

Local expression of ϵ germline gene transcripts and RNA for the ϵ heavy chain of IgE in the bronchial mucosa in atopic and nonatopic asthma

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Background: The demonstration of ϵ germline gene (C ϵ) transcripts and mature mRNA for the ϵ heavy chain gene (I ϵ) in the nasal mucosa suggested that IgE synthesis may occur in allergic rhinitis.

Objective: In view of our previous demonstration of increases in IL-4 mRNA⁺ cells in asthmatic subjects, we assessed whether local IgE synthesis may also be a feature of bronchial asthma.

Methods: Fiberoptic bronchoscopic mucosa biopsy specimens were obtained from 9 atopic asthmatic subjects and 10 nonatopic normal (intrinsic) control subjects. To control for atopy, we also studied 9 nonatopic asthmatic subjects and 10 atopic nonasthmatic control subjects. Tissue was processed for immunohistochemistry for B cells (CD20) and in situ hybridization for I ϵ and C ϵ RNA⁺ cells and IL-4 mRNA⁺ cells. **Results:** B-cell numbers in the bronchial mucosa were similar for asthmatic subjects compared with control subjects, whereas significantly higher numbers of I ϵ RNA⁺ ($P = .02$ and $P = .04$, respectively), C ϵ RNA⁺ ($P = .01$ and $P = .03$, respectively), and IL-4 mRNA⁺ ($P = .001$ and $P = .001$, respectively) cells were observed in atopic asthmatic subjects and nonatopic asthmatic subjects, respectively, but not in atopic control subjects compared with nonatopic control subjects. In asthmatic subjects there were significant correlations between I ϵ RNA⁺ cells ($r = 0.54$, $P = .02$) and C ϵ RNA⁺ cells ($r = 0.48$, $P = .05$) when compared with the number of IL-4 mRNA⁺ cells.

Conclusion: Increases in I ϵ and C ϵ RNA⁺ cells, but not B-cell numbers, in the bronchial mucosa provide evidence for local IgE synthesis in both atopic and nonatopic asthma. These changes appear to relate to asthma rather than atopy per se and, at least in part, may be under the regulation of IL-4. (*J Allergy Clin Immunol* 2001;107:686-92.)

Key words: Atopic asthma, nonatopic asthma, local IgE, IL-4

The critical step in the commitment of B cells to the synthesis of IgE is referred to as heavy chain switching and comprises somatic recombination within the Ig heavy chain gene cluster to transform the ϵ germline gene into the rearranged gene. This then gives rise to mRNA for the ϵ heavy chain of IgE.¹ Two signals are required for heavy chain switching to IgE both in vitro and in vivo. IL-4 and IL-13 target the ϵ gene for recombination; cross-linking of CD40 on the B-cell membrane then initiates recombination to proceed in a proportion of the cells.² IL-4 is released from cytoplasmic stores on activation of mast cells³ and, like IL-13,⁴ is secreted by T_H2 cells.⁵ CD40 ligand is constitutively expressed by tissue mast cells and induced on T cells after activation by antigen.^{6,7}

Our previous results suggest that local IgE synthesis and cytokine regulation of heavy chain switching to IgE occur in the nasal mucosa.⁸⁻¹⁰ In this study we have used RNA probes to detect whether resident B cells have the capacity to express IgE in the bronchial mucosa in asthma. We have used the combination of I ϵ , C ϵ , and IL-4 probes to determine the concentrations of B cells expressing ϵ germline gene transcripts and ϵ chain mRNA and the number of IL-4 mRNA⁺ cells. We have also used double immunohistochemical staining with differentially labeled antibodies to colocalize I ϵ and C ϵ to CD20⁺ B cells.

We examined bronchial biopsy specimens from patients with well-documented atopic asthma and compared them with specimens from normal nonatopic control subjects. We also included patients with intrinsic (nonatopic) asthma and atopic subjects without asthma to control for the effects of asthma and atopy per se.

METHODS

Patients

The study was performed with the approval of the Ethics Committees of the Royal Brompton Hospital (London, United Kingdom) and the Hochgebirgsklinik (Davos Wolfgang, Switzerland). Written informed consent was obtained.

