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Expression of ϵ germ-line gene transcripts and mRNA for the ϵ heavy chain of IgE in nasal B cells and the effects of topical corticosteroid

We have studied the expression of the gene encoding the ϵ heavy chain of IgE in nasal B cells of hayfever patients. We developed probes to detect transcripts of the ϵ germ-line gene and the rearranged gene by *in situ* hybridization of biopsy sections from the nasal mucosa. We compared tissue from hayfever patients out of season with that of normal controls, and also of hayfever patients treated with topical corticosteroid (fluticasone propionate) or placebo for 6 weeks and then challenged with antigen. ϵ chain mRNA was expressed in an unexpectedly high proportion of nasal B cells of both hayfever patients and normal subjects. However, although similar numbers of B cells were found in both groups, the proportion of cells that express ϵ chain mRNA was several times higher in the hayfever patients. No transcripts of the ϵ germ-line gene were detected in either group before allergen challenge. When hayfever patients were administered antigen locally, early (10–30 min) and late (1–24 h) symptoms ensued. After 24 h, coincident with an increase in the number of cells expressing mRNA for IL-4 in the tissue, ϵ germ-line gene transcripts appeared in the nasal B cells. The induction by allergen of IL-4 mRNA and ϵ germ-line gene transcripts was suppressed by fluticasone propionate treatment. Our results suggest that local IgE synthesis and cytokine regulation of heavy chain switching to IgE occur in the nasal mucosa.

1 Introduction

1.1 Background

Immunoglobulin E (IgE) mediates allergic reactions by binding to its high-affinity receptor, Fc ϵ RI, on mast cells and basophils. Cross-linking of the attached IgE by multivalent antigen then triggers the allergic response. IgE antibodies on the mast cells of the nasal mucosa are cross-linked by airborne allergens, leading to the symptoms of hayfever. The provenance of the IgE on nasal mast cells (and indeed on mast cells at other mucosal surfaces, such as the lung, gut and skin) is unknown [1]. IgE may be secreted by B cells and bound to receptors on mast cell precursors and basophils elsewhere and transported to the nasal mucosa, or it may be produced by B cells within the nasal mucosa itself. IgE is the least abundant class of Ab (~ 100 ng/ml in normal serum, compared to 10 mg/ml for IgG). 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 immunoglobulin heavy chain gene cluster to transform the ϵ germ-line gene into the rearranged gene. This then gives rise to mRNA for the ϵ heavy chain of IgE [2]. If B cells do

provide a local source of IgE in the nasal mucosa, then there are two further possibilities. B cells may switch to IgE before or after they migrate to the tissue.

Evidence in favor of local IgE synthesis has recently come from studies of cultured B cells derived from human nasal secretions, which express IgE at both the mRNA and the protein level [3–5]. It is not clear, however, that these cells ever resided in the nasal mucosa. Heavy chain switching occurs in B cells within lymphoid tissue *in vivo* [6, 7] and in isolated B cells *in vitro*. It is not known whether B cells within non-lymphoid tissues ever undergo switching.

Two signals are required for heavy chain switching to IgE both *in vitro* and *in vivo*. IL-4 and the recently described IL-13 are agents that 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 [8]. IL-4 is released from cytoplasmic stores on activation of mast cells [9] and, like IL-13 [10], is secreted by a subset of T helper cells (Th2 cells) [11]. CD40 ligand (CD40L) is constitutively expressed by tissue mast cells and induced on T cells, following activation by antigen [12, 13]. IL-4 is required for the differentiation of T cells into the Th2 phenotype, which secrete their own IL-4, and thereafter auto-crine production of IL-4 supports cell proliferation [14]. Thus it has been inferred that activation of mast cells by allergen may promote the differentiation of T cells into Th2 cells and initiate switching to IgE in local B cells [12, 15–18]. The resulting Th2 cells, upon activation by antigen, could then continue the process.

