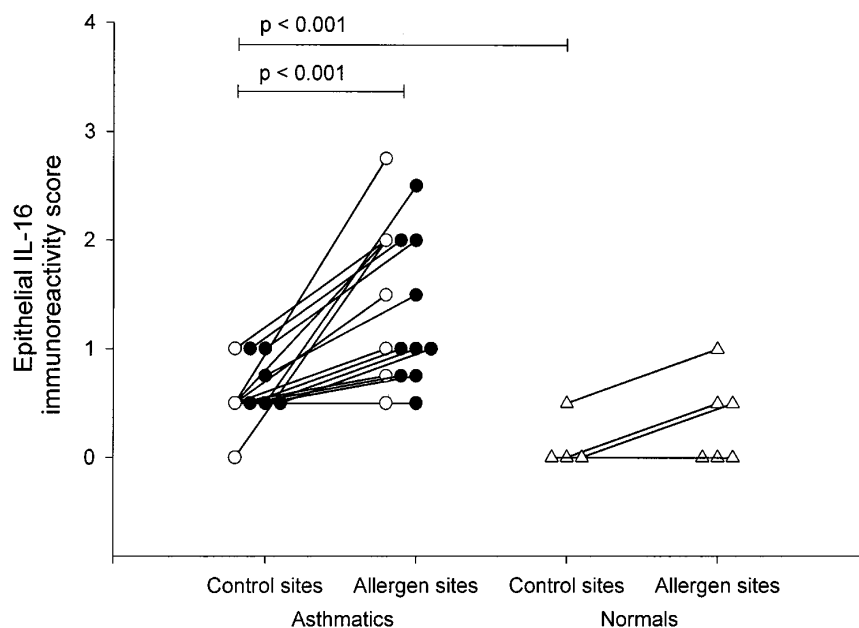


FIG 1. Effects of allergen challenge on epithelial IL-16 immunoreactivity score in normal and asthmatic subjects. Asthmatic subjects were identified as subjects with either a PC₂₀FEV₁ value < 4 mg/mL (closed circles) or PC₂₀FEV₁ ≥ 4 mg/mL (open circles). Results are expressed as a percentage of positive cells/total cells (score 0 to 4, see Methods).

nique of segmental allergen bronchoprovocation to investigate the role of IL-16 in the development of allergic inflammation. Our data show that specific allergen challenge was associated with an influx of inflammatory cells, particularly eosinophils. Increases in the numbers of both airway epithelial and nonepithelial IL-16-immunoreactive cells in the bronchial mucosa were observed in atopic asthmatic subjects but not in normal control subjects. Colocalization studies revealed that the predominant IL-16-immunoreactive cells in the airways after allergen challenge were epithelial cells, T cells, and eosinophils. Increased airway expression of IL-16 in response to allergen provocation was also associated with release of bioactive IL-16 in the airways of subjects with asthma.

A number of the functional characteristics of IL-16 led us to study its contribution to allergen-driven airway inflammation. IL-16 is secreted by airway epithelial cells after stimulation with histamine, a mast cell-derived mediator released after IgE cross-linking.²⁴ IL-16 induces the migration of CD4⁺ cells in vitro, including CD4⁺ T cells and CD4-bearing eosinophils, dependent on cell surface expression of CD4.^{16,17} In addition, the ability of IL-16 to activate CD4⁺ T cells to express IL-2R and MHC class II molecules in vitro is in concert with the activation state of CD4⁺ T cells infiltrating the bronchial mucosa in asthma. Consistent with a potential role of IL-16 in allergic inflammation, we have previously shown that allergen challenge induced upregulation of epithelial and subepithelial IL-16 expression in the nasal mucosa of subjects with atopic rhinitis.²⁵ Furthermore,