Bronchial biopsy specimens were taken from atopic asthmatic subjects and nonatopic control subjects. We also included a group of nonatopic asthmatic subjects and atopic nonasthmatic control subjects (Table I).¹¹ Asthma was defined as a clear history of current asthma symptoms and evidence of more than 20% reversibility of FEV₁ either spontaneously or after administration of inhaled β_2 -agonists, a histamine PC₂₀ provocation test result of less than 8 mg/mL in the previous 2 weeks, or both. Atopy was defined as a positive skin

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Received for publication August 3, 2000; revised January 3, 2001; accepted for publication January 17, 2001.

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0091-6749/2001 \$35.00 + 0 1/83/114339
doi:10.1067/mai.2001.114339

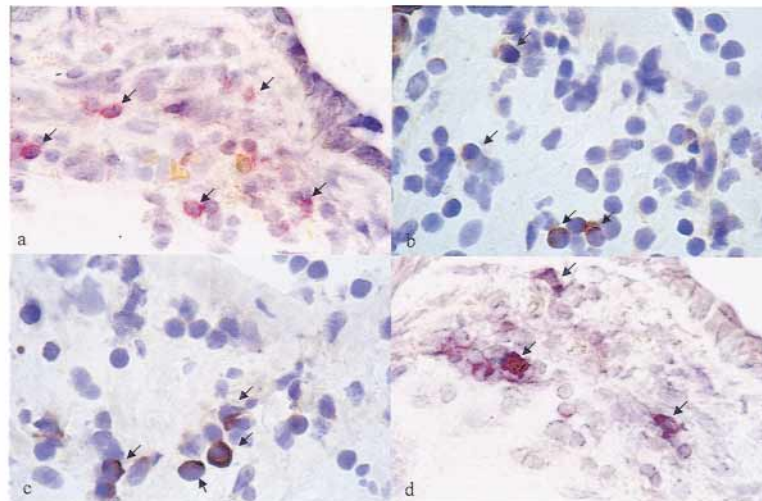


FIG 1. Immunohistochemistry/in situ hybridization studies of the bronchial mucosa. **A**, **B** lymphocytes immunostained with anti-CD20 antibody. Positive cells stain red (arrows). **B** and **C**, Individual Cε and Iε RNA⁺ cells detected by using digoxin-labeled antisense RNA probes. Positive cells stain dark blue (arrows). **D**, Cells stained sequentially with anti-CD20 followed by hybridization with an antisense RNA probe against Cε. Double-positive cells stain purple (arrows).

TABLE I. Clinical features of study participants

	Atopic asthmatic subjects	Nonatopic asthmatic subjects	Atopic control subjects	Nonatopic control subjects
No.	9	9	10	10
Sex (M:F)	4:5	4:5	7:3	8:2
Age (y)	41 ± 3	56 ± 2	31 ± 2	29 ± 2
FEV ₁ (% predicted)	85.8 ± 7.2	78.9 ± 6.3	103.7 ± 4.6	106 ± 3
Histamine PC ₂₀ (mg/mL)	1.8 ± 0.5	1.5 ± 0.9	>16	>16
Total IgE (IU/mL)	606 ± 394	66.5 ± 11	232 ± 69.7	25.0 ± 8.3

Results are given as means ± SEM where shown.

prick test response (wheal size ≥ 3 mm greater than that obtained with the diluent control) to extracts of one or more of 11 common aeroallergens (house dust mite [*Dermatophagoides pteronyssinus*], timothy grass pollen, birch pollen, mixed tree pollens, mixed weeds, cat dander, dog dander, horse dander, *Aspergillus* species, *Cladosporium* species, and *Alternaria* species). All atopic subjects demonstrated elevated serum allergen-specific IgE concentrations (CAP RAST >0.7 IU/mL) to one or more of these allergens. Nonatopic subjects were defined on the basis of negative skin prick test responses to the above range of aeroallergens in the presence of a positive histamine control response. The nonatopic control subjects also exhibited serum IgE concentrations within the normal range of our laboratory (0–150 IU/mL) and negative RAST test results to the above aeroallergens. Nonatopic (intrinsic) asthmatic subjects were significantly older than their atopic counterparts and had no detectable specific IgE antibodies in their serum against common inhaled allergens. Atopic control subjects were asymptomatic or had mild allergic rhinitis with no history of wheeze or chest tightness, normal baseline lung function with less than 10% reversibility of the FEV₁ either spontaneously or after administration of inhaled β_2 -agonists, and histamine PC₂₀ provocation test results of greater than 16 mg/mL. All subjects were non-smokers and had not taken oral corticosteroids for the previous 2 months or inhaled corticosteroids for the previous 2 weeks.