We observed earlier that allergen provocation in the nasal mucosa of hayfever patients resulted in early (0–60 min) and late (1–24 h) nasal symptoms, accompanied by the recruitment of Th2 cells (predominantly CD4⁺ Th2 lymphocytes) expressing IL-4 [19, 20] and IL-13 [21], assessed

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at 24 h. We and others [22] have also found that nasal tissue harbors a population of B cells, the extent of which is unaffected by allergen challenge. In a double-blind trial we further showed that pretreatment with the topical corticosteroid, fluticasone propionate (FP), inhibits allergen-induced early and late nasal responses, as well as the associated recruitment of Th2 cells and expression of IL-4 [23]. This suggested that the release of IL-4 by mast cells and/or T cells on activation by allergens may induce heavy chain switching to IgE *in situ* and that some of the committed cells may go on to secrete IgE.

To resolve this question in respect of nasal tissue, we have re-examined the number of B cells and IL-4-expressing cells in the nasal mucosa of hayfever patients exposed to allergen, including those pretreated with FP, using previously described methods of immunohistology and *in situ* hybridization on nasal biopsy sections [19, 20, 24]. In addition we have used new RNA probes to detect whether resident B cells have the capacity to express IgE or undergo allergen-induced local switch recombination to IgE.

1.2 Experimental approach

IL-4 and IL-13 direct heavy chain switching to IgE by stimulating the transcription of the ϵ germ-line gene [8]. Germ-line gene transcripts contain a specific sequence, the I ϵ sequence, which is spliced to the sequence that codes for the constant region of the ϵ chain (C ϵ 1–C ϵ 4) (Fig. 1). However, the I ϵ exon contains a number of stop codons and hence the germ-line gene transcript is not translated into protein. The I ϵ exon is deleted in the switch, so that the I ϵ sequence is absent from ϵ chain mRNA transcribed from the rearranged gene. Thus it provides a specific probe for the detection of the ϵ germ-line gene transcripts. We have been able to determine in this way whether the increase in IL-4 mRNA (and presumably protein), induced by allergen challenge in the nasal mucosa of hayfever patients, indeed leads to the appearance of ϵ germ-line gene transcripts in neighboring B cells, as we predicted.

Probing for transcripts of the rearranged gene presents greater difficulty. We have used an ϵ chain constant region probe, the C ϵ probe, complementary to the sequence in ϵ chain mRNA that codes for the C ϵ 2–C ϵ 4 region of the ϵ chain. However, this sequence also occurs in germ-line gene transcripts. These contain a single copy of I ϵ and C ϵ 2–C ϵ 4 sequences (Fig. 1), and the two probes which we have prepared are of similar lengths (650 and 526 nucleotides, respectively; Sect. 2.3). When labeled under the same conditions they should therefore afford comparable sensitivity. Sensitivity may not be an issue anyway, since we measured the number of positive cells, not the amounts of RNA per cell. All cells that contained RNA in excess of the lower limit of detection were counted. The difference between the numbers of cells detected with the C ϵ and I ϵ probes was then the number that express ϵ chain mRNA. Several experimental observations justified the subtraction procedure. Thus, double *in situ* hybridization of nasal biopsy sections with the I ϵ and C ϵ probes revealed that essentially all I ϵ ⁺ cells are also C ϵ ⁺, but not *vice versa* (Sect. 3.4). Moreover, we found that nasal biopsy samples from hayfever patients between seasons and normal individuals contained only C ϵ ⁺ cells, but after allergen challenge

there was an *increase* in the number of C ϵ ⁺ cells above this background, equal to the total number of I ϵ ⁺ cells (Sect. 3.4 below).

We have used the combination of I ϵ , C ϵ and IL-4 probes to determine the concentrations of B cells expressing ϵ germ-line gene transcripts, ϵ chain mRNA and IL-4 mRNA in the nasal mucosa of hayfever patients before and 24 h after allergen challenge, and after 6 weeks treatment with FP. Parallel experiments were carried out with a probe for γ chains (the C γ probe) to compare the effects of allergen challenge and steroid treatment on expression of ϵ and γ chain genes. We have also used double immunohistochemical staining with differentially labeled antibodies against IgE plus either CD20 (for B cells), CD38 (for plasma cells) or tryptase (for mast cells) in an attempt to distinguish IgE associated with these cell types in the nasal biopsy sections.

