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Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients

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Production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients. A. KleinJan, J.G. Vinke, L.W.F.M. Severijnen, W.J. Fokkens. ©ERS Journals Ltd 2000.

ABSTRACT: Allergic diseases are characterized by allergic complaints in the shock organ and specific immunoglobulin (Ig)E in serum. Literature data indicate that the nasal mucosa itself could produce at least a large part of the specific IgE in allergic rhinitis patients.

In order to investigate this hypothesis, nasal mucosal biopsies from the inferior turbinate were taken from symptomatic grass pollen allergic rhinitis patients, symptomatic house dust mite allergic rhinitis patients and nonallergic healthy controls, confirmed by radioallergosorbent test and skin-prick test. Immunohistochemical double-staining was performed for B-cells (CD19) with IgE, plasma cells (CD138) with IgE and plasma cells with biotinylated allergens.

Significantly more IgE-positive B-cells and IgE-positive plasma cells were found in the nasal mucosa of allergic patients than in that of nonallergic controls. Double staining with biotinylated allergens and plasma cells showed allergen-positive plasma cells in the nasal mucosa of allergic patients and no allergen-positive plasma cells in the nasal mucosa of nonallergic patients. Blocking experiments using polyclonal antibodies directed against IgE showed a significant reduction in the number of allergen-positive cells in contrast to experiments using polyclonal antibodies directed against IgG, IgA or IgM.

This study describes new evidence that specific immunoglobulin E is produced locally in the nasal mucosa in patients with seasonal allergic rhinitis and perennial allergic rhinitis, but not in nonallergic controls.
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To date, it has not been known where the production of specific immunoglobulin (Ig)E takes place in allergic rhinitis patients. Some studies have suggested that the nasal mucosa itself is able to produce at least a large part of the IgE [1–5]. HUGGINS and BROSTOFF [6] reported patients who had specific IgE in their nasal secretions and no detectable levels of specific IgE in their serum. In addition, specific IgE is present on cells in the nasal mucosa [7]. The nasal mucosa itself contains all the cell types (dendritic cells, T-cells and B-cells [8]) and cytokines (interleukin (IL)-4 and IL-13 [9–12]) necessary for an IgE immunoresponse. Other studies have suggested that IgE synthesis takes place in the downstream cervical lymph nodes [13, 14] or upper respiratory and lower respiratory lymph nodes [15]. However no information has been obtained as to whether the IgE comes from the circulation (transudate) or from the mucosal glands (exudatate).

ZURCHER *et al.* [16] demonstrated that functional B-cells, isolated from the nose and cultured in a CD40-stimulating system, could synthesize IgE. DURHAM *et al.* [17] reported that local allergen provocation induces ε germline transcripts in nasal B-cells. They were not able to detect IgE-positive B-cells. PAWANKAR *et al.* [18] suggested novel and critical roles for mast cells obtained from allergic rhinitis patients in amplifying IgE production, within the local microenvironment of the nasal mucosa.

In this study, the possibility of determining the presence of IgE-positive B-cells, IgE-positive plasma cells and allergen-binding plasma cells in the nasal mucosa of seasonal allergic rhinitis patients, perennial allergic rhinitis patients and nonallergic healthy controls was investigated. Double-staining experiments were performed to verify whether all allergen-positive cells were also positive for IgE. Blocking experiments using polyclonal antibodies directed against Igs were performed to analyse the specificity of the allergen-binding cells for IgE.

Materials and methods

Patients and controls

Nasal biopsy (2.5 mm) was performed using Gerritsma forceps [19]. Twelve grass pollen allergic rhinitis patients (six male/six female), median age 30 yrs (range 15–45 yrs), with a history of clear seasonal rhinitis for 2 yrs and a skin-prick test with a reaction of 3+ for grass pollen and a radioallergosorbent test (RAST) score median of 4+ (range 3+–5+) for grass pollen. The biopsy samples from

the seasonal grass pollen allergic rhinitis patients were taken during the grass pollen season. Sixteen perennial allergic rhinitis patients (10 male/six female) median age 25 yrs range 18–51 yrs) yielded a positive skin-prick test for house dust mite (HDM), 3+, and a RAST score median of 3+ (range 3+–5+) for *Dermatophagoides pteronyssinus*. Patients had to have two or more symptoms of perennial rhinitis (nasal blockage, rhinorrhoea, sneezing) and to have required medication for perennial rhinitis for 1 yr. The biopsy samples from the perennial allergic rhinitis patients were taken between October and January (inclusive) during the HDM season. The control biopsy samples were taken from twelve healthy volunteers (seven male/five female), median age 36 yrs (range 18–62 yrs) without nasal complaints or nasal abnormalities on ear, nose and throat examination and a negative RAST.