Fiberoptic bronchoscopy

Bronchoscopy was performed in all subjects (asthmatic and control subjects), and 1 to 2 biopsy specimens were taken from the right middle, lower, and upper lobe bronchi. This procedure was well tolerated. Safety aspects of our bronchoscopy protocol have previously been reported.¹² Bronchial biopsy specimens were processed immediately in the bronchoscopy room and stored at -80°C before use for immunohistochemistry and in situ hybridization.

Tissue processing

Immunohistology of bronchial biopsy specimens was performed on 6- μm cryostat sections by using a modified alkaline phosphatase–antialkaline phosphatase method (Fig 1, A).¹³ In situ hybridization for Cε and Iε was performed on 10- μm cryostat sections prefixed in 4% paraformaldehyde and hybridized with digoxin-labeled antisense riboprobes, as previously described (Fig 1, B and C).¹⁴ For detection of IL-4 mRNA⁺ cells, a sulfur 35-labeled antisense riboprobe was used, as previously described.¹⁵ Sections were exposed to a photographic emulsion, and IL-4 mRNA⁺ cells appeared as clusters of silver grains overlying discreet individual cells. Controls were performed with sense probes (having an identical sequence to mRNA) and with antisense probes after pretreatment of sections with RNase.

TABLE II. B lymphocytes, ϵ heavy chain RNA⁺ cells, and IL-4 mRNA⁺ cells in the bronchial mucosa

Cells	Atopic asthmatic subjects (n = 9)	Nonatopic asthmatic subjects (n = 9)	Atopic nonasthmatic control subjects (n = 10)	Nonatopic control subjects (n = 10)
CD20	14 (5-42)	20 (4-29)	15 (3-26)	16 (8-34)
C ϵ RNA	20 (3-54)	19 (0-38)	1 (0-11)	0 (0-1)
I ϵ RNA	10 (2-33)	11 (0-31)	1 (0-10)	0 (0-2)
IL-4 mRNA	24 (5-115)	70 (38-148)	1 (0-25)	1 (0-39)

Values are given as median cells per square millimeter (interquartile range). C ϵ ⁺ ($P = .01$ and $P = .03$), I ϵ ⁺ ($P = .02$ and $P = .04$), and IL-4⁺ ($P = .001$ and $P = .001$) cells were increased in atopic asthmatic and nonatopic asthmatic subjects, respectively, but not atopic control subjects compared with nonatopic control subjects.

Double immunohistochemistry/in situ hybridization was performed sequentially on the same sections (Fig 1, D). Immunohistology was first performed with an anti-CD20 antibody, and positive cells were visualized by using the alkaline phosphatase–antialkaline phosphatase technique. Positive cells stained red. In situ hybridization was then performed on the same sections by using a digoxigenin-labeled complementary RNA probe and visualized by using 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate, which stained cells dark blue, as previously described.¹⁶ Individual double-positive cells were evident as purple-staining cells at 400 $\times$ magnification (Fig 1, D).

Tissue sections were assessed with an Olympus BH2 microscope with an eyepiece graticule at 200 $\times$ magnification. Counts were made along the whole length of bronchial biopsy specimens. Counts were expressed per square millimeter of whole biopsy sections. The mean $\pm$ SD surface areas of biopsy sections counted for each of the 4 groups varied from 3.6 ± 0.4 to 3.7 ± 0.5 mm², with within-group coefficients of variation from 11.9% to 13.8%. These surface areas were equivalent to mean $\pm$ SD basement membrane lengths of biopsy sections counted from 5.0 ± 0.9 to 5.2 ± 1.1 mm, with within-group coefficients of variation of 18.2% to 20.8%. The between-observer coefficients of variation for duplicate counts for all 4 markers assessed (CD20, C ϵ , I ϵ , and IL-4) for all 4 groups varied from 1.0% to 3.8%. Coefficients of variation between 6-step duplicate sections from the same biopsy specimen for all 4 markers examined for all 4 subject groups varied from 12.9% to 22.8%.