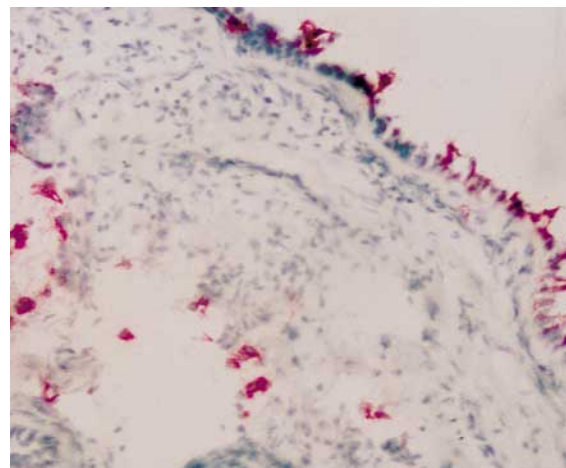


FIG 2. IL-16 immunoreactivity in a bronchial biopsy specimen obtained 24 hours after allergen provocation in an asthmatic subject. IL-16-immunoreactive cells were localized both in the epithelium and in the subepithelial layer.

bioactive IL-16 was identified in BAL fluid obtained 6 hours after segmental allergen challenge in atopic asthmatic subjects but not in normal control subjects or atopic nonasthmatic subjects.²³ The current data extend these previous observations and further strengthen the association between IL-16 expression and development of atopic inflammation.

In agreement with prior studies,²⁰ we here demonstrated constitutive IL-16 expression in epithelial cells. The epithelial IL-16 immunoreactivity score in asthmatic

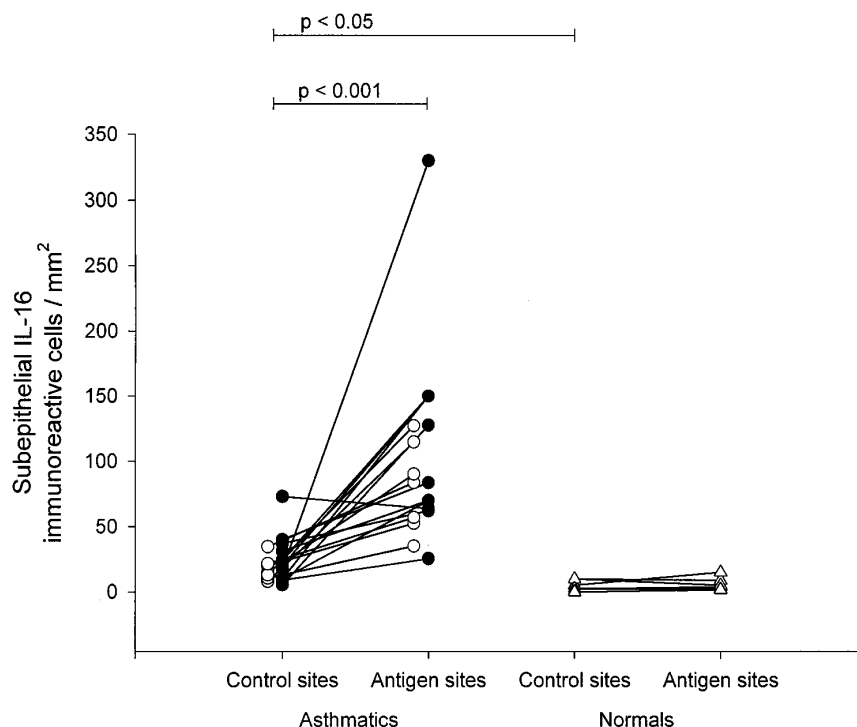


FIG 3. Effects of allergen challenge on subepithelial IL-16 immunoreactivity in normal and asthmatic subjects. Asthmatic subjects were identified as subjects with either a PC₂₀FEV₁ value < 4 mg/mL (*closed circles*) or PC₂₀FEV₁ ≥ 4 mg/mL (*open circles*). Results are expressed as the numbers of IL-16-immunoreactive cells per square millimeter.

