

Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma

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Background: Class switching from IgM/IgG/IgA to IgE is required for B cells to express IgE. This requires class switch recombination in the Ig heavy-chain gene locus. It is generally believed that class switch recombination occurs in lymphoid tissue, but it was recently shown that class switching to IgE occurs in the nasal mucosa in allergic rhinitis.

Objective: We aimed to determine whether class switching to IgE also occurs in the bronchial mucosa in asthma, and to look for possible differences/similarities between atopic and nonatopic asthma.

Methods: We have used RT-PCR to examine ϵ immunoglobulin heavy-chain germline gene transcripts (GLTs; ϵ GLTs), ϵ circle transcripts (CTs; $I\epsilon$ -C μ CT or $I\epsilon$ -C γ CT), and mRNA encoding the heavy chain of IgE (ϵ mRNA) and activation-induced cytidine deaminase (AID) in bronchial biopsies from atopic patients with asthma, nonatopic patients with asthma, atopic controls without asthma, and nonatopic controls without asthma (10 subjects in each group).

Results: The ϵ GLT and AID mRNA were detectable in the bronchial mucosa of subjects in all 4 groups. In contrast, $I\epsilon$ -C μ

CT, $I\epsilon$ -C γ CT, and ϵ mRNA were detectable in the bronchial mucosa of the majority of both atopic and nonatopic patients with asthma, but rarely in the controls without asthma.

Conclusion: The bronchial mucosa is a site primed in all individuals for class switching to IgE, because of B-cell expression of ϵ GLT and AID mRNA. However, it is only in patients with asthma, regardless of atopic status, that class switching to IgE occurs.

Clinical implications: Our findings reveal prospects for local targeting of the Ig class switch mechanism in the management of atopic and nonatopic asthma. (*J Allergy Clin Immunol* 2007;119:213-8.)

Key words: IgE, class switch recombination, asthma, mucosa

Asthma is a debilitating disease affecting one fifth of the population of the developed world. Severe asthma is a major cause of hospitalization and health care costs. In clinical practice, asthma is classified as atopic or nonatopic, according to the presence or absence of circulating IgE directed against local aeroallergens detected by skin prick test (SPT) or *in vitro* techniques (RAST or ELISA). These IgE antibodies interact with the high-affinity IgE receptor (Fc ϵ RI) on mast cells, which may result in immediate hypersensitivity on allergen provocation and acute exacerbation of disease.¹

About one third of adult patients with asthma are classified as nonatopic.^{2,3} They tend to have more severe disease, often associated with chronic rhinosinusitis, but apart from their lack of acute reactivity to allergens, their disease is clinically similar.³⁻⁵

IgE is characterized by its ϵ heavy-chain. It is produced after heavy-chain switching in B cells from IgM, IgG, or IgA to IgE, which proceeds in 3 stages: germline gene transcription, DNA recombination within the heavy-chain locus producing ϵ circle transcripts (CTs), and synthesis of ϵ -chain mRNA that is translated into protein.¹ The switch to IgE is initiated by the cytokines IL-4 or IL-13, produced principally by T_H2 cells, which drive ϵ germline gene transcription.^{6,7} IL-4 also increases the expression of activation-induced cytidine deaminase (AID),⁸ an enzyme required for class switch recombination (CSR).⁹ AID expression and CSR, well known to occur in the germinal centers of lymphoid tissue,¹⁰ have recently also been

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Abbreviations used

AA: Atopic patient with asthma
 AC: Atopic control without asthma
 AID: Activation-induced cytidine deaminase
 CSR: Class switch recombination
 CT: Circle transcript
 GLT: Germline gene transcript
 NA: Nonatopic patient with asthma
 NC: Nonatopic control without asthma
 SPT: Skin prick test
 UK: United Kingdom

demonstrated within the nasal mucosa of patients with allergic rhinitis.¹¹⁻¹³

Molecular immunopathological comparisons of bronchial biopsies from atopic and nonatopic patients with asthma have demonstrated conditions conducive to IgE switching (elevated IL-4 and IL-13), expression of ϵ germline gene transcripts (GLTs; ϵ GLTs), and Fc ϵ RI mRNA (suggestive of IgE synthesis because IgE upregulates its own receptor) in both groups of patients.^{1,14-18} In view of these data, we hypothesized that the environment of the bronchial mucosa induces B-cell IgE switching leading to IgE synthesis in patients with asthma, regardless of their atopic status.