Genetic probes

The I ϵ probe corresponded to the sequence from the start site of ϵ germline gene transcription to the end of the I ϵ exon^{8,17} at position 650. This was generated by cloning the indicated *Bam*HI fragment (accession No. x56797) into Bluescript Ksp. The plasmid, linearized with *Xba*I or *Hind*III, was transcribed in the presence of digoxigenin-UTP with T7 polymerase for the antisense probe and with T3 polymerase for the sense probe used as a negative control.¹⁷

The C ϵ probe was generated from an ϵ chain cDNA clone, as previously described.^{8,18} The sequence corresponded to a 1150 base *Hind*III fragment from pSC213, encompassing 24 bases of C ϵ 1 and the rest of the C ϵ sequence. This fragment was cloned into pGEM,¹⁵ cut at an *Sma*I site and transcribed with SP6 polymerase for the antisense probe (526 nucleotides), cut at the *Bam*HI site, and transcribed with T7 polymerase for the sense probe used as a negative control.

The IL-4 probe was prepared by inserting a human IL-4 cDNA of 500 bp (generously provided by Dr J-Y Bonnefoy, Glaxo Institute for Molecular Biology, Geneva, Switzerland) into pGEM, as described.⁸

Statistical analysis

All biopsy samples were coded in random order, and sections were counted blind, independent of the clinical protocol. The primary analysis was the comparison of ϵ RNA⁺ cells in atopic asthmatic patients with normal nonatopic control subjects. Secondary outcome measures were comparisons between the nonatopic asthma group and

the nonatopic normal control group and between the atopic and nonatopic normal control groups. Correlations were performed by using the Spearman rank method. Unpaired analyses were performed with the 2-tailed Mann-Whitney *U* test. Analyses were performed with a statistical package (Minitab Statistical Software, Minitab, Inc, Pa). *P* values of less than .05 were considered as significant.

RESULTS

Individual CD20⁺ B lymphocytes were seen scattered throughout the bronchial mucosa, mainly within the lamina propria, as discreet individual cells rather than cell aggregates (Fig 1, A). There was a wide scatter from 0 to 90 cells/mm² in both atopic and nonatopic asthmatic subjects and in atopic and nonatopic healthy subjects, with no significant differences among the groups (Table II).

Cells expressing RNA for the sterile ϵ germline gene transcript (I ϵ) and mature RNA for the ϵ heavy chain gene (C ϵ) are shown in Fig 1, B and C, and Fig 2. RNA staining was evident as a perinuclear rim in discreet digoxigenin-positive mononuclear cells scattered throughout the lamina propria. Very little intraepithelial staining was detected, and the overall distribution corresponded to that observed for B lymphocytes. The median number of I ϵ RNA⁺ cells observed for the atopic asthmatic subjects was higher than those for the nonatopic normal control subjects ($P = .02$). Similarly, there were significantly higher numbers of I ϵ RNA⁺ cells observed in the nonatopic asthmatic subjects compared with those found in the nonatopic control subjects ($P = .04$). In contrast, no significant elevations in I ϵ ⁺ cells in the bronchial mucosa were observed in the atopic nonasthmatic subjects compared with those found in the nonatopic normal control subjects ($P = .35$). Similarly, there were significantly higher numbers of C ϵ RNA⁺ cells in atopic asthmatic subjects ($P = .01$) and in nonatopic asthmatic subjects ($P = .03$) but not in atopic nonasthmatic subjects ($P = .25$) compared with biopsy specimens from the nonatopic normal control subjects (Fig 2 and Table II).

To determine the phenotype of ϵ RNA-expressing cells, bronchial mucosa tissue sections from 10 asthmatic subjects (5 atopic and 5 nonatopic subjects) were subjected to sequential immunohistochemistry and in situ hybridization. Greater than 90% C ϵ and I ϵ RNA⁺ cells were CD20⁺ B cells in both the atopic (C ϵ , 92% $\pm$ 3%; I ϵ , 91% $\pm$ 8%) and nonatopic asthmatic (C ϵ , 92% $\pm$ 2%; I ϵ , 92% $\pm$ 2%) subjects. Conversely, when the percentage of CD20⁺ B cells, which expressed IgE heavy chain RNA, were deter-

