

Increases in Allergen-Specific IgE in BAL after Segmental Allergen Challenge in Atopic Asthmatics

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IgE is important in both early and late allergic responses. Increases in the numbers of RNA transcripts coding for IgE have been observed in the bronchial mucosa of asthmatics and in the nasal mucosa of hay fever patients both during natural allergen exposure and after nasal allergen challenge, suggesting that IgE may be synthesized locally in the mucosa. In this study we have examined bronchoalveolar lavage (BAL) taken before and 24 h after bronchoscopic segmental allergen challenge from 18 atopic asthmatic patients, looking for evidence of increases in IgE protein. Allergen-specific IgG and total and allergen-specific IgE were measured in BAL using a fluoroenzyme immunoassay. There was a significant increase in allergen-specific IgE (Ku/L) in the BAL after allergen challenge [before [median (interquartile range)] 0 (0, 0); after 0.35 (0, 1.87): $p = 0.009$] which was not observed for allergen-specific IgG ($p = 1.0$) or for IgE specific to an allergen to which the subject was sensitized but was not used for provocation ($p = 1.0$). Correction for corresponding increases in total IgE, albumin, and urea in BAL did not affect the observed changes in allergen-specific IgE. These data indicate that allergen provocation results in a selective local accumulation of isotype-specific and allergen-specific IgE antibody within the bronchi, independent of alterations in circulating IgE.

Keywords: IgE; asthma; bronchoalveolar lavage; allergen challenge

Atopic individuals are defined by elevated serum levels of allergen-specific IgE or positive immediate responses to skin-prick testing to one or more common inhaled allergens. Cross-linking, by allergen, of membrane-bound IgE on the surface of mast cells triggers the release of histamine and other inflammatory mediators, which results in the immediate allergic response. Interleukin (IL)-4, which is simultaneously released from mast cells, stimulates the T helper cell, type 2 (Th2) differentiation of T lymphocytes, which then preferentially secrete cytokines such as IL-4 (1) and IL-5 (2) which orchestrate IgE antibody production and the eosinophil-rich cellular infiltration that is characteristic of the late-phase response.

The process by which B cells become committed to IgE production is termed isotype switching, during which somatic recombination of the immunoglobulin heavy chain cluster gives rise to messenger RNA (mRNA) for the ϵ heavy chain. We have previously observed elevated numbers of mature ϵ -chain mRNA (Ce)-containing cells in the nasal mucosa of

patients with allergic rhinitis (3) and increased numbers of ϵ germ line gene transcripts in the bronchial mucosa of both atopic and nonatopic asthmatics (4). We have also demonstrated that the numbers of ϵ germ line transcripts in the nasal mucosa are increased by natural allergen exposure (5) or after an experimental allergen challenge (3) and that these increases are inhibited by topical corticosteroids (5). We have not, however, previously examined IgE protein levels.

Fiberoptic bronchoscopy allows a specific area of the bronchial mucosa to be challenged with allergen, and bronchoalveolar lavage (BAL) permits the epithelial lining fluid in that area to be examined. In this study, we have sought further evidence in support of local IgE synthesis in the bronchial mucosa by measuring allergen-specific IgE in BAL fluid (BALF) taken before and 24 h after bronchoscopic segmental allergen challenge, in atopic asthmatic subjects. Advantages of this approach as compared with previous RNA studies are that it enables analysis of IgE specificity and of the biologically active mature protein.

METHODS

Patients

Eighteen nonsmoking atopic patients with a clinical history of house dust mite or grass pollen-sensitive asthma took part in the study. Atopy was confirmed by a positive (> 5 mm) skin-prick test to either timothy grass pollen (*Phleum pratense*) or house dust mite (*Dermatophagoides pteronyssinus*) extracts (Soluprick; ALK Abelló, Denmark) and the presence of serum-specific IgE antibodies to the same allergen. All patients had a resting FEV₁ greater than 70% predicted. Asthma was defined by clinical history and either documented reversibility of air-flow obstruction or increased methacholine airway responsiveness (i.e., provocation concentration of methacholine required to cause a 20% reduction in FEV₁ [PC₂₀] of less than 16 mg/ml [6]). Subjects were excluded if they had received oral glucocorticoids within 6 mo or immunotherapy within the previous 5 yr. Fourteen of the 18 patients had not used inhaled corticosteroids on a regular basis and none had used leukotriene receptor antagonists. The study was performed with the approval of the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust and the written informed consent of all the participants.