To test the hypothesis that local CSR to IgE and IgE synthesis occurs in the bronchial mucosa of patients with asthma, we have performed RT-PCR to look for ϵ GLTs, CTs (I ϵ -C μ CT and I ϵ -C γ CT), ϵ mRNA, and AID mRNA in bronchial biopsies from atopic and nonatopic patients with asthma and atopic and nonatopic controls without asthma.

METHODS

Clinical protocol

Bronchial biopsies were obtained at fiberoptic bronchoscopy from atopic patients with asthma (AAs), nonatopic patients with asthma (NAs), atopic controls without asthma (ACs), and nonatopic controls without asthma (NCs; n = 10 in each group). Asthma was defined as a clear history of relevant symptoms with either reversible airways obstruction ($\geq 15\%$ variability in the FEV₁ either spontaneously or after inhaled albuterol, 200 μ g), and/or a histamine PC₂₀ provocation test result < 8 mg/mL documented in the previous 6 months. Nonasthma was defined as a lifelong absence of symptoms, FEV₁ within the predicted range, and $< 5\%$ reversible with bronchodilator. Atopy was defined as a positive SPT and RAST to 1 or more of a full range of common aeroallergens (house dust mite, mixed grasses, mixed trees, mixed molds, cat and dog dander). Nonatopy was defined as uniformly negative SPT (in the presence of appropriate controls) and RAST to the same aeroallergens. Five of 10 of the atopic and 4 of 10 of the nonatopic patients with asthma were taking inhaled corticosteroids, but none was taking systemic corticosteroids. Smokers and subjects with any chronic disease other than asthma or rhinitis were excluded. The characteristics of the study participants are shown in Table I.

Fiberoptic bronchoscopy was performed as previously described.¹⁷ Eight to 10 biopsies were obtained from first-generation or second-generation right middle or lower lobe bronchi. Any wheezing after the bronchoscopy was treated with additional albuterol as

TABLE I. Characteristics of study participants

Patient group	Number (n)	Male: female	Age (y)*	Total serum IgE (kU/L)*	SPT $\dagger$
AA	10	5:5	33.5 (21-54)	242 $\dagger$ (89-4150)	All positive
NA	10	4:6	34.5 (24-61)	23.5 (0-621)	All negative
AC	10	4:6	23 (21-30)	86 (23-241)	All positive
NC	10	7:3	22 (20-40)	17.4 (7.5-81)	All negative

*Data are expressed as medians and ranges.

$\dagger$ Data presented from 9 donors (not available for 1 patient).

$\ddagger$ Skin prick test with all common aeroallergens: house dust mite, mixed grasses, mixed trees, mixed molds, and cat and dog dander.

required. Nasal prongs were used to deliver supplementary oxygen. Oxygen saturation was monitored throughout the procedure.

The study was performed with the approval of the Guy's Hospital Ethics Committee and the patients' written informed consent.