2 Materials and methods

2.1 Patients

The study was performed with the approval of the Royal Brompton Hospital Ethics Committee and the patients' written informed consent. Twenty-one patients with summer hayfever were studied. Inclusion criteria for the patients included a history of at least 2 years of summer hayfever and a positive skin prick test (greater than 5 mm) to Timothy grass pollen extract (*Phleum pratense*, Soluprick, ALK, Denmark). Patients were all non-smokers. None had received topical or oral medication in the previous 6 months, or immunotherapy in the previous 5 years. Normal subjects were non-atopic (negative skin test) non-smokers with no nasal symptoms. Baseline blood samples and nasal biopsies were performed out of season at a time when the patients were asymptomatic. Patients were randomized to receive 6-week pretreatment with topical nasal corticosteroid (200 mg FP, two sprays per day) or a matched placebo nasal spray containing the aqueous diluent. After treatment all patients underwent local nasal provocation with grass pollen extract, followed by a second nasal biopsy after 24 h. Nasal provocation was performed with 1000 biological U of grass pollen extract (*P. pratense*, ALK, Denmark) applied on a 4-mm filter paper disc to the under-surface of the inferior nasal turbinate. Nasal biopsies were taken from the site of allergen challenge using 2.5-mm diameter cup-and-ring forceps (Gerritsma forceps) as described [19].

2.2 Immunohistology

The mAb used were anti-CD20 for B cells (Dako Ltd., High Wycombe, GB), anti-IgE (Vectra Ltd., GB), anti-tryptase antibody G3 for mast cells (kindly provided by Dr. L. Schwartz, Richmond, VA), and anti-CD38 antibody for plasma cells (Dako).

Immunohistology of nasal biopsies was performed on 6- μ m cryostat sections using a modified alkaline phosphatase anti-alkaline phosphatase (APAAP) method [24] (Fig. 3A). *In situ* hybridization was performed on 10- μ m cryostat sections, pre-fixed in 4 % paraformaldehyde and hybridized

with antisense riboprobes, following pretreatment of sections with RNase as described [19]. Controls were performed with sense probes (having an identical sequence to messenger RNA) (Fig. 3B, C, E, F).

Immunohistochemical staining for IgE was performed on biopsy specimens from 9 grass pollen-sensitive patients and 12 non-atopic normal subjects outside the pollen season. Double immunohistochemistry was performed on biopsy specimens from 7 pollen-sensitive patients using anti-IgE (immunoperoxidase method, in which positive cells stain brown), followed by the phenotypic Ab markers (APAAP method, in which positive cells stain red), resulting in double-positive cells, which stain red-brown [25, 26].

Tissue sections were assessed using an Olympus BH2 microscope with an eyepiece graticule at 200 × magnification. Counts were made along the whole length of nasal biopsy samples to a depth of 0.45 mm (including the whole samples lamina propria, where the majority of inflammatory cells were located). Counts were expressed per high-power field (hpf, 0.202 mm²). The coefficient of variation between observers was <5 % for *in situ* hybridization and 8 % for immunohistology counts.

2.3 Genetic probes

Our Iε probe (Fig. 1) corresponded to the sequence from the start site of ε germ-line gene transcription, referenced as nucleotide 1, to the end of the Iε exon at position 650. This was generated by cloning the indicated Bam HI fragment (top line; accession no. X56797) into Bluescript Ksp. The plasmid, linearized with Xba I or Hind III, was transcribed in the presence of [³²P]UTP with T7 polymerase for the antisense probe and with T2 polymerase for the sense probe used as a negative control [27].

The Cε probe was generated from an ε chain cDNA clone [28]. The sequence corresponded to a 1150-base Hind III fragment from pSC213, encompassing 24 bases of Cε1 and the rest of the Cε sequence. This fragment was cloned into pGEM [23], cut at a Sma I site and transcribed with SP6 polymerase for the antisense probe (526 nucleotides), and cut at the Bam HI site and transcribed with T7 polymerase for the sense probe, used as a negative control.

For the consensus γ chain constant region probe (Cγ), a 5' end-labeled 30-base oligonucleotide was used, corresponding to the Cγ2 region (IgG1, nucleotide 1206–1235 of accession no. J00228; IgG2, nucleotide 1204–1233 of J00230; IgG3, nucleotide 1785–1814 of X03604; IgG4, nucleotide 1205–1234 of K01316). The consensus Cγ probe had complete homology to all four IgG subclasses.

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 above.