Study Protocol

Subjects were admitted to hospital for 24 h during which two fiberoptic bronchoscopies were performed—before and 24 h after segmental allergen challenge. Inhaled corticosteroid drugs were discontinued for 2 wk before the first bronchoscopy. Blood was taken immediately before the performance of both bronchoscopies.

Fiberoptic Bronchoscopy

Bronchoscopy was performed between 1 and 2 P.M. in all cases. The procedure for bronchoscopy, BAL, and segmental allergen provocation was as previously described (7). During the first bronchoscopy BAL was taken from the right upper lobe. A second bronchoscopy was performed 24 h later. BAL was obtained from the site of allergen challenge (right middle lobe) and, where relevant, the saline control challenge (lingula). In 5 subjects endobronchial biopsies were taken

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from the right middle lobe immediately after BAL—both before and after allergen challenge in three patients and before challenge only in a further two patients. These biopsy samples underwent explant culture for *in vitro* assessment of IgE production. For all participants peak expiratory flow rate (PEFR) was monitored hourly, for at least 8 h, and at 22 to 24 h after the allergen challenge procedure.

Processing of BAL and Blood Samples

BAL samples were filtered through sterile gauze and centrifuged. Cytospins were prepared from resuspended cells, and BAL supernatants were divided into 1-ml aliquots and stored at -80°C until use. A differential cell count was performed on cytopins of BAL cells using May-Grünwald-Giemsa stain. Blood was centrifuged at 3,000 rpm for 10 min, then serum was removed and divided into 1-ml aliquots and stored at -80°C until used.

Measurement of Immunoglobulins, Urea, and Albumin in BAL and Serum

Aliquots of 1 ml of frozen BAL and serum were transported on dry ice to the Allergy Diagnostic Laboratory where assays were performed for total IgE, specific IgE (to the allergen used in the segmental challenge and, as a control, to an allergen to which the patient had a raised specific IgE in the serum but which had not been used in the challenge procedure), and specific IgG to the allergen used in the challenge procedure. All immunoglobulin assays were performed using an automated fluoroenzyme assay (uniCAP 100; Pharmacia Diagnostics, Uppsala, Sweden).

Assays for albumin and urea in BAL and serum were performed at the Biochemistry Department of the Royal Brompton and Harefield Hospitals NHS Trust. Serum albumin was measured with a Bromocresol Purple technique using a Beckman LX20 Analyzer (Beckman-Coulter Instruments UK Ltd., High Wycombe, Bucks, UK). BAL albumin was measured by a chemiluminescent immunoassay using the DPC Immulite analyzer (EuroDPC Ltd., Llanberis, Wales, UK). Urea concentrations in serum and BAL were measured conductometrically using the Beckman LX20 Analyzer.

Immunohistochemistry

BAL cytopins were processed for immunohistochemistry using an alkaline phosphatase–antialkaline phosphatase (APAAP) technique previously described (8). The monoclonal antibody (mAb) used was CD138 (Serotec Ltd, Oxford, UK). Briefly cytopins were incubated with the primary antibody for 30 min at room temperature, followed by 30-min incubations with unconjugated rabbit anti-mouse immunoglobulin and APAAP complex (both Dako Ltd, Cambridge, UK). The reaction was visualized with fast red alkaline phosphatase substrate.

Explant Culture Conditions

Each biopsy was washed in Hanks' balanced saline and cultured in 200 μL of Yssel's media in a 96-well plate under standard conditions (5% CO_2 and 37°C). After 24 h the supernatant was removed and stored at -20°C for analysis of total IgE by ELISA.