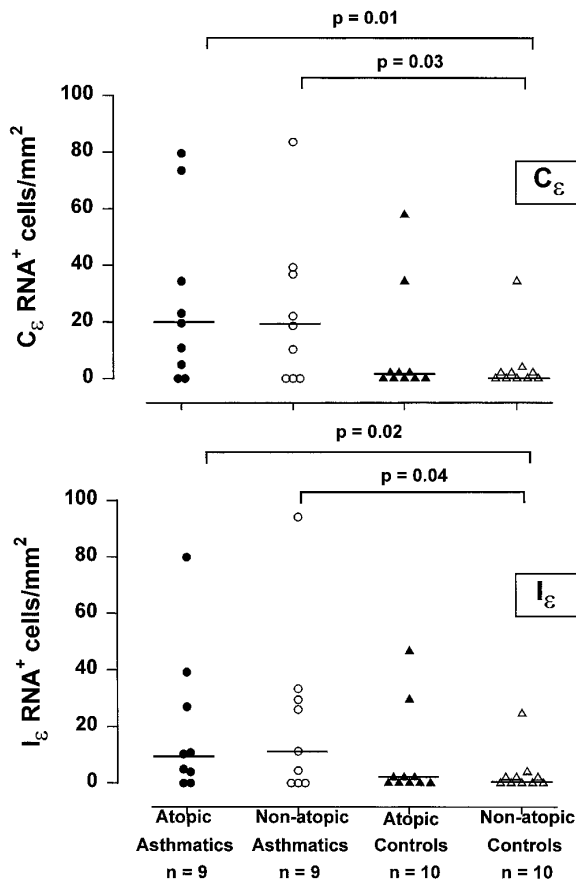


FIG 2. I ϵ and C ϵ expression in the bronchial mucosa in asthmatic and control subjects. Results are expressed as the number of RNA⁺ cells per square millimeter of bronchial mucosa. Circles refer to atopic (filled circles) and nonatopic (open circles) asthmatic subjects. Triangles refer to atopic (filled triangles) and nonatopic (open triangles) nonasthmatic control subjects. Bars represent median values. Levels of statistical significance compared with the nonatopic control group are shown (Mann-Whitney U test).

mined, the results were CD20/C ϵ of $70\% \pm 11\%$ and CD20/I ϵ of $74\% \pm 8\%$ of B cells in atopic asthmatic subjects and CD20/C ϵ of $63\% \pm 8\%$ and CD20/I ϵ of $58\% \pm 12\%$ of B cells in nonatopic asthmatic subjects.

For the asthmatic subjects, the numbers of I ϵ RNA⁺ and C ϵ RNA⁺ cells were compared with the number of IL-4 mRNA⁺ cells within the bronchial mucosa. When data for the atopic and nonatopic asthmatic subjects were combined, significant correlations were observed for both I ϵ ($r = 0.54$, $P = .02$) and C ϵ ($r = 0.48$, $P = .05$; Fig 3). When atopic and nonatopic asthmatic subjects were considered separately, there was a significant association for atopic asthmatic subjects between I ϵ RNA⁺ cells and IL-4 mRNA⁺ cells ($r = 0.75$, $P = .02$) and a trend for an association between C ϵ and IL-4 ($r = 0.58$, $P = .1$). Similar trends were observed for the nonatopic asthmatic group (I ϵ vs IL-4 and C ϵ vs IL-4: both $r = 0.58$ and $P = .1$).