RNA isolation and RT-PCR

Total RNA was isolated from whole biopsies using the Qiagen RNA isolation kit (Qiagen, Crawley, United Kingdom [UK]) following the manufacturer's instructions. RNA was suspended in nuclease-free water and quantified by spectrophotometry at 260 nm. RNA was reverse transcribed to cDNA, and ϵ GLT, CT, and AID mRNA were PCR-amplified as described elsewhere.¹³ Briefly, for RT-PCR, 2.5 μ g total RNA was first denatured at 100°C for 2 minutes and then cooled on ice for 2 minutes before being added to a 20- μ L reaction mix containing 5x First strand buffer (Invitrogen Ltd, Paisley, UK), 0.1 mol/L dithiothreitol, 10 mmol/L dNTP mix, oligo dT primer (0.5 μ g/ μ L), 40 U/ μ L RNase OUT (Invitrogen), random d(N)₁₀ primers (2 μ g/ μ L), and 200 U/ μ L Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen Ltd, Paisley, UK). The reaction was incubated at 37°C for 10 minutes and 42°C for 40 minutes, with a final incubation of 10 minutes at 50°C. Finally, the cDNA reaction was diluted with 80 μ L nuclease-free water and incubated at 100°C for 2 minutes. cDNA was stored at -20°C until further needed.

ϵ mRNA was amplified by seminested PCR using a forward primer in the variable-region of the heavy-chain (VH) and reverse primers in the heavy-chain of IgE. The first round of ϵ mRNA PCR was conducted in a 30- μ L reaction containing 20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L KCl, 0.1 mmol/L EDTA, 2.0 mmol/L MgCl₂, 10 mmol/L dNTP mix; 50 pmol/L each primer: forward primer VF2 (5'-AGTCTGGAGCAGAGGTG3') and reverse primer C ϵ R1 (5'-CAGGACGACTGTAAGATCTTCACG3'); and 2.5U Taq polymerase (Promega Life Sciences, Southampton, UK). The first cycle was conducted at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 66°C for 1 minute and 72°C for 1.5 minute, and a final extension at 72°C for 5 minutes. A second round of PCR was performed on a 3- μ L aliquot of the first-round PCR product under the same conditions as described, except that an inner C ϵ R2 reverse primer (5'-GGGGTGAAGTCCCTGGAGC3') and annealing temperature of 62°C were used. PCR amplification of mRNA isolated from tonsil B cells stimulated with IL-4 and anti-CD40 was used as positive control for ϵ GLT and ϵ CT PCR. cDNA from an IgE-expressing cell line (AF10) was used as a positive control for ϵ mRNA PCR. PCR amplification of cDNA from an AID-expressing cell line (RAMOS) was used as a positive control for AID mRNA. Nuclease-free water was substituted for cDNA in negative controls.

PCR products were cloned and sequenced to confirm their identity. The identities of ϵ CT were further confirmed by Southern blot

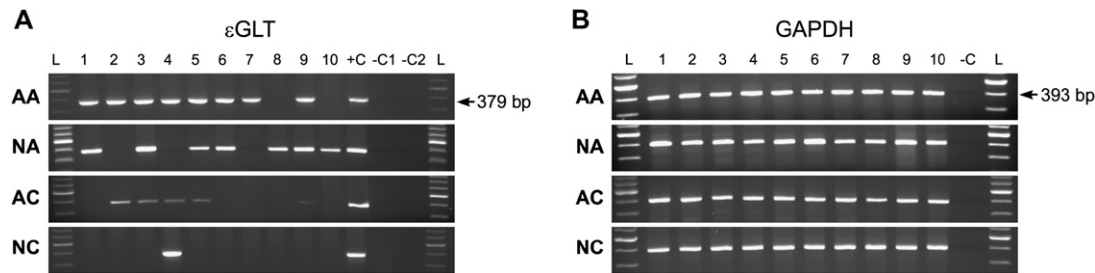


FIG 1. Expression of ϵ GLT in the bronchial mucosa. **A**, Distribution of ϵ GLT (379 bp) in 10 subjects (1-10) representing AA, NA, AC, and NC. **B**, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to control cDNA loading. Lanes +C, -C, -C1, -C2, and L correspond to PCR-positive control, PCR-negative control, the first-and second-round PCR-negative controls, and 100-bp DNA ladder, respectively.