Detection of Total IgE in Explant Supernatants by ELISA

Plates were coated with a 1:1 mixture of two anti-IgE mAbs prepared from hybridoma cell lines, mAb 7.12 (cell line ATCC:HB-236) and mAb 4.15 (ATCC:HB-235), and incubated at 4°C overnight. Plates were then blocked with 2% marvel (Premier Brands, Wirral, UK)/phosphate-buffered saline (PBS) and incubated at room temperature for 1 h, followed by several washes in PBS-0.1% Tween (PBS-T). Samples from a given time point plus doubling dilutions of purified standard human IgE (in house) were added in duplicate to the plate and incubated at 4°C overnight. The in-house IgE standard was validated against a World Health Organization (WHO) international reference preparation of human serum IgE (75/502) supplied by the National Institute for Biological Standards and Control (N.I.B.S.C.), Potters Bar, UK (data not shown). Plates were again washed in PBS-T and incubated in horseradish peroxidase–labeled anti-IgE antibody (Dako, Cambridge, UK) for 5 h at room temperature, and washed in PBS-T before incubation in *o*-phenylenediamine dihydrochloride (OPD) solution (Sigma,

Dorset, UK) for 15 to 30 min at room temperature in the dark. The reaction was quenched with 3 M HCl and read at 492 nm using an automated ELISA reader (ICN Flow Multiskan Plus; Biological Instrumentation Services, Kirkham, Lancashire, UK).

Analysis

Data were analyzed using a statistical software package (Minitab Inc., State College, PA). Comparisons between measurements taken before and after allergen challenge were performed using the Wilcoxon matched-pairs signed-ranks test (9). Values of $p < 0.05$ were considered statistically significant.

RESULTS

The clinical characteristics of the 18 patients who participated in the study are summarized in Table 1. All participants completed the study protocol.

Clinical Response to Segmental Allergen Challenge

After the allergen challenge, 14 of the 18 subjects developed a late (> 4 h) fall in their PEFR of greater than 20% from pre-bronchoscopy baseline [mean (SD) maximal fall 24% (9.6)]. In contrast the mean (SD) maximal fall in PEFR after the second (nonchallenge) bronchoscopy was significantly lower [9.7% (9.9); $p = 0.001$]. During an earlier 24-h period of PEFR monitoring, readings did not vary by more than 10% in any individual.

The volumes of BAL recovered before [mean (SD); 57.8 (20.7) ml] and after [61.1 (22.1) ml] allergen challenge were not significantly different ($p = 0.8$). Results of all BAL and serum measurements are summarized in Table 2.

Allergen-Specific IgE Concentrations in BAL and Serum

IgE specific to the allergen used in challenge was not detectable in the BAL at baseline for any of the 18 subjects studied. After allergen challenge there were substantial increases in allergen-specific IgE in nine of the 18 subjects (Figure 1A). Eleven of the 18 subjects were sensitized (positive skin-prick test and RAST) to another allergen with which they were not challenged. IgE specific to this “irrelevant” allergen was not detectable in the BAL either before or after challenge (Figure 1B). In the serum there was a small increase (6%) in specific IgE to the challenge allergen after allergen provocation, compared with serum obtained before. This was not seen for the “irrelevant” allergen (Figures 1A and 1B).

Allergen-Specific IgG in Serum and BAL

There was no allergen-specific IgG to the allergen used in challenge present in the BAL either before or after allergen challenge. Similarly there was no change in specific IgG in serum (Figure 1C).

Total IgE in Serum and BAL

In the BAL there was a significant increase in total IgE in 13 of 18 subjects ($p = 0.03$, Table 2). The proportion of this total

TABLE 1. PATIENT CHARACTERISTICS (n = 18)

Age, mean (range)	31 (21–44)
Sex, M:F	7:11
FEV ₁ , L/min	3.6 (0.8)*
% predicted	110 (18)*
PC ₂₀ , mg/ml methacholine	3.4 (1.4, 15) [†]
RAST, Ku/L [‡]	54 (36)*

Results are expressed as * mean (SD), [†] median (interquartile range).