None of the patients had structural nasal abnormalities, undergone nasal surgery in the past 6 months, acute respiratory or sinus infection, serious or unstable concurrent disease, proven allergy for an other relevant inhalant allergen, undergone treatment with systemic or inhaled/intranasal corticosteroids or inhaled/intranasal sodium cromoglycate in the preceding month or used astemizole in the previous 6 weeks.

The study was approved by the Ethics Committee. All patients gave informed written consent.

Allergens

The allergens were covalently bound to a soluble polymer/copolymer matrix labelled with biotin (Diagnostic Products Corporation, Apeldoorn, the Netherlands) [20]. GP1 (50 mg·mL^{-1}), a mixture of biotinylated grass pollen (orchard Kentucky, blue, rye and timothy grasses), and D1 (20 mg·mL^{-1}) biotinylated HDM were used.

Blocking experiment

In order to determine whether the allergen binding was specific to IgE, cryostat sections were preincubated with blocking polyclonal antibodies directed against IgA, IgE, IgG, and IgM (5 mg·mL^{-1}) central laboratory of the Netherlands Red Cross blood transfusion service (CLB), the Netherlands) and phosphate-buffered saline (PBS, pH 7.8), followed by the normal allergen staining procedure. These polyclonal antibodies blocked the cell-bound antibodies of different types. The blocking was based on spheric hindrance or the idiotypes of the antibodies were masked, so that the allergen could not bind. In order to determine the amount of blocking, the number of allergen- positive cells were counted and the signal intensities ranked in a blinded fashion.

Staining procedure

The antibodies used for the staining procedure are detailed in table 1. Frozen sections (6-µm thick) were fixed in acetone for 10 min at room temperature (22±C) and rinsed in PBS (pH 7.8). Endogenous peroxidase blocking was carried out by means of treatment for 30 min in 1% sodium azide and 0.01% hydrogen peroxide in PBS. The slides were then placed in a semi-automatic stainer (Sequenze; Shandon, Amsterdam, the Netherlands).

Double staining plasma cells or B-cells and immunoglobulin E

The sections were subsequently incubated for 10 min with PBS containing bovine serum albumin (BSA, 1%), normal goat serum (10%), and normal rabbit serum (10%) (CLB, Amsterdam, the Netherlands) and then for 60 min with the antibodies to CD138 (plasma cells syndican I; Serotec (Diagnostic Products Corporation, the Netherlands). They were then rinsed with PBS for 5 min, incubated with biotinylated goat antimouse (Biogenix, (Klinipath, Duiven, the Netherlands)) for 30 min, rinsed once more with PBS for 5 min, incubated with alkaline phosphatase-conjugated streptavidin (Biogenix, (Klinipath) for 30 min, rinsed with PBS for 5 min, and incubated for 60 min with FITC-conjugated rabbit antihuman IgE. After rinsing with PBS, they were incubated for 30 min with peroxidase-conjugated rabbit-anti FITC (Dako (ITK, Uithoorn, the Netherlands)). Finally, the slides were rinsed with PBS and tris-hydroxymethyl-aminomethane (Tris) (0.2 M, pH 8.5) for 5 min each, incubated for 10 min with 1.0 mM Fast