2.4 In situ hybridization

Double immunostaining and *in situ* hybridization [20] (see Fig. 3D) were performed with anti-CD20 Ab, visualized

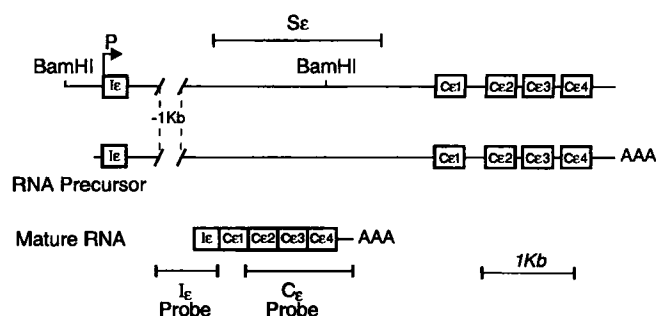


Figure 1. Identity of the Iε and Cε and probes. Top line: the ε germ-line locus (DNA), showing sites of digestion with Bam HI, the start site of transcription (P), the switch region (Sε) and the Iε exon and the exons encoding the four ε chain constant regions (Cε1–4). Middle line: the unprocessed ε germ-line gene transcript. Bottom line: the mature RNA ε germ-line gene transcript after RNA splicing, and the sequences corresponding to the Iε and Cε probes. Exposure to IL-4 induces transcription of the ε germ-line gene from the promoter site (P). Transcription proceeds through the ε chain constant region-coding exons Cε1–Cε4, but RNA splicing joins the Iε and Cε1 exons. When genetic recombination occurs, the DNA between the recombined sequences in the homologous switch regions (Sε), including the Iε exon, are deleted and replaced by a transcription unit containing the ε chain variable region coding exons. The Iε probe thus detects the transient appearance of ε germ-line gene transcripts, which precedes rearrangement of the gene and the synthesis of mRNA. The Cε probe detects both the ε germ-line transcript and ε chain mRNA.

by diaminobenzidine (DAB) brown staining. *In situ* hybridization was then performed using a ³⁵S-labeled complementary RNA probe, followed by autoradiography. Individual double-positive cells were evident as brown cells within a clump of black silver grains at 400 × magnification.

Simultaneous *in situ* hybridization with probes coding for Cε and Iε was performed as described [29]. In brief, the Cε probe was labeled with digoxigenin and the Iε probe with ³⁵S-labeled UTP. The probes were applied simultaneously to 10-μm cryostat sections of nasal biopsy samples from four patients who had received placebo nasal treatment at 24 h after allergen provocation with grass pollen extract. Hybridization was performed at 42°C for 12 h. The Cε probe was visualized with antibody to digoxigenin labeled with chromogen. Slides were then exposed for 10 days and Iε RNA was visualized by autoradiography.

2.5 Statistical analysis

Patients were stratified according to their level of skin prick test sensitivity to grass and hayfever severity before random selection to receive either FP or matched placebo nasal spray. This resulted in closely matched groups for comparison (Table 1). All biopsy samples were coded in random order and sections counted blind, independently of the clinical protocol. Within-group comparisons (baseline/allergen) were based on the Wilcoxon matched-pairs signed-ranks test. Between-group comparisons (magnitude of changes between baseline and allergen for subjects on FP compared with those on placebo nasal spray) were performed using an unpaired analysis (Mann-Whitney U test). Correlations were performed using Spearman's

rank method. Statistics were performed using a statistical package (Minitab Statistical Software, Minitab, In. PA 16801-2756, USA). p values less than 0.05 were taken as significant.

3 Results

3.1 Clinical data

The clinical features of the 21 patients with summer hayfever examined in this study are shown in Table 1. Patients treated with placebo or topical corticosteroid (FP) were well matched for age, gender and circulating grass pollen-specific IgE concentration. Clinical results of nasal allergen provocation are summarized in Fig. 2. Allergen provocation resulted in early and late increases in sneezing, nasal blockage and secretions (all $p < 0.01$) in the placebo-treated group. These effects were all markedly reduced in the topical steroid-treated group.