TABLE II. Distribution of ϵ GLT, ϵ CT, ϵ - and AID mRNA in the bronchial mucosa

Markers	Patient no.										Total
	1	2	3	4	5	6	7	8	9	10	
AA											
εGLT	•	•	•	•	•	•	•		•		8/10
Iε-Cμ. CT		•	•		•	•			•		5/10
Iε-CγCT		•		•			•	•		•	5/10
ε mRNA	•	•	•			•	•	•	•	•	8/10
AID mRNA		•	•			•	•	•	•	•	7/10
NA											
εGLT	•		•		•	•		•	•	•	7/10
Iε-Cμ. CT		•				•	•	•		•	5/10
Iε-CγCT		•			•			•			3/10
ε mRNA	•	•	•	•	•	•		•	•		8/10
AID mRNA	•	•				•		•	•		5/10
AC											
εGLT		•	•	•	•				•		5/10
Iε-Cμ. CT											0/10
Iε-CγCT											0/10
ε mRNA				•		•					2/10
AID mRNA								•			1/10
NC											
εGLT				•							1/10
Iε-Cμ. CT											0/10
Iε-CγCT											0/10
ε mRNA					•						1/10
AID mRNA			•		•	•	•				4/10

hybridization. This procedure has been described in detail previously.¹³ The results presented are representative of at least 3 reproducible independent PCR amplifications performed on each subject sample.

Statistical analysis

The χ^2 test was used for comparison of marker frequencies in AA versus AC and NA versus NC. *P* values less than .05 were considered statistically significant.

RESULTS

ϵ GLT expression in the bronchial mucosa

ϵ Germline transcription marks the first step in the commitment of B cells to the synthesis of IgE. We sought ϵ GLT in bronchial mucosal biopsies from 10 patients in each of the 4 subject groups. As shown in Fig 1, A, and Table II, ϵ GLT expression was detected in 8 of 10 AAs, 7 of 10 NAs, 5 of 10 ACs, and 1 of 10 NCs.

ϵ CT expression in the bronchial mucosa

DNA recombination, producing ϵ CT, marks the irreversible step in the commitment of B cells to the synthesis of IgE. We sought ϵ CT in the same biopsies used to detect GLT. IgM to IgE (I ϵ -C μ) CTs were detected in the bronchial biopsies from 5 of 10 AAs and 5 of 10 NAs (Fig 2, A). IgG to IgE (I ϵ -C γ) CTs were amplified from biopsies from 5 of 10 AAs and 3 of 10 NAs (Fig 2, B). In biopsies from 1 AAs and 2 NAs, both I ϵ -C μ CT and I ϵ -C γ CT were detected together (Table II). In contrast, no CTs were observed in biopsies from any of the control subjects without asthma (data not shown). Overall, CTs (I ϵ -C γ or I ϵ -C μ) were observed in biopsies from 9 of 10 AAs compared with 0 of 10 ACs (*P* = .0001) and 6 of 10 NAs compared with 0 of 10 NCs (*P* = .01). To confirm their identity, PCR products from the positive biopsies were cloned and sequenced. The expected sequences were obtained in all cases (data not shown).

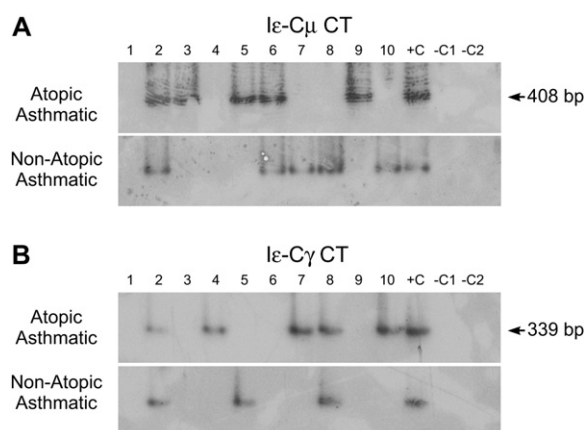


FIG 2. Expression of ϵ CT in the bronchial mucosa. PCR-amplified ϵ CT products detected by agarose gel electrophoresis were probed with a radiolabeled probe (I ϵ) by Southern blotting.¹³ **A and B,** Distribution of I ϵ -C μ CT and I ϵ -C γ CT in atopic and nonatopic patients with asthma. Lanes 1-10 correspond to 10 patients in each group. Lane +C shows the PCR-positive control. Lanes -C1 and -C2 are the first-round and second-round PCR-negative controls.