[‡] IgE to allergen used in challenge.

TABLE 2. SUMMARY OF RESULTS

	Before Challenge Median (IQ range)	After Challenge Median (IQ range)	Median Difference* (95% CI)	p*
BAL				
Specific IgE (challenge [†]), Ku/L [‡]	0 (0, 0)	0.35 (0, 1.9)	0.9 (0, 2.01)	0.009
Specific IgE (irrelevant [†]), Ku/L [‡]	0 (0, 0)	0 (0, 0)	0 (0, 0)	NS
Specific IgG (challenge [†]), Mg/L [§]	0 (0, 0)	0 (0, 0)	0 (0, 0)	NS
Total IgE, Ku/L [‡]	6.1 (5.3, 6.5)	7.1 (5.5, 10.7)	1.5 (0.1, 6)	0.03
Specific IgE/Total IgE, %	0 (0, 0)	4.6 (0, 16)	8.2 (0, 11.6)	0.009
Albumin, g/L	0.04 (0.03, 0.06)	0.1 (0.06, 0.15)	0.06 (0.04, 0.08)	< 0.001
Urea, mmol/L	0.3 (0.2, 0.3)	0.4 (0.3, 0.5)	0.1 (0, 0.2)	0.09
Sp IgE (challenge [†])/albumin	0 (0, 0)	0 (0, 25)	7.5 (0, 17)	0.02
Sp IgE (challenge [†])/urea	0 (0, 0)	1.8 (0, 5)	2.5 (0, 3.4)	0.02
Serum				
Specific IgE (challenge [†]), Ku/L [‡]	36 (21, 93)	37 (25, 94)	2.3 (0.2, 5.9)	0.05
Specific IgE (irrelevant [†]), Ku/L [‡]	4.7 (1, 19)	4.8 (1, 18)	0.04 (−0.1, 3.1)	0.7
Specific IgG (challenge [†]), Mg/L [§]	7.1 (4, 26)	6.7 (3, 22)	−0.6 (−1.4, 0.2)	0.1
Total IgE, Ku/L [‡]	231 (111, 404)	260 (115, 484)	13.6 (5, 49)	0.003
Specific IgE/total IgE, %	21 (11, 38)	23 (11, 35)	0.2 (−1.43, 2.43)	0.8
Albumin, g/L	44 (40, 44)	41 (38, 45)	−1 (−2, 0.5)	0.2
Urea, mmol/L	4.2 (2.8, 4.8)	3.6 (2.4, 4.1)	−0.45 (−1, 0.1)	0.1

Definition of abbreviation: NS = not significant.

* Before/after comparisons by Wilcoxon matched-pairs signed-ranks test.

[†] "Challenge" refers to the allergen used for segmental bronchial provocation. "Irrelevant" refers to an allergen to which the subject was sensitized but which was not used for provocation.

[‡] Detection limit of IgE assay 0.35 Ku/L.

[§] Detection limit of IgG assay 0.02 Mg/L.

IgE that was specific for the allergen used in challenge also increased, as shown by an increase in the specific IgE/total IgE ratio. The increases in allergen-specific IgE in BAL accounted for 60% (0.9 of 1.5 Kilo units [Ku]/L) of the observed increases in total IgE (Figure 2A, Table 2).

By contrast, in the serum the observed rises in specific IgE (10 of 18 patients, median rise 7.3, $p = 0.052$) and total IgE (13 of 18 patients, median rise 13.6, $p = 0.003$) were much smaller (approximately 6%), and there was no change in the specific IgE/total IgE ratio, with specific IgE accounting for only 17% of the increase in total IgE (Table 2).

Albumin and Urea in Serum and BAL

Compared with baseline there were no significant changes in the concentrations of albumin or urea in the serum after allergen challenge. Conversely the mean concentrations for both

albumin and urea in the BAL tended to increase after allergen challenge (Table 2). Correcting for both of these factors did not affect the observed increases in specific IgE in BAL (Figures 2B and 2C). In contrast to the increases in allergen-specific IgE/albumin, the ratio of total IgE to albumin in BAL decreased significantly after challenge [median (interquartile range) before 126 (107, 188); after 74 (39, 123), $p = 0.004$].