3.2 Colocalization of ϵ gene transcripts to B cells

CD20⁺ B cells were present in the nasal epithelium and submucosa in about the same numbers in atopic and normal subjects and did not change after allergen challenge (Table 1, Fig. 3A). Each C ϵ ⁺ cell was identified by a discrete cluster of silver grains (Fig. 3C). In three individuals, double immunostaining/*in situ* hybridization showed that more than 95 % of C ϵ ⁺ cells were identified as CD20⁺ B cells (Fig. 3D). From typical sections such as this it was apparent that a high proportion of the B cells in this tissue express the ϵ chain gene.

3.3 Expression of C ϵ and I ϵ RNA in hayfever patients and normal controls

The numbers of C ϵ ⁺ and I ϵ ⁺ cells in the nasal mucosa were examined in hayfever patients out of season and compared with corresponding cell counts in normal healthy control subjects. No cells positive for I ϵ were detected in either group. Interestingly, however, the mean value for the number of C ϵ ⁺ (mRNA⁺) cells was several times higher in hayfever patients than in normal controls (Fig. 4).

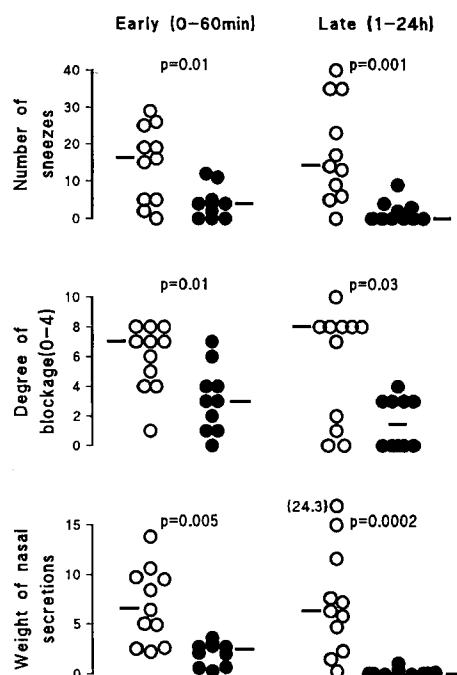


Figure 2. Nasal allergen provocation tests. Data are presented as individual values after FP (●) or placebo (○) nasal spray. All symptoms (early and late) were inhibited by topical corticosteroid (all $p < 0.01$, except late blockage $p < 0.03$; Mann-Whitney U Test).

3.4 Effect of local allergen challenge on expression of I ϵ , C ϵ , C γ and IL-4 RNA in hayfever patients

Patients with hayfever showed a marked increase in the number of C ϵ ⁺ cells 24 h after allergen challenge (Fig. 5A) and I ϵ ⁺ cells became clearly detectable (Fig. 5B). There was a significant positive correlation between the number of I ϵ ⁺ cells and the increase in C ϵ ⁺ cells ($r = 0.76$, $p < 0.01$); *i.e.* the number of I ϵ ⁺ cells was similar to the increase in the number of C ϵ ⁺ cells in individual patients. Simultaneous *in situ* hybridization of allergen-challenged biopsy samples from four patients with C ϵ and I ϵ probes revealed that all I ϵ ⁺ cells were also C ϵ ⁺. However, only 65 % (60–70 %) of C ϵ ⁺ cells were C ϵ ⁺ and I ϵ ⁺, *i.e.* a mean of 35 % of C ϵ ⁺ cells were C ϵ ⁺ and I ϵ [−]. These findings demonstrate that the two probes are equally sensitive and that I ϵ [−] C ϵ ⁺ RNA is ϵ chain mRNA (Sect. 1.2).

Table 1. Clinical features of 21 patients with summer hayfever

	Placebo	FP
Number	11	10
Age (years)	31.3 (2.7)	30.0 (2.4)
Gender	6M, 5F	4M, 6F
Serum total IgE (KU/l)	50.9 (17)	92 (23)
Serum grass pollen-specific IgE (Phadebas scale 0–5)	2.7 (0.4)	3.0 (0.3)
B cells (CD20 ⁺) in nasal Mucosa (cells/hpf) ^a		
Before allergen	7 (3–17)	7 (1–21)
After allergen	2 (1–30)	2 (1–12)

a) B cell counts are expressed as medians (interquartile ranges) per high power field (hpf).