Table 1. – Antibodies used to stain biopsy specimens

Antibody	Concentration µg·mL^{-1} dilution	Source
Anti CD19	1.3	Immunotech (Coulter, the Netherlands)
Anti CD138 (plasma cells syndican I)	1	Serotec (DPC, Apeldoorn, the Netherlands)
FITC-labelled anti-IgE	50	CLB (Amsterdam, the Netherlands)
Biotinylated grass pollen	50	DPC
Biotinylated *der* PI	20	DPC
Biotinylated goat antimouse	1:50	Biogenix (Klini Path, Duiven, the Netherlands)
Alkaline phosphatase/peroxidase-labelled streptavidine	1:50	Biogenix (Klini Path)
Alkaline phosphatase-labelled goat antibiotin	1:50	Sigma (the Netherlands)
Alkaline phosphatase-labelled streptavidine/biotin complex	1:50	Vector (Brunschwig Chemie, the Netherlands)
Peroxidase-labelled rabbit anti-FITC	1:50	Dako (ITK, Uithoorn, the Netherlands)
Peroxidase-labelled rabbit antimouse peroxidase	1:100	Sigma
Peroxidase-labelled mouse antiperoxidase	1:100	Sigma

IgE: immunoglobulin E; FITC: fluorescein isothiocyanate; DPC: Diagnostic Products Corporation; CLB: central laboratory of the Netherlands Red Cross blood transfusion service.

Blue substrate, rinsed with sodium acetate (0.2 M, pH 4.6) for two periods of 5 min, incubated with 1% 3-amino-9-ethylcarbazole (AEC) for 30 min, rinsed in distilled water and mounted in glycerine/gelatin (1:1).

Double staining immunoglobulin E and biotinylated allergen

The sections were subsequently incubated for 10 min with PBS containing BSA (0.5–1%), normal rabbit serum (10%) and normal goat serum (10%) (CLB), followed by 100 min with biotinylated allergen (AlaSTAT, DPC) [7] and thereafter for 60 min with the polyclonal antibodies to IgE. They were then rinsed with PBS for 5 min, incubated with alkaline phosphatase-conjugated goat-anti-biotin (Sigma) for 30 min After rinsing with PBS, they were incubated for 30 min with peroxidase-conjugated rabbit anti-FITC (Dako, (ITK), Uithoorn, the Netherlands). Finally, the samples were rinsed with PBS for 5 min, rinsed with Tris (0.2 M, pH 8.5) for 5 min, incubated for 10 min with 1.0 mM Fast Blue substrate, rinsed with sodium acetate (0.2 M, pH 4.6) for two periods of 5 min, incubated with AEC for 30 min, rinsed in distilled water and mounted in glycerine/gelatin (1:1).

Double staining plasma cells and biotinylated allergen

The sections were subsequently incubated for 10 min with PBS containing BSA (0.5–1%) and normal rabbit serum (10%) (CLB), followed by 60 min with the antibodies to plasma cells and thereafter for 100 min with biotinylated allergen. They were then rinsed with PBS for 5 min, incubated with peroxidase-conjugated Rabbit antimouse (Sigma) for 30 min, rinsed once more with PBS for 5 min and incubated with peroxidase-conjugated mouse antiperoxidase for 30 min. They were then rinsed with PBS for 5 min, and incubated with alkaline phosphatase-conjugated goat antibiotin (Sigma) for 30 min. Finally, the samples were rinsed with PBS for 5 min, rinsed with Tris buffer (0.2 M, pH 8.5) for 5 min, incubated for 10 min with 1.0 mM Fast Blue substrate, rinsed with sodium acetate (0.2 M, pH 4.6) for two periods of 5 min, incubated with AEC for 30 min, rinsed in distilled water and mounted in glycerine/gelatin (1:1).

Controls

The controls used were biopsy sections from nonallergic patients and those stained immunohistochemically with irrelevant mouse IgG subclasses. In these sections, no positivity, nonspecific binding of the conjugates or endogenous enzyme activity were found.

Statistical analysis

The blocking experiments were analysed using the Friedman two-way analysis of variance (ANOVA) test. The distribution of cells in the epithelium and in the lamina propria was not symmetrical and the variances were unequal. For statistical analysis, Kruskal-Wallis one-way ANOVA was used to calculate the overall p-value. A p-value of <0.05 was considered to indicate a significant difference between groups of nasal mucosal biopsies. The nonparametric Mann-Whitney U-test was performed to analyse each group with respect to each other.

Results

Blocking experiment via *preincubation with polyclonal antibodies*

In nasal mucosal sections from mite allergic patients (n=6) and grass pollen allergic patients (n=10), the number of allergen-positive cells was always lower after preincubation with polyclonal antibodies directed against IgE ($p<0.0005$, Friedman two-way ANOVA). The intensity of the signal was also decreased by preincubation with antibodies directed against IgE. No significant effect was seen after preincubation with the other Igs or PBS on the number of allergen-positive cells or the intensity of the specifically stained cells (fig. 1).