subjects, although higher than that observed in normal control subjects, was somewhat lower compared with that observed in our initial study.²⁰ A likely explanation for this difference may be related to previous use of regular inhaled steroid therapy by some subjects recruited for the present protocol. In contrast, the subjects included in our initial study had newly diagnosed asthma or mild disease, which had not required previous use of anti-inflammatory drugs. Because bronchial epithelial cells are the first cell type to come in contact with inhaled glucocorticoids, the effects of these drugs on epithelial cytokine expression may be more profound. In this regard, we have previously shown that glucocorticoids inhibited allergen-induced epithelial IL-16 expression.²⁵ It is therefore possible that prolonged effects of inhaled steroids in some study subjects, although discontinued at least 2 weeks before the study, may have contributed to the relatively low expression of epithelial IL-16. Nevertheless, bronchoprovocation with allergen was associated with a significant increase in epithelial IL-16 expression similar to that observed in the nose.²⁵ Given that allergen exposure was associated with increased expression of IL-16 in epithelial cells, it is likely that allergen induced cytokine synthesis *per se* locally. As such, epithelial cells are not motile cells that could be recruited after allergen challenge. In addition, we are not aware of any data suggesting that bronchial epithelial cells may proliferate after allergen provocation. Previous data have also shown

that allergen-induced IL-16 expression occurs at both the messenger RNA and the protein levels in the nasal mucosa, suggesting local cytokine synthesis.²⁵ IL-16 gene expression has been shown to be upregulated in bronchial epithelial cell lines after stimulation with histamine, IL-1, or tumor necrosis factor- α .^{24,26} mediators that can be released in the allergic response.

Apart from the airway epithelial cells, IL-16 has been shown to be synthesized by a variety of cells including T cells, eosinophils, and mast cells.^{16,24,27,28} We recently reported that, in asthmatic subjects, CD3⁺ lymphocytes were the major nonepithelial cellular source of IL-16.²² Eosinophils and mast cells were also significant cellular sources of IL-16. Colocalization studies performed on biopsy specimens obtained at 24 hours after allergen challenge demonstrated that the majority of the nonepithelial IL-16-immunoreactive cells were T cells and eosinophils. The nature of this study precluded the identification of the cellular sources of IL-16 at different time points after challenge. However, it is plausible that epithelial cells and mast cells may represent an early source of IL-16 after challenge, whereas resident and newly recruited inflammatory cells such as T cells and eosinophils may contribute to perpetuate the production and release of IL-16 in airways. The regulation of IL-16 synthesis by different cell types awaited complete investigation. Increases in the numbers of subepithelial IL-16-immunoreactive cells after allergen exposure may be

attributed to either newly recruited inflammatory cells that express IL-16 or induction of IL-16 synthesis by resident inflammatory cells, or both. In that regard, we cannot exclude the possibility that increases in the numbers of infiltrating eosinophils may partly contribute to the increased expression of IL-16 observed after allergen provocation. Unfortunately, it is not possible to differentiate the production of IL-16 by resident inflammatory cells and newly recruited cells using the present protocol. Nevertheless, it is likely that both resident (epithelial or nonepithelial) and newly recruited cells contribute to the exaggerated amounts of IL-16 detected in asthmatic airways after allergen provocation.

The current data raised the question whether allergen-induced airway expression of IL-16 is a feature of atopy or asthma per se. To precisely answer this question, similar types of experiments would need to be performed in atopic nonasthmatic subjects. A surrogate for these experiments is to examine the effects of allergen provocation on airway IL-16 expression in asthmatic subjects with seasonal asthma studied when they are asymptomatic (outside season) with a PC₂₀FEV₁ value ≥ 4 mg/mL. Our data show that allergen provocation was associated with an increase in the numbers of IL-16-immunoreactive cells compared with baseline in subjects with PC₂₀FEV₁ ≥ 4 mg/mL as was observed in subjects with PC₂₀FEV₁ < 4 mg/mL. Thus allergen provocation was associated with upregulation of IL-16 expression regardless of the presence of asthma symptoms and airway hyperresponsiveness at the time of the study. This may indicate that allergen-induced airway expression of IL-16 is a feature of atopy.