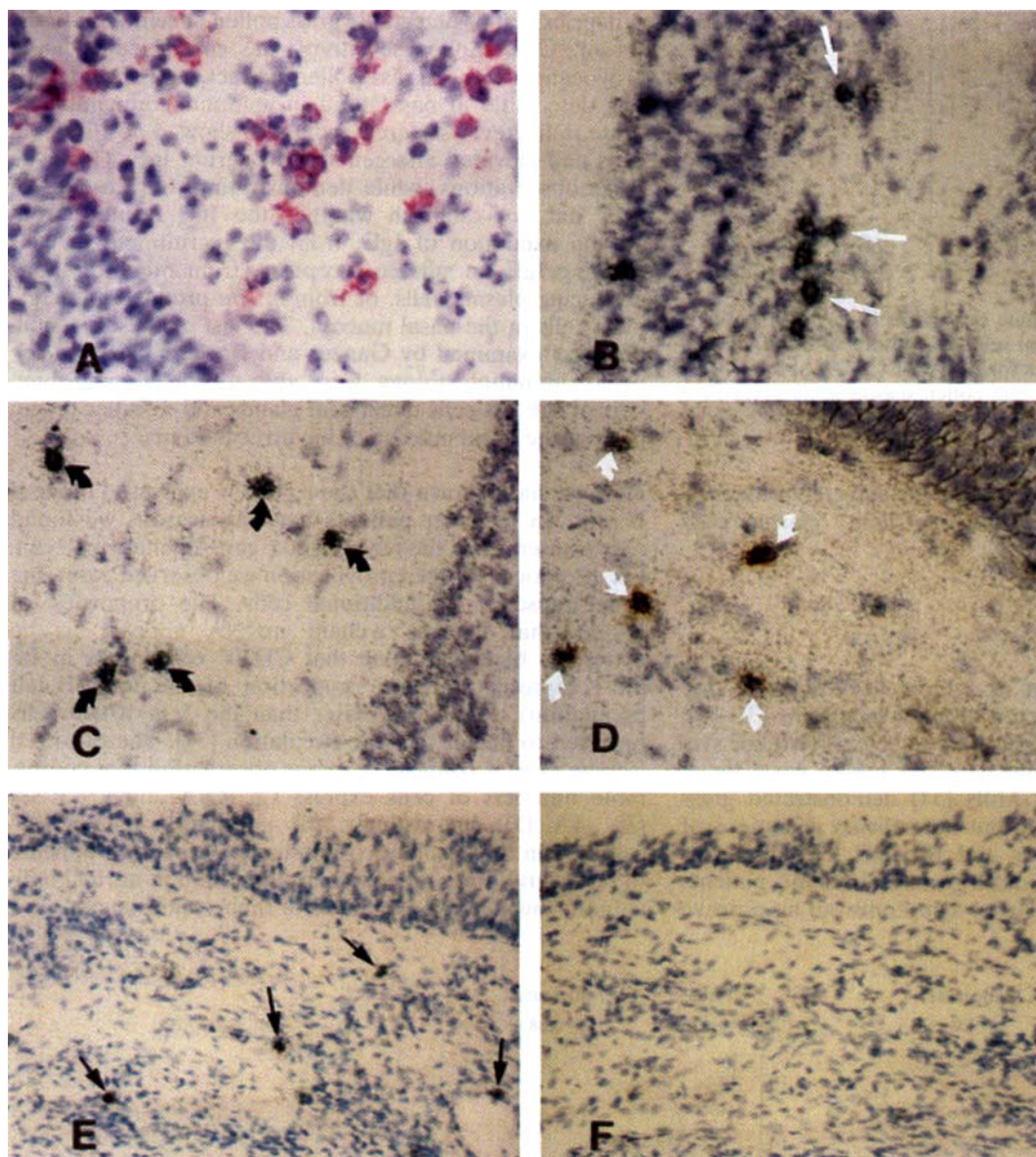


Figure 3. Immunohistology and *in situ* hybridization of nasal biopsy specimens. (A) CD20⁺ B cells, (B) IL-4 mRNA⁺ cells, (C) IgE Cε⁺ cells after allergen challenge which are colocalized to CD20⁺ cells, (D) by use of double immunohistochemistry/*in situ* hybridization. IgE expression after allergen is compared in placebo and topical corticosteroid-treated patients in panels (E) and (F).