Immunohistochemical staining

Single staining. The number of IgE-positive cells·mm^2 section of nasal mucosa is given in table 2 for the epithelium and lamina propria. No IgE-positive cells were observed in the epithelium of healthy control biopsy samples. Significantly (10 fold) more IgE-positive cells were found in the nasal mucosa of allergic patients (grass pollen or mite allergic patients) than in healthy nonallergic control subjects. No differences were observed in the numbers of B-cells (CD19) or CD138-positive cells (plasma cells). Allergen-positive cells were found in allergic patients only, not in controls. GP1-positive cells were only found in patients with only grass pollen allergy. D1-positive cells were only found in the nasal mucosa of patients with perennial allergic rhinitis (allergic to HDM). No allergen-positive cells were found in nonallergic controls.

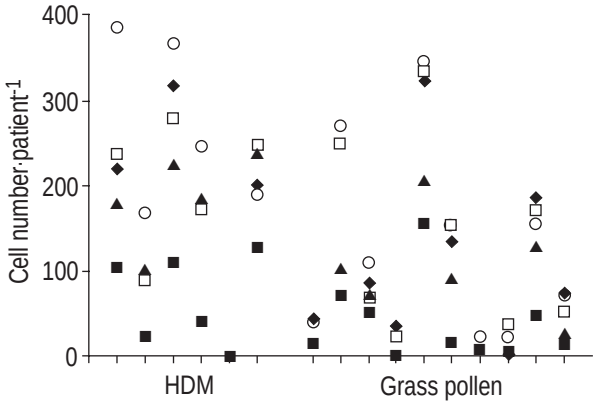

Fig. 1. – Blocking experiments using polyclonal antibodies directed against immunoglobulin (Ig) E (■) showed a significant reduction in allergen-positive cells in contrast to those using polyclonal antibodies directed against IgG (□), IgA (▲) or IgM (♦) and phosphate-buffered saline (○). Biopsy samples from each patient house dust mite (HDM), n=6; grass pollen, n=10) are plotted separately.

Table 2. – Number of cells found in the nasal mucosa of grass pollen allergic patients, perennial allergic patients and healthy controls

Cell type	Seasonal allergics	Perennial allergics	Controls	ANOVA p-value*
Epithelium				
IgE-positive cells	4(1–41)	14 (2–32)	0 (0–0)	0.0003
CD19 (B-cells)	0 (0–0)	0 (0–6)	0 (0–3)	0.2
IgE-positive B-cells	0 (0–1)	0 (0–0)	0 (0–0)	0.05
Lamina propria				
IgE-positive cells	417 (273–590)	209 (97–330)	22 (7–37)	0.0001
CD19 (B-cells)	225 (144–293)	152 (81–233)	142 (114–203)	0.2
Plasma cells	43 (18–100)	78 (60–113)	122 (46–151)	0.06
Allergen-positive cells	45 (32–85)	30 (16–57)	0 (0–0)	0.0000
IgE-positive B-cells	8 (4–16)	6 (3–13)	1 (1–5)	0.02
IgE-positive plasma cells	8 (3–18)	9 (6–16)	0 (0–6)	0.0007
Allergen-positive plasma cells	5 (3–8)	2 (2–7)	0 (0–0)	0.0000

*: Kruskal-Wallis one way analysis of variance. Data are presented as median (interquartile range).

Double staining. Immunoglobulin E-positive B-cells. Double staining with polyclonal antibodies directed against IgE and monoclonal antibodies directed against B-cells resulted in the easy identification of red IgE-positive cells, blue B-cells and mixed-colour (red and blue) IgE-positive B-cells (fig. 2a). In two of the controls, no IgE-positive B-cells were found in the lamina propria. Allergic patients were found to have four times as many IgE-positive B-cells in the lamina propria ($p<0.02$) than nonallergic controls. Grass pollen allergic patients had significantly more IgE-positive B-cells in the epithelium ($p<0.02$) and in the lamina propria ($p<0.05$) than nonallergic controls. Perennial (mite) allergic patients were found to have more IgE-positive B-cells ($p<0.05$) in the lamina propria than nonallergic controls. No significant differences were observed between hay fever patients and perennial mite allergic patients (fig. 3a).