IgE heavy chain mRNA expression in the bronchial mucosa

IgE synthesis by B cells is the third and final step in the production of IgE. We observed ϵ mRNA in biopsies from 8 of 10 AAs compared with 2 of 10 ACs ($P = .020$) and 8 of 10 NAs compared with 1 of 10 NCs ($P = .006$; Fig 3, Table II). There was no correlation between serum IgE levels and presence or absence of ϵ mRNA in patients with asthma (data not shown).

AID mRNA expression in the bronchial mucosa

Activation-induced cytidine deaminase expression is required for CSR, and thus occurs in lymphoid tissue¹⁰ and has been observed in the nasal mucosa of patients with allergic rhinitis.^{12,13} We obtained a 335-bp band corresponding to the expected size of AID mRNA, the identity of which was confirmed by sequencing, in 7 of 10 AAs, 5 of 10 NAs, 1 of 10 ACs, and 4 of 10 NCs, as shown in Fig 4 and Table II. Like the presence ϵ GLT, that of AID mRNA demonstrates that the bronchial mucosa is primed to undergo class switching to IgE, and that this is unrelated to atopic or asthmatic status.

DISCUSSION

We have investigated B-cell IgE switching and synthesis in the bronchial mucosa of patients with asthma, and provide the first direct and definitive evidence that bronchial mucosal B cells undergo class switch recombination from IgM and IgG to IgE *in situ*. Our data would suggest that this phenomenon is a particular feature of asthma, and occurs irrespective of the atopic status of the patients with asthma as assigned by conventional SPT or *in vitro* testing to detect allergen-specific IgE. The expression of ϵ CT is a

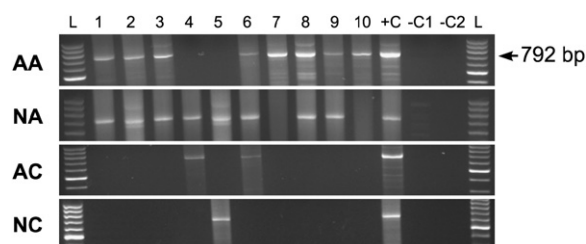


FIG 3. Expression of ϵ mRNA in the bronchial mucosa. ϵ mRNA was PCR-amplified from the bronchial mucosa of subjects with and without asthma. Lanes marked 1-10 represent 10 patients in each of the 4 groups. Lanes marked +C, -C1, -C2 and L are the PCR-positive control, first-round and second-round PCR-negative controls, and DNA ladder, respectively.

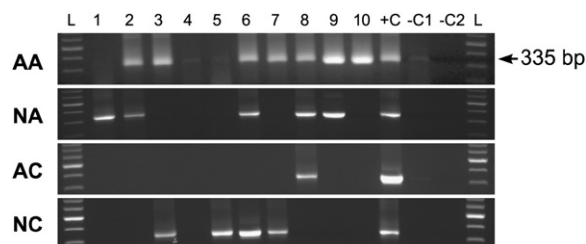


FIG 4. Expression of AID mRNA in the bronchial mucosa. AID mRNA was amplified by nested PCR reaction. Lanes 1-10 show 10 patients in each of the 4 experimental groups. Lane +C shows the PCR-positive control. Lanes -C1 and -C2 are the first-round and second-round PCR-negative controls. Lane L is the 100-bp DNA marker ladder.

marker of inevitable commitment of B cells to IgE synthesis and, unlike ϵ GLT, cannot be uniquely identified by other techniques such as *in situ* hybridization. Our data show that B cells within the asthmatic bronchial mucosa undergo CSR from both IgM and/or IgG to IgE. A previous study also provided evidence for ϵ CT production in the nasal mucosa of patients with allergic rhinitis.¹³ This propensity of B cells to undergo CSR in asthma is set against a background in which the bronchial mucosa appears to be generally conducive to Ig class switching.