Increases in the number of cells expressing Iε and Cε RNA were accompanied by a rise in the number expressing IL-4 RNA (Fig. 5C). However, the magnitudes of these effects were not correlated, reflecting the fact that IL-4 expression occurs in different cell types, whereas the parallel increases in numbers of Cε⁺ and Iε⁺ cells are likely to refer not only to the same cell type but to identical cells.

A Cγ probe was used to compare the effect of allergen challenge on expression of ε and γ chain genes in the hayfever patients. In contrast to the ratio of the immunoglobulins in serum (IgG:IgE = 10 000:1), the populations of Cγ⁺ and Cε⁺ cells in the nasal mucosa before allergen challenge were about equal. Furthermore, allergen challenge resulted in a significant *decrease* in the Cγ⁺ population, contrasting to an *increase* in Cε⁺ cells (Fig. 5A, D).

3.5 Effects of topical corticosteroid therapy in hayfever patients

Topical corticosteroid therapy resulted in inhibition of these allergen-induced changes within the nasal mucosa (Fig. 5A–D). Thus, in FP-treated patients there were no significant changes in either Cε, Iε, IL-4 or Cγ RNA⁺ cells. The differences between them and placebo-treated patients were all highly significant (Fig. 5).

3.6 Immunolocalization of IgE

IgE protein-positive cells were more numerous in biopsy samples from atopic, grass pollen-sensitive patients outside the pollen season when compared with non-atopic normal subjects (Fig. 6). Double immunohistochemistry revealed that the majority of these cells were mast cells

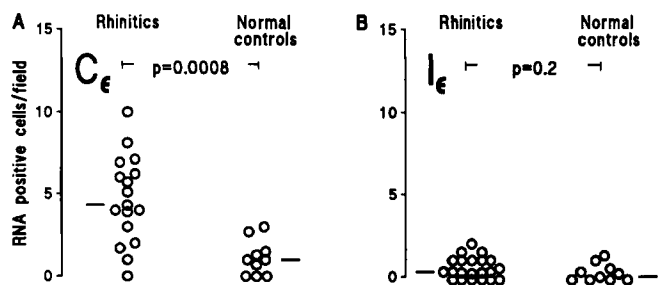


Figure 4. Comparison of ϵ gene transcripts in hayfever patients and non-atopic normal control subjects. Results of *in situ* hybridization using antisense probes for RNA for $C\epsilon$ (A) and $I\epsilon$ (B) of nasal biopsy samples outside the pollen season in hayfever patients and non-atopic normal control subjects are shown.

(tryptase positive), and there was no detectable localization to either $CD20^+$ B cells or $CD38^+$ plasma cells (Fig. 7).

4 Discussion

Following the recognition of IgE as the "reaginic antibody" [30], IgE was detected in nasal secretions [31–34]; this raised the possibility that there might be local IgE synthesis. Ohashi et al. further detected IgE in nasal tissue homogenates [35]. Platts Mills [34] demonstrated grass pollen-specific IgE in nasal but not salivary secretions of hayfever patients. Huggins and Brostoff [31] suspected local IgE synthesis on clinical grounds in some patients with clear-cut seasonal hayfever symptoms but no positive

skin prick test or detectable grass pollen-specific IgE in the serum. Grass pollen sensitivity was confirmed by nasal provocation with a grass pollen extract, and specific IgE was detectable in nasal fluid in amounts comparable to those in hayfever sufferers, who also showed positive skin tests and elevated allergen-specific serum IgE. However, these observations, while demonstrating the presence of IgE, did not establish whether the IgE resulted from plasma exudation of IgE, from the recruitment of cells carrying IgE on surface receptors, from migratory IgE-producing plasma cells, or from *in situ* production of IgE by B cells in the nasal mucosa. The last of these possibilities was examined by Ganzer and Bachert [36] who, by use of immunohistology, were able to detect small numbers of IgE^+ B cells in cervical glands and tonsillar tissue, but not the nasal mucosa of hayfever patients.