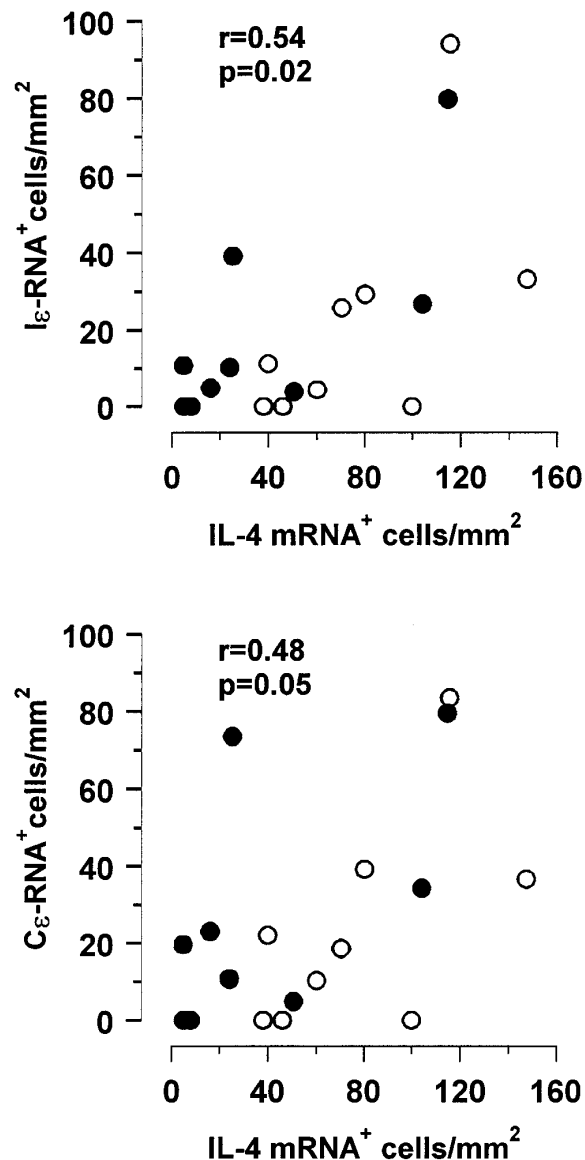


FIG 3. Relationship between IL-4 and ϵ gene expression in the bronchial mucosa in asthma. Atopic asthmatic subjects are represented by filled circles. Nonatopic asthmatic subjects are represented by open circles. Correlation was done with the Spearman rank method.

DISCUSSION

In patients with bronchial asthma compared with normal control subjects, despite comparable numbers of CD20⁺ B lymphocytes within the bronchial mucosa, we detected elevated numbers of cells expressing the ϵ germline gene transcript (I ϵ) and cells expressing C ϵ , which detects either the sterile transcript or mature ϵ heavy chain messenger RNA. These elevations were detected in biopsy specimens from both atopic and nonatopic asthmatic subjects but not in biopsy specimens from atopic nonasthmatic subjects compared with those

from nonatopic, normal, healthy control subjects. Furthermore, there was an association between the number of $C\epsilon^+$ and $I\epsilon^+$ cells and the number of cells expressing IL-4 mRNA. Taken together, these data confirm, at least at the RNA level, that local IgE expression in the bronchial mucosa is a feature of both atopic and nonatopic asthma. Atopy, as defined by positive skin test responses and elevated circulating allergen-specific IgE concentrations, appears neither necessary nor sufficient for local IgE synthesis to occur locally within the bronchial mucosa in asthma. The results both demonstrate the presence of B cells locally with potential to switch in favor of IgE synthesis ($I\epsilon/CD20^+$ cells) and are consistent with occurrence of local IgE heavy chain class switching.

Although we observed significant differences in cell counts for $C\epsilon$ and $I\epsilon$ RNA $^+$ cells between asthmatic and control subjects, our findings may be questioned on the basis of the relatively low numbers of subjects (ie, 9-10 per group) and the variability in cell counts we observed (Fig 2). Several factors may have contributed to such variability, including observer variability, variation in cell counts between sections within and between biopsy specimens, variability between different lung regions and within-subject (biologic) variability with time and in relation to medication, smoking history, and recent viral infection. Our subjects were all nonsmokers, and their medication was strictly standardized, including no oral steroids for 2 months or inhaled steroids for at least 2 weeks before the study. Cell counts were performed blind and in duplicate and were standardized in relation to the total surface area counted and the equivalent basement membrane length (>5 mm, within the 5-10 mm recommended in a recent study).¹⁹ Between-observer variability for cell counts was 1% to 3%, and our between-section variability was 12.9% to 22%, which is consistent with previous reports.^{20,21} We did not assess between-region variability, although previous reports from our own²² and other groups²³ have found no differences between different lung regions. One careful study demonstrated that the variation in cell counts within subjects with time was greater than that observed between lung regions.²⁴ Despite the variability we observed in cell counts, $C\epsilon^+$ and $I\epsilon^+$ cells were present in 7 of 9 atopic asthmatic subjects and 6 of 9 nonatopic asthmatic subjects, as opposed to only 2 of 10 atopic control subjects and 1 of 10 normal nonatopic control subjects. Our results are internally consistent for $C\epsilon$, $I\epsilon$, and IL-4 and are further supported by the significant correlations between $C\epsilon$ and $I\epsilon$ RNA $^+$ cells and the increases in IL-4 mRNA $^+$ cells, the putative cause for the observed increases in $C\epsilon$ and $I\epsilon$ expression.