Cytospins

The total number of cells (stained with May-Grünwald-Giemsa stain) in the BALF increased 4-fold after allergen challenge [before [median (interquartile range)] 6 (4, 9), after 26 (16, 70); $p = 0.001$]. The proportion of eosinophils [before 1% (0, 1), after 14% (8, 22); $p = 0.002$] and neutrophils [0 (0, 2.5), 7.5 (4, 21); $p = 0.003$] increased substantially, whereas the proportion of lymphocytes [5 (3, 9), 7.5 (3, 10); $p = 0.4$] did not.

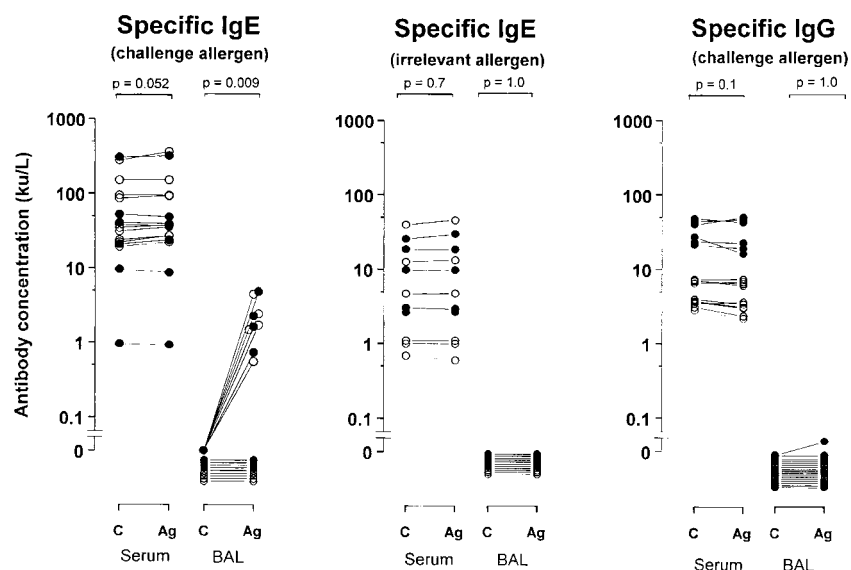


Figure 1. Allergen-specific IgE and allergen-specific IgG in BAL and serum at baseline (C) and 24 h after allergen challenge (Ag). (Right panel) Specific IgE to allergen used in segmental challenge. (Middle panel) Specific IgE to an irrelevant allergen to which patient was sensitive but was not used in challenge procedure. (Left panel) Specific IgG to allergen used for challenge. Closed circles represent challenges with *D. pteronyssinus* (house dust mite); open circles represent challenges with *P. pratense* (timothy grass pollen).

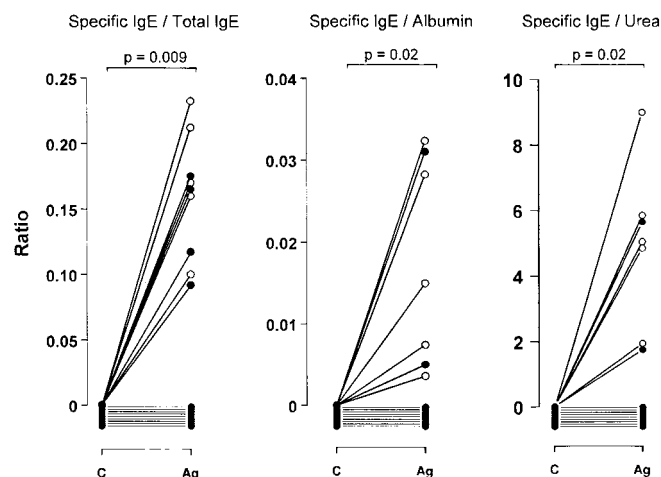


Figure 2. Ratio of allergen-specific IgE to (left panel) total IgE, (middle panel) albumin, and (right panel) urea in BAL before (C) and 24 h after (Ag) segmental allergen challenge. Closed circles represent challenges with *D. pteronyssinus*; open circles represent challenges with *P. pratense*.