In this study, IL-16 immunoreactivity was assessed using an mAb that was raised against the C-terminal peptide of the molecule and that recognizes both the precursor and the secreted bioactive form of IL-16. Unfortunately, there is no antibody available that recognizes only the smaller active secreted fragment of IL-16. Therefore we cannot distinguish which form of IL-16 is being induced to accumulate in tissues in our experiments. Regulation of pro-IL-16 is, however, a crucial regulatory step in IL-16 synthesis at least in bronchial epithelial cells, which express little or no constitutive IL-16 messenger RNA and protein. To investigate whether allergen challenge induced the release of bioactive IL-16 in the airways, BAL fluid samples were assessed for T-cell chemoattractant activity. Our data show that BAL fluid obtained from asthmatic subjects after allergen provocation has chemotactic activity for T cells, which could be partly attributable to IL-16. Thus increase in airway IL-16 immunoreactivity after allergen provocation was associated with release of bioactive IL-16 within the airways of subjects with asthma.

A potential criticism of our study may reside in the use of 2 different protocols for our study subjects. All normal control subjects underwent dual diluent/allergen challenge. Within the asthmatic group, 4 subjects underwent dual diluent/allergen challenge, whereas the remaining subjects underwent antigen challenge only associated

TABLE II. Phenotype of IL-16-immunoreactive cells in asthmatic subjects

Subject	IL-16-immunoreactive cells (%)			
	CD3+	MBP+	CD68+	Tryptase+
1	58.0	35.0	9.0	11.1
2	33.2	50.0	4.8	7.0
3	50.8	30.4	10.8	12.3
4	42.0	40.0	15.9	8.0
5	45.0	38.0	12.1	13.4
Mean $\pm$ SD	45.8 $\pm$ 9.3	38.7 $\pm$ 7.3	10.5 $\pm$ 4.1	10.4 $\pm$ 2.9

TABLE III. Numbers of EG2⁺ cells, CD4⁺ cells, and CD25⁺ cells in bronchial biopsy specimens

Study group	EG2 ⁺ cells (/mm ²)	CD4 ⁺ cells (/mm ²)	CD25 ⁺ cells (/mm ²)
Asthmatic subjects			
Control sites	30.6 $\pm$ 24.3*	140.5 $\pm$ 71.9	9.4 $\pm$ 7.3
Allergen sites	61.2 $\pm$ 32.1†	170.7 $\pm$ 87.6	14.4 $\pm$ 13.7
Normal subjects			
Control sites	8.2 $\pm$ 8.2	130.3 $\pm$ 91.4	3.5 $\pm$ 6.8
Allergen sites	12.6 $\pm$ 11.1	122.7 $\pm$ 55.7	3.7 $\pm$ 5.7

Values are expressed as mean $\pm$ SD.

* $P < .05$ compared with control sites in normal subjects.

† $P < .01$ compared with control sites in asthmatic subjects.

TABLE IV. T-cell migration in response to BAL fluid*

BAL fluid sample	Cell migration (% of control)
Baseline	103.8 $\pm$ 33.4
Baseline + anti-IL-16†	94.0 $\pm$ 23.3
Allergen	178.8 $\pm$ 28.6‡
Allergen + anti-IL-16†	130.7 $\pm$ 18.7§

*T-cell migration induced by unconcentrated BAL fluid samples obtained from asthmatic subjects ($n = 12$) at baseline and 24 hours after allergen provocation. Cell migration is expressed as percentage of control cell migration in presence of control buffer alone.

†BAL fluid samples were preincubated with neutralizing anti-IL-16 antibody.

‡ $P = .005$ compared with T-cell migration induced by BAL fluid samples obtained at baseline.