Immunoglobulin E-positive plasma cells. Double staining with polyclonal antibodies directed against IgE and monoclonal antibodies directed against plasma cells resulted in the easy identification of red IgE-positive cells, blue plasma cells and mixed-colour (red and blue) IgE-positive plasma cells (fig. 2b). Plasma cells were only evaluated in the lamina propria because CD138 is also expressed weakly in epithelial cells (fig. 2b). Cells positive for IgE alone, IgE-positive plasma cells and non-IgE-positive plasma cells were found in allergic patients as well as in nonallergic controls. However, in seven of the controls, no IgE-positive plasma cells were found. In the lamina propria of the nasal mucosal biopsies from allergic patients, significantly eight-fold ($p<0.0007$) more IgE-positive plasma cells were present than in biopsy samples from nonallergic controls (fig. 3b).

Immunoglobulin E-positive allergen positive cells. IgE-positive cells stained red, the allergen-positive IgE-positive cells stained mixed red/blue and hardly any cells positive for allergen alone (blue, if present) were found. All allergen-positive cells were also positive for IgE, except in one of the grass pollen allergic patients (median 100% (range 71–100%) and one of the mite allergic patients (median 100% (range 96–100%)).

Allergen positive plasma cells. Double staining was performed using biotinylated allergens (blue) and antibodies

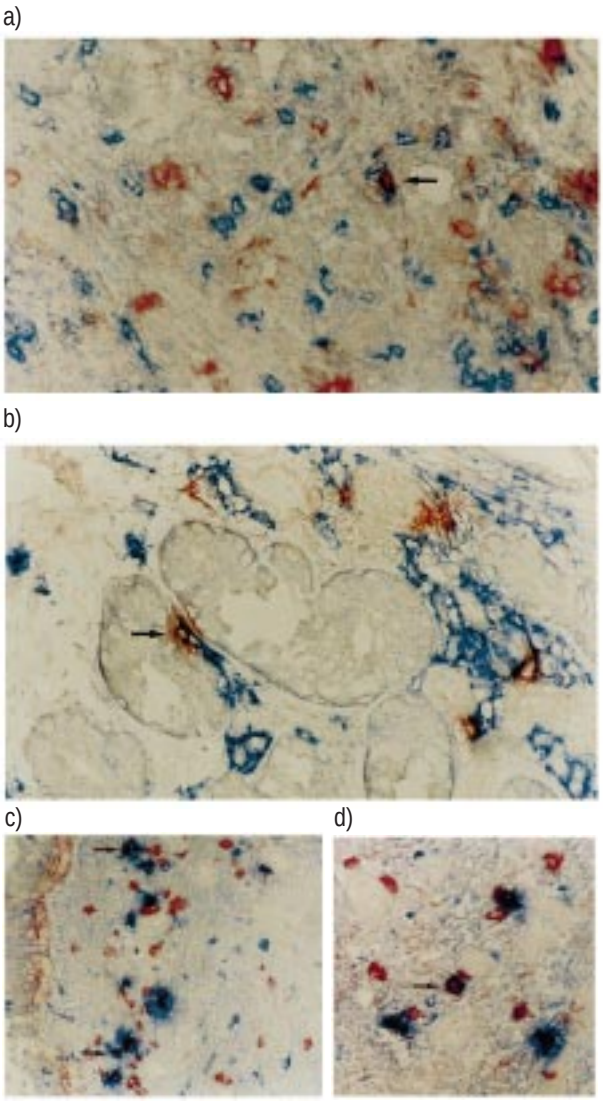

Fig. 2. – Photomicrographs of four nasal mucosal biopsy sections obtained from allergic patients, and double stained immunohistochemically with antibodies directed against immunoglobulin (Ig) E (red) and: a) B-cells (blue) (arrows indicate IgE-positive B-cells) and ; b) plasma cells (blue) (arrows indicate IgE-positive plasma cells); and c, d) double staining using biotinylated allergens (blue) and antibodies directed against plasma cells (red) (arrows indicate allergen-positive plasma cells). (Internal scale bars=36 μm(a–c); 72 μm (d).)