The specificity of the $C\epsilon$ and $I\epsilon$ probes were supported by the finding of discreet perinuclear staining confined to mononuclear cells, the absence of significant background staining, and negative results with both RNase pretreatment and sense probes. Double immunohistochemistry and in situ hybridization also confirmed that ϵ gene expression was confined almost exclusively ($>90\%$ to B cells). Although we could detect IgE protein-positive cells within the bronchial mucosa of asth-

matic patients, this was confined largely to mast cells, and we were unable to detect IgE protein staining of CD20 $^+$ B cells or CD138 $^+$ plasma cells (data not shown). This may be a consequence of low levels of IgE expression, of secretion rather than intracellular accumulation of a major portion of the product, or both. It may be that relatively few committed B cells go on to secrete IgE in large amounts. Alternatively, there may have been too few cells secreting large amounts of IgE to be detected in our biopsy samples. Very few cells may, however, secrete large amounts of IgE. Chvatchko et al²⁵ were unable to detect IgE in sections from isolated murine lungs but found that IgE was detected in supernatants after the culture of isolated lung tissue. Detection of mRNA synthesis by B cells within the bronchial mucosa is supportive evidence of protein synthesis.

It has been suggested that IgE is produced by B cells in the bone marrow or regional lymph glands and that IgE reaches mucosa surfaces by diffusion or through migrating cells.²⁶ However, several lines of evidence support local IgE synthesis at mucosa sites.⁸ IgE has been detected in local nasal secretions in patients with symptoms of allergic rhinitis in the absence of positive skin test responses or detectable allergen-specific IgE in serum.^{27,28} Allergen-specific IgE was detectable in nasal secretions but not in salivary secretions of patients with hay fever.²⁹ IgE concentrations in nasal lavage fluid have been shown to rise more rapidly than in the serum of asthmatic children after a period of allergen avoidance followed by re-exposure to allergen.³⁰ Our previous findings in patients with allergic rhinitis of local increases in ϵ gene transcripts after both experimental⁸ and natural⁹ allergen exposure and the recent detection of both switch circles and IgE protein production from B cells in nasal lavage fluid^{31,32} support the concept of local IgE synthesis in the nasal mucosa. The present findings in the bronchial mucosa, which are also consistent with local IgE synthesis, may explain, at least in part, why some atopic subjects have asthma and others have rhinitis or eczema or have no clinical manifestations of their atopic state.

Ever since the first description of intrinsic asthma,³³ there has been debate about the relationship of this variant of the disease to atopy.³⁴⁻³⁶ One suggestion is that intrinsic asthma represents a form of autoimmunity or autoallergy triggered by infection because a respiratory influenza-like illness often precedes onset. Some authors have suggested that intrinsic asthmatic subjects are allergic to an as-yet undetected allergen and could therefore benefit from allergen avoidance, as previously demonstrated in atopic asthmatic subjects. Our viewpoint is that although intrinsic asthma has a different clinical profile from extrinsic asthma, it does not appear to be a distinct immunopathologic entity.³⁶ In a series of bronchial biopsy studies with in situ hybridization, immunohistochemistry, or both, both variants of the disease were characterized by the following: (1) infiltration of eosinophils³⁷ and T_H2 cells secreting IL-4 and IL-5¹¹ and (2) the presence of CC chemokines and Fc ϵ RI $^+$ cells.^{38,39} Thus there appears to be more similarities than differences between

extrinsic and intrinsic asthma. Both could be IgE-mediated diseases, although in the intrinsic patients the putative antigen or allergen is unknown, and IgE production appears to be local rather than generalized, as in atopy.

IgE has been recognized as an important potential therapeutic target in bronchial asthma. Recent studies with a humanized recombinant murine monoclonal anti-human IgE in bronchial asthma supports this view. Anti-IgE therapy was effective in inhibiting both early and late asthmatic responses after allergen challenge.^{40,41} A recent controlled clinical trial in patients with moderately severe bronchial asthma demonstrated a modest reduction in asthma symptoms and improvement in quality of life in patients with moderately severe atopic asthma.⁴² Our findings support the view that a trial of anti-IgE therapy may be indicated in patients with so-called intrinsic late-onset asthma. Furthermore, they suggest that an alternative to a systemic (intravenous) approach may be the use of topical anti-IgE therapy both for rhinitis and bronchial asthma.

In summary, we have confirmed that in both atopic and nonatopic asthma, B lymphocytes have the potential to switch in favor of IgE heavy chain production. Our data are consistent with the occurrence of IgE heavy chain switching occurring locally within the bronchial mucosa, although confirmation will require in vitro studies of cultured human bronchial tissue, with examination of the time course of generation of IgE heavy chain RNA and protein production.

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