We have demonstrated ϵ GLT expression in mucosal B cells of patients with asthma and controls, consistent with a previous study,¹⁶ as well as expression of the enzyme AID, which is required for the process of CSR⁹ and which is more conventionally associated with the germinal centers of lymphoid tissue.¹⁰ This may reflect a general requirement for B cells to switch to production of Igs of classes other than IgM, such as IgE or IgA, at mucosal surfaces in the course of immune surveillance or defense against attack by microorganisms. The expression of ϵ GLT and AID demonstrates that the bronchial mucosa is primed for class switching to IgE.

The mechanisms whereby AID is expressed at mucosal surfaces are not clear. Of relevance to asthma, AID expression is induced by IL-4,⁸ which is highly expressed in the bronchial mucosa of both atopic and nonatopic patients with asthma but is also detectable along with IL-13¹⁵ in controls without asthma.¹⁸ This low level of IL-4 production may nevertheless be sufficient to induce

AID expression in normal controls. There were instances in which CT was expressed in the absence of AID; this could be explained by the threshold of AID detection. Clearly, additional as yet undefined regulatory mechanisms may also operate.

Our data also show evidence of mature ϵ heavy-chain mRNA production that occurs with significantly greater incidence in patients with asthma compared with subjects without asthma, regardless of atopic status. Again, this is confirmatory of an earlier study showing elevated C ϵ mRNA expression in atopic and nonatopic patients with asthma using *in situ* hybridization.¹⁶ It is reasonable to assume that this reflects ongoing, elevated synthesis of mature IgE in the asthmatic bronchial mucosa by local B cells. ϵ mRNA in occasional biopsies from subjects without asthma most likely originates from B cells already committed to IgE synthesis elsewhere. Local production of allergen-specific IgE has in addition been demonstrated directly in the nasal mucosa of the upper respiratory tract.¹⁹ Theoretical calculations¹ suggested that the rate of local IgE synthesis in the nasal mucosa greatly exceeds that required to saturate IgE receptors on local leukocytes such as mast cells. Thus, local IgE synthesis may well account for most, if not all, biologically significant IgE production, and circulating IgE may reflect spillover of IgE from mucosal sites, rather than passage of IgE or IgE-producing B cells from more remote lymphoid tissue.

Mucosal production of IgE by B cells may be part of a wider process of clonal expansion of these cells locally within the mucosa of the respiratory tract. Evidence has previously been presented suggesting clonal expansion of B cells in the nasal¹² and bronchial mucosa.²⁰ There is similar evidence of IgE production by local germinal centers at other mucosal surfaces.²¹

Our data suggesting that CSR and bronchial mucosal IgE synthesis are features of atopic and nonatopic asthma add to the mounting evidence that atopic and nonatopic asthma are strikingly similar in terms of their cellular and molecular immunopathology.^{14,15,18} If it is assumed that a significant proportion of ongoing IgE synthesis derives from the bronchial mucosa of patients with asthma, then it is interesting to speculate why some have positive SPT and others do not. This may simply be a question of quantity, and that IgE spillover is not always detectable in the periphery. It is conceivable that all IgE synthesis is directed against as yet unknown allergens in nonatopic patients with asthma. A further possibility is that IgE is somehow sequestered within the mucosa in some patients, for instance, by autoantigens or commensal bacteria. Whatever the case, these data further narrow the gap between atopic and nonatopic asthma in terms of pathophysiology.

The clinical efficacy of monoclonal anti-IgE antibodies such as omalizumab in ameliorating some aspects of asthma²² supports the concept that ongoing IgE synthesis plays a significant role in determining asthma severity, but no study has demonstrated that removal of IgE that is directed specifically against aeroallergens is critical for this effect. Thus, regardless of the specificity of the IgE

produced in nonatopic patients with asthma, anti-IgE therapy may well be efficacious for these patients.

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