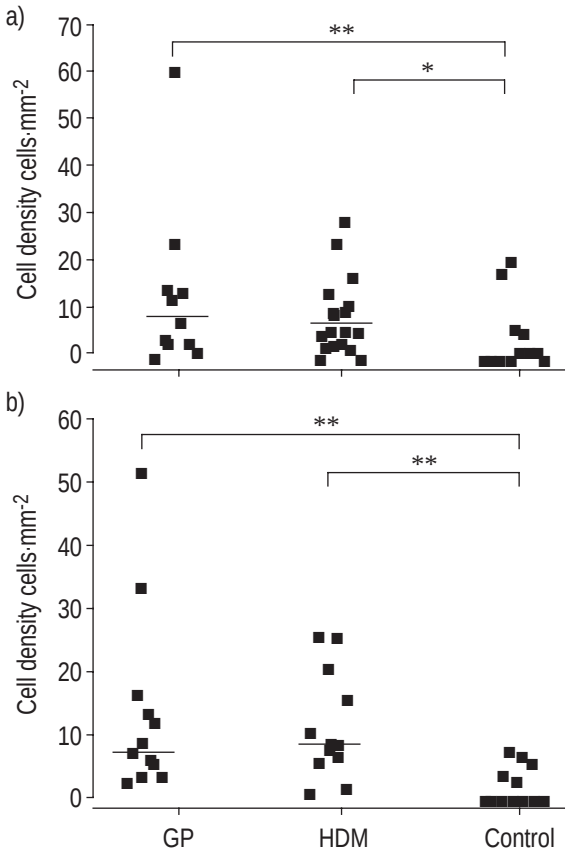

Fig. 3. – Density of: a) immunoglobulin (Ig) E-positive B-cells; and b) IgE-positive plasma cells in the nasal mucosa (lamina propria) of grass pollen allergic patients (GP, n=11), perennial allergic patients (HDM; a) n=16, b) n=12) and healthy controls (n=12). *: $p<0.05$; **: $p<0.01$.

directed against plasma cells red. The allergen-positive cells generally showed a membrane-staining pattern, with the allergen bound on the surface of the cell. However, another staining pattern was found for the allergen-positive plasma cells, which also showed blue staining in and around the cell (fig. 2c). This pattern of staining was observed in all grass pollen allergic patients. However, in two of the mite allergic patients, no allergen-positive plasma cells were found.

Discussion

Local production of specific IgE in the nasal mucosa has been hypothesized by several authors [1–5]. DURHAM *et al*. [21] recently described the expression of ε germ line gene transcripts indicating isotype-switching B-cells and an increase in the level of messenger ribonucleic acid (mRNA) encoding the heavy chain of IgE in nasal B-cells in the nasal mucosa after local allergen provocation. ZURCHER *et al*. [16] demonstrated the possibility of IgE synthesis by nasal epithelial B-cells; functional B-cells can be isolated from the nose, and, after stimulation with IL-4 and antibodies directed against CD40, IgE-protein synthesis could be induced. DIAZ-SANCHEZ *et al.* [22] also found IgE-producing B-cells and mRNA encoding IgE in nasal lavages fluid from allergic rhinitis patients.

In this biopsy study, the production of IgE by B-cells and plasma cells *in vivo* was evaluated in naturally provoked allergic rhinitis patients. This study showed significantly more IgE-positive B-cells and IgE-positive plasma cells in allergic patients than in nonallergic controls. IgE-positive B-cells and plasma cells were also observed in healthy controls. An explanation for these IgE-positive cells could be the smoking of some of the patients, (environmental) tobacco smoke increases the number of IgE-positive cells [23, 24]. The allergen-positive plasma cells (specific IgE) were observed only in allergic patients, in accordance with previous observations of allergen-positive cells occurring only in the nasal mucosa of allergic rhinitis patient and not in nonallergic controls [7]. The observation of the presence of allergen-positive plasma cells and the finding that all allergen-positive cells were also IgE-positive indicate that the local production of allergen-specific IgE takes place in the nasal mucosa. Blocking experiments showed that the binding was specific for IgE but not for IgG, IgA or IgM, meaning that specific IgE is the critical factor for allergen binding to cells and that this binding was not based on the allergen binding to IgG, IgA or IgM. IgE antibodies remain firmly fixed to mast cells for an extended period. IgA antibodies function by inhibiting the adherence of coated microorganisms to the surface of mucosal cells, thereby preventing entry into the body tissues. IgG has properties with respect to the neutralization of bacterial toxins and binding to microorganisms to enhance their phagocytosis. The locally-produced IgA or IgG levels were not high enough to neutralize the allergen binding to specific IgE-positive cells.