§ $P = .005$ compared with T-cell migration induced by BAL fluid samples obtained after allergen challenge.

with baseline biopsy specimens. The low expression of IL-16 in normal subjects after diluent challenge was in line with our previous data showing low constitutive IL-16 expression in normal subjects.²⁰ Furthermore, within the asthmatic group, the numbers of IL-16-immunoreactive cells identified in the diluent-challenged sites were in the same range as numbers at baseline. The magnitude of the observed effect of antigen challenge on the numbers of IL-16-immunoreactive cells did not differ when data were compared with control biopsy specimens taken either at baseline or from the diluent-challenged sites.

The functional significance of elevated expression of IL-16 in airways in response to antigen remains to be fully elucidated. IL-16 was originally identified as a CD4⁺ cell chemoattractant factor. However, its ability to induce CD4⁺

cell migration in vivo has not been established yet. We did find an association between the expression of IL-16 and the numbers of CD4⁺ cells in asthmatic subjects with PC₂₀FEV₁ < 4 mg/mL. This may suggest IL-16 has a direct chemotactic effect on CD4⁺ cells, or alternatively may reflect IL-16 production by T cells themselves. Furthermore, allergen challenge is associated by release of bioactive IL-16 in asthmatic airways after challenge as confirmed by the presence of T-cell chemotactic activity in BAL fluid, which is neutralized by anti-IL-16 antibody. This is in agreement with our previous studies showing a positive correlation between airway IL-16 expression and CD4⁺ cell infiltration both in subjects with ongoing asthma and in subjects during late nasal responses.^{20,25} However, we cannot draw any firm conclusions on the putative role of IL-16 as a chemoattractant for CD4⁺ cells in vivo. To directly implicate IL-16 in CD4⁺ cell recruitment within lower airways, experiments investigating the effects of IL-16 instillation in airways would need to be performed. However, because of ethical issues, these types of experiments are difficult to perform in human beings. Despite the parallel increase in the numbers of EG2⁺ eosinophils and IL-16-immunoreactive cells after antigen challenge in atopic asthmatic patients, no correlation was found between IL-16 expression and the numbers of infiltrating EG2⁺ eosinophils. Although IL-16 is chemotactic for CD4-bearing eosinophils in vitro, the in vivo significance of this finding is unclear because the expression of the CD4 surface molecule by tissue eosinophils remains to be confirmed.

Apart from its chemoattractant and activation properties, IL-16 more recently was found to exert certain inhibitory effects, including inhibition of one-way mixed lymphocyte reaction and induction of reversible functional anergy in CD4⁺ cells after T-cell receptor/CD3 costimulation.^{29,30} These latter findings suggest that IL-16 might have potential important immunoregulatory properties. Whether the anti-inflammatory role of IL-16 may be more important or relevant than its role as a proinflammatory cytokine in the pathogenesis of airway allergic inflammation is as yet unknown. Sparse data exist in the literature to elucidate the in vivo role of IL-16 in allergen-induced airway responses. The administration of anti-IL-16 prevents the antigen-induced airway hyperresponsiveness, but not antigen-induced airway eosinophilia, in mice through mechanisms that remain to be elucidated.³¹ The results of the current study, as well as our previous data showing an association between inflammation and increased tissue expression of IL-16 in allergic rhinitis and atopic dermatitis, are consistent with a proinflammatory role of IL-16 in allergic diseases.^{25,32} Further studies are needed to investigate the effects of IL-16 in allergen-induced T-cell responses in atopic subjects and on the production of cytokines by T cells in particular. More information on the functional activities of IL-16 is also needed before the precise role of this cytokine in various inflammatory processes can be fully understood. In that regard, definitive studies must await the development of IL-16 knockout mice and IL-16 transgenic mice.

In summary, we have shown that the numbers of IL-16-immunoreactive cells are increased in bronchial mucosa from atopic asthmatic patients 24 hours after antigen provocation in vivo. Epithelial cells, as well as T cells and eosinophils, were the predominant cellular sources of IL-16 in asthmatic airways in response to antigen. IL-16 may contribute to the immunoregulation of the inflammatory infiltrate in the airways in response to antigen. Further investigation will be required to fully ascertain the functional roles of this cytokine in allergic airway diseases.

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