There were no CD138⁺ plasma cells detected by immunohistochemistry in any of the BAL cytopins either before or after allergen challenge. In contrast, sections from nasal biopsies employed as positive controls contained CD138⁺ cells.

IgE Production by Explants of Bronchial Mucosa

Bronchial biopsies obtained at the same time as BAL from five subjects—three both before and 24 h after allergen provocation and two before provocation only—were cultured *in vitro* for estimation of IgE protein production. Both biopsy specimens from one subject produced significant amounts of IgE after 24 h incubation. This subject also demonstrated increased specific and total IgE in the BAL after allergen challenge (Table 3). In contrast, all of the other explants produced no detectable IgE after 24 h incubation. Correspondingly, none of these subjects had detectable IgE in BAL at the time of biopsy.

DISCUSSION

We have shown an increase in allergen-specific IgE concentrations in BALF obtained from atopic asthmatic subjects at 24 h after segmental allergen challenge. This IgE was allergen-specific, isotype-specific and was disproportionately raised in comparison to total IgE concentrations in BAL of the same subjects. Correction for the effects of vascular leakage (increases in albumin) and variable dilution of BAL (changes in urea) during the late response did not alter the observed increases in allergen-specific IgE. In addition, bronchial biopsies taken simultaneously with BAL samples and cultured for 24 h produced IgE *in vitro* when obtained from a patient in whom IgE was detectable in BAL but not in patients in whom IgE was undetectable in BAL *in vivo*.

These data clearly demonstrate the presence of allergen-specific IgE in BAL 24 h after segmental allergen challenge. Increases were not observed in all subjects, which may represent a heterogeneity of response in patients with differing allergen sensitivity to a uniform dose of allergen, a different time-course of the response in different individuals after a single dose of allergen, or the failure of postallergen BAL IgE concentrations to reach detectable levels. The source of the antibody remains to be confirmed. One possibility is that the IgE may enter the epithelial lining fluid directly from the

TABLE 3. COMPARISON OF IgE IN BAL AND BRONCHIAL EXPLANTS

Patient No.	Challenge	Serum-specific IgE (Ku/L)	Specific IgE in BAL (Ku/L)		Total IgE in BAL (Ku/L)		Total IgE in Explants* (Ku/L)	
			Pre	Post	Pre	Post	Pre	Post
8	PP	100	0	0.56	5.5	5.6	< 1	—
9	PP	44	0	0	5.7	7.0	< 1	—
10	DP	100	0	0.7	6.1	8.1	7.0	7.9
11	PP	40	0	0	7.1	7.2	< 1	< 1
12	PP	26	0	0	6.4	5.2	< 1	< 1

Definition of abbreviations: DP = *Dermatophagoides pteronyssinus*; PP = Phleum pratense. * Explants from bronchial biopsies cultured *in vitro* for 24 h.

plasma. The observed small increase in total IgE (by 15%) in BAL after allergen challenge supports this argument, but the percentage of this total IgE that was allergen-specific remained highly significantly increased in the BAL in contrast to the serum, where both specific and total IgE increased slightly with no change in their relative proportions.