In a previous study, it was observed that monoclonal antibodies directed against IgE recognize >80% of all allergen-positive cells [7]; the present study used rabbit polyclonal antibodies directed against IgE, and showed that all allergen-positive cells were also IgE-positive. The significantly higher number of IgE-positive B-cells in the nasal mucosa of allergic patients compared to the nasal mucosa of nonallergic controls and the observation of no difference in numbers of B-cells expressing CD40 (data not shown) are an indication that, the ε germ line switches to IgE-positive B-cell early in life. These switched B-cells require only IL-4 to respond with persistent IgE formation [25]. The significantly higher numbers of IgE-positive plasma cells in the allergic nasal mucosa compared to the controls suggests that the maturation of IgE-positive B-cells to IgE-producing plasma cells takes place locally in the nasal mucosa. B-cells express CD 40 during the mature/activated stage [26]. It is not possible to differentiate between IgE-positive B-cells if they are IgE-positive memory B-cells or at least activated B-cells [27]. Once formed, the switched IgE-positive memory B-cells are long-lived cells, but not all IgE responses lead to the formation of IgE-positive memory B-cells [28]. The present data suggest that the maturation of IgE-expressing B-cells (activated or memory) to IgE-producing plasma cells takes places in the nose. It is noteworthy that many of the cytokines induced by allergen provocation such as IL-4, IL-6 and IL-13 are also B-cell proliferation factors [11, 12, 29]. The nasal mucosa might also be the place of maturation of B-cells. In the nasal mucosa are germinal centres containing dendritic cells, T-cells and B-cells. The availability of IL-4 and IL-13 produced in the nasal

mucosa makes it possible for isotype switching of B-cells to IgE-positive B-cells and proliferation and maturation of B-cells to IgE-producing plasma cells to occur. Moreover, recent work done by the group of DURHAM *et al*. [17] reports an increase in the number of B-cells expressing ε germ line transcripts locally in the nasal mucosa of hay fever patients after allergen provocation. This increase in ε germ line transcripts could be suppressed by local steroid treatment. Similar observations of isotype switching have been made by SAXON *et al*. [22] in nasal mucosal cells. Not only T-cells but also mast cells and basophils have the capacity to stimulate IgE synthesis by B-cells, as producers of IL-4 and IL-13 and by the interaction of mast cells CD154 (CD40 ligand) with B-cell CD40 [18, 30, 31]. The mast cell/basophilic induction and stimulation of B-cell IgE-production indicate that immunoglobulin switching, previously thought to take place only in lymph node germinal centres, may also occur in peripheral organs such as the nose. However, it is not clear what impact this mast cell/B-cell interaction has on the amount of IgE produced.

Comparing the present upper airway results with data from the lower airways, CHVATCHKO *et al*. [32] describe antigen-driven differentiation of B-cells *via* induction of a follicular dendritic cell network in mice, with germinal centres occurring in the parenchyma of inflamed lungs. These germinal centres would then provide a local source of immunoglobulin E-secreting plasma cells, contributing to the release of factors mediating inflammatory processes in the lung [32]. Transplantation literature case reports describe nonasthmatic recipients of asthmatic lungs who develop asthma after transplantation; however, asthmatic recipients of normal lungs do not develop asthma for up to 3 yrs after transplantation. This supports the local characteristic of lung disease in asthma, [33]. It is unclear which portion of specific immunoglobulin E is produced in the nasal mucosa of allergic rhinitis patients because lymphoid tissues may also be involved in the production of immunoglobulin E [4, 15, 34]. However, this study clearly demonstrates that B-cells/plasma cells in the nasal mucosa of allergic rhinitis patients produce (specific) immunoglobulin E. This concurs with the hypothesis put forward in the 1970s [1–3, 6].

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