Here we have shown that the ϵ gene is expressed in nasal B cells. In hayfever patients between seasons we found only transcripts of the rearranged ϵ gene (ϵ chain mRNA), but after local allergen provocation we observed germ-line gene transcripts in additional cells. The proportion of B cells that express ϵ chain mRNA appears to be extremely high (assuming that $CD20^+$ cell counts in Table 1 represent a large proportion of the total B cell population), certainly greater than the 1 in 10000 cells reported to be IgE^+ in the circulation [37]. The distorted isotype ratio is confirmed by our observation that comparable numbers of cells express $C\epsilon$ RNA and $C\gamma$ RNA (Fig. 4A, D). Interestingly, the population of $mRNA^+$ cells within the nasal mucosa in hayfever patients appears to be several times greater than that found in normal individuals: this may be diagnostic of their condition.

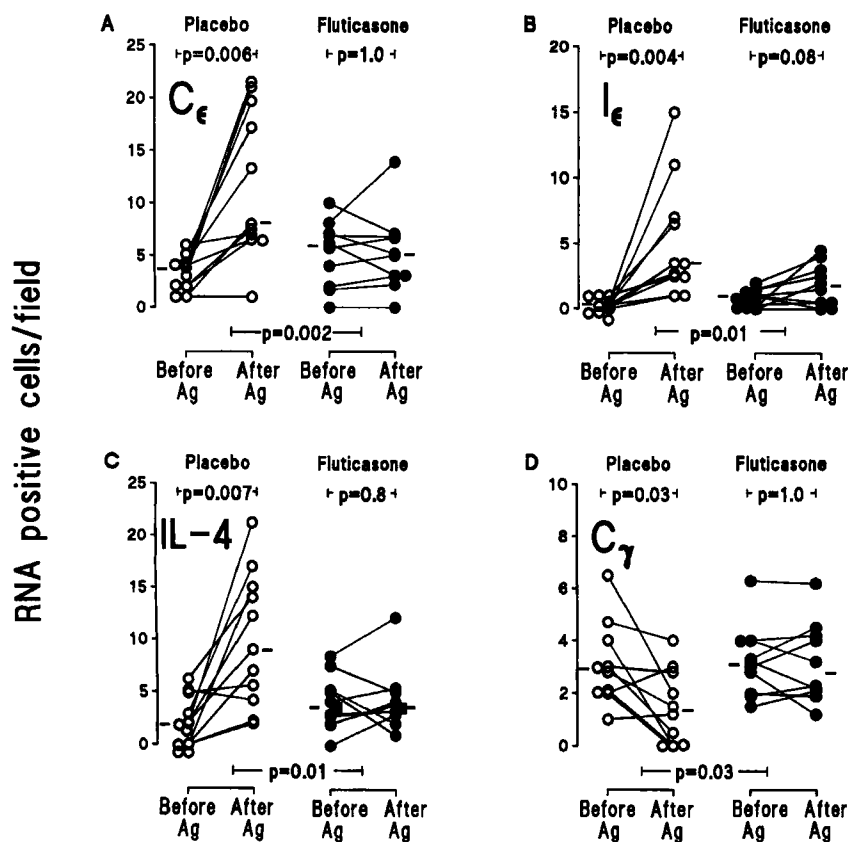


Figure 5. *In situ* hybridization using antisense probes for RNA in nasal biopsy samples obtained before and after nasal allergen provocation. (A, B) $C\epsilon$ and $I\epsilon$ in placebo- and topical corticosteroid-treated patients. $C\gamma$ and IL-4 mRNA $^+$ cells were also examined in the same samples (C, D). Solid horizontal bars represent median values. Within-group comparisons (before/after allergen) were made using the Wilcoxon test. Between-group comparisons (FP vs. placebo) were made using the Mann-Whitney U Test. Levels of statistical significance are shown.

Our work provides strong evidence that local regulation of IgE synthesis by IL-4 may occur in nasal B cells. Further studies are needed to strengthen this conclusion, but there are several implications: local regulation of IgE synthesis could account for the efficacy of topical steroid treatment in hayfever patients, and could be the basis for the development of new therapeutic strategies. It may also explain why only a proportion of "atopic" individuals (with a genetic predisposition to exhibit exaggerated IgE responses) develop hayfever, while others have either no clinical manifestations or develop atopic disease (such as asthma or eczema) in other anatomical locations.

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