Albumin concentration in the BALF increased 2.5-fold 24 h after allergen challenge, reflecting the increase in vascular permeability that occurs during both early and late phases of the allergic response. There remained, however, a persistent increase in specific IgE in BAL after correction for albumin. This is consistent with previous studies showing a higher ratio of IgE to albumin in nasal polyp fluid than in serum (10) and increases in IgA in BAL, even after correction for albumin, after segmental provocation with a highly soluble immunogenic antigen (11). It is probable that some IgE will have entered the epithelial lining fluid as a result of exudation from plasma, with the fall in the ratio of total IgE to albumin in BAL after allergen challenge reflecting the relative speed of movement of the two molecules resulting from their respective molecular weights (albumin: 70,000; IgE: 170,000) (10). Taken together these data suggest that, although some simple leakage of antibody will occur, it is an insufficient explanation for the disproportionate increase observed in allergen-specific IgE over and above total IgE and albumin after segmental allergen provocation. It is possible that specific IgE produced in the bronchial mucosa in response to the presence of allergen may subsequently enter, and be detectable in, the blood, partially explaining the increases in serum IgE.

In contrast to the IgE response to the allergen that was used for bronchial challenge, the concentration of allergen-specific IgE to an allergen to which the patient was sensitized but which was not used for challenge did not change. This strongly supports the specificity of the local IgE response in the bronchi for the provoking allergen. There was also no increase in allergen-specific IgG, which confirms the class-specificity of the response, again indicating the importance of the local IgE response to the provoking allergen.

A previous study examined changes in BAL immunoglobulin levels in ragweed-sensitive patients. Similar increases in ragweed-specific IgE were found in 11 of 19 patients, which correlated with concentrations of eosinophil cationic protein (ECP). However, this study did not examine the allergen-specificity of the response or compare the response with total IgE, albumin, or urea (12). In a more recent study using whole lung allergen challenge, ragweed-specific IgE was detectable in BAL before challenge in four of 16 subjects in whom larger late asthmatic responses were observed. However, no post-challenge IgE measurements were documented and the source of the IgE detected in BAL was not investigated (13).

There is additional evidence supporting local IgE production in the respiratory mucosa available from nasal studies in humans. Several studies in hay fever patients have shown the presence of allergen-specific IgE in nasal secretions despite the absence of salivary IgE (14), serum IgE (15), or positive skin-prick tests (16). *D. pteronyssinus*-specific IgE was found to increase significantly in the nasal secretions of Italian children during periods of allergen exposure and to decrease with allergen avoidance despite only small and inconsistent changes in serum IgE (17). Compared with normal control subjects, the nasal mucosa of allergic patients contains abundant cells that stain with biotinylated allergen (18) and significantly more allergen-specific IgE⁺ B cells and plasma cells (19).

We and others have shown that IgE is expressed by B cells at RNA level in the nasal mucosa (3, 20) and in the bronchial mucosa of asthmatics compared with normal control subjects (4). In the current study, we have demonstrated in five subjects that IgE synthesis by bronchial explants corresponded to specific IgE concentrations in BAL, i.e., IgE synthesis was detectable in one of the two subjects who had increases in specific IgE in BAL and in none of the three who did not. Although this is currently an isolated observation, it is suggestive that IgE measurements in BAL may reflect ongoing local mucosal IgE synthesis which has previously been shown to precede systemic synthesis (17).

Earlier studies (21) suggest that the time-course of B-cell switching is substantially longer than 24 h making it likely that the increase in IgE we have observed is caused by synthesis of IgE by B cells that are already switched and committed to allergen-specific IgE production activated by the presence of allergen rather than by newly switched B cells. A different approach, involving the demonstration of S ϵ /S μ deleted switch circular DNA in cultured explants, is required to test whether switching and local IgE synthesis is also induced by allergen challenge.

The importance of clearly defining the role of IgE, whether locally or systemically produced, in allergy lies in the potential for new antiallergic treatments. Intravenous treatment with monoclonal anti-IgE antibody resulted in small improvements in asthma symptom scores and reduced corticosteroid use in patients with perennial allergic asthma (22). In contrast topical (inhaled) anti-IgE therapy did not block allergen-induced late responses (23), possibly as a consequence of failure of mucosal penetration using a high-molecular-weight antibody based therapy. The evidence supporting an important role for local IgE synthesis, enhanced by the present study does, however, suggest that further attempts at targeting IgE synthesis, probably using small molecular inhibitors of IgE/Fc ϵ R1 interaction (24), are indicated.

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