

Expression of IL-4, C ϵ RNA, and I ϵ RNA in the nasal mucosa of patients with seasonal rhinitis: Effect of topical corticosteroids

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Background: Nasal allergen provocation has demonstrated that allergen-induced rhinitis is associated with an increase in local IL-4 mRNA and IgE heavy chain (C ϵ) and IgE heavy chain promoter (I ϵ) RNA and that pretreatment with topical glucocorticosteroids inhibits the increase in these transcripts.

Objective: This study was undertaken to determine whether observations made after acute allergen provocation can be extended to the case of chronic exposure experienced during the pollen season.

Methods: Biopsy specimens were obtained from the inferior turbinate of 33 pollen-sensitive subjects with allergic rhinitis before and during pollen season. Patients were randomized in a double-blind fashion and treated with either topical steroids (200 μ g fluticasone propionate twice daily; $n = 16$) or matched placebo nasal spray ($n = 17$) before the pollen season. Alkaline phosphatase anti-alkaline phosphatase immunocytochemistry was used to identify B cells (CD20⁺), and in situ hybridization was used to detect IL-4, C ϵ , and I ϵ RNA⁺ cells.

Results: Baseline examination revealed IL-4 and C ϵ RNA but virtually no I ϵ RNA⁺ cells in the nasal mucosa. Analysis revealed a significant difference in the expression of C ϵ and I ϵ RNA⁺ cells ($p < 0.001$). Biopsy specimens taken after antigen exposure exhibited highly significant increases in placebo-treated ($p < 0.001$) but not steroid-treated patients. In both groups, the number of CD20⁺ cells was unchanged when preexposure and postexposure biopsy specimens were compared.

Conclusions: These results show strong support for the hypothesis that IgE class switching occurs locally within the nasal mucosa of subjects with seasonal allergic rhinitis and that this response can be inhibited through strategies directed against local IgE production. (*J Allergy Clin Immunol* 1998;101:330-6.)

Key words: IgE, C ϵ , I ϵ , IL-4, allergic rhinitis, topical corticosteroids

Abbreviation used

FP: Fluticasone propionate

Allergic rhinitis is characterized by eosinophilic inflammation of the nasal mucosa and local infiltration of CD4⁺ T lymphocytes expressing T_{H2}-type cytokines, including IL-4.^{1,2} In addition to its integral role in the differentiation of T cells toward a T_{H2}-type profile of cytokine expression,³ IL-4 has been shown to initiate immunoglobulin isotype switching to IgE in B cells.⁴⁻⁶ In the presence of costimulatory signals such as CD40 activation, this DNA recombination event results in rearrangement of the immunoglobulin heavy chain genes so as to favor IgE production over the synthesis of other immunoglobulin isotypes.

IL-4 plays a key role in the induction of IgE switching by stimulating transcription from the germline promoter site of IgE (I ϵ), located upstream of the unrearranged IgE heavy chain gene (C ϵ).^{6,7} Transcription through I ϵ results in the production of untranslatable germline transcripts containing the I ϵ region spliced to C ϵ (I ϵ -C ϵ), and this transcriptional activity allows access of the cellular recombinase machinery to switch sequences upstream of C ϵ . In the process of isotype switching, I ϵ is deleted, and the C ϵ gene is positioned directly downstream of the VDJ (genes which encode for antigen specificity), forming the mature ϵ -mRNA (VDJ-C ϵ).⁸⁻¹¹ I ϵ RNA⁺ cells are thus considered to be B cells targeted for antibody class switching to IgE. In view of this, C ϵ RNA is expressed by both B cells undergoing isotype switching (I ϵ -C ϵ ⁺) as well as those producing mature ϵ -mRNA (VDJ-C ϵ). The use of riboprobes complementary to both these transcripts (I ϵ and C ϵ) and examination of the difference in the number of positive cells will enable us to distinguish B cells actively isotype switching to IgE.

The production of IgE has been ascribed to B cells within the lymph nodes, spleen, bone marrow, and blood.¹² However, the presence of IgE in nasal secretions from individuals with allergic rhinitis^{13,14} and the identification of B cells within the nasal mucosa have suggested a local synthesis of this immunoglobulin

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within the tissue. In support of this hypothesis, we have recently shown that nasal allergen provocation is associated with an increased local expression of IL-4, C ϵ , and I ϵ RNA.¹⁵ Furthermore, pretreatment of these individuals with topical corticosteroids before specific antigen challenge inhibited the early and late phase responses and the number of IL-4, C ϵ , and I ϵ RNA⁺ cells.¹⁶⁻¹⁸ However, it is still not clear whether these changes observed after experimental allergen provocation can be extrapolated to the case of chronic exposure experienced during the pollen season.

In this study we examined B-cell numbers, IL-4, and C ϵ RNA expression as well as the expression of I ϵ sterile transcripts in the nasal mucosa of 33 subjects with seasonal rhinitis before and during the pollen season and in response to topical glucocorticosteroid treatment. We also examined the presence of C γ RNA to compare the effect of seasonal exposure on the expression of C ϵ and C γ RNA transcripts. Nasal biopsy specimens were obtained from the inferior turbinate of pollen-sensitive subjects with allergic rhinitis before and during the pollen season, and patients were randomized in a double-blind fashion to receive treatment with topical steroids (fluticasone propionate [FP], 200/ μ g twice daily) or matched placebo nasal spray. B cells were identified in nasal biopsy sections by immunocytochemistry with an anti-CD20 monoclonal antibody. In situ hybridization with antisense, radiolabeled riboprobes was performed to identify IL-4, C γ , C ϵ , and I ϵ RNA⁺ cells.

METHODS

Patient selection

Thirty-three patients with seasonal allergic rhinitis were recruited from the Allergy Clinic at the Sahlgrenska Hospital, Gothenburg, Sweden. Individuals included in the study were nonsmokers, exhibited a positive skin prick test reaction to Timothy grass pollen extract (wheal >5 mm), and had a history of moderate to severe hay fever for at least 2 years. Patients having perennial nasal symptoms and those receiving immunotherapy within the last 5 years or oral or topical steroids within the last 6 months were excluded from the study. All methods were approved by the Hospital Ethics Committee, and patients provided written informed consent.

Study design and tissue preparation

Patients were recruited out of the grass pollen season and randomized to receive either a 6-week course of FP aqueous nasal spray (200 μ g) twice daily or a matched placebo control. Weekly nasal visual analogue scores were used to indicate the patients' overall hay fever symptoms. This was done by asking each patient to respond to the question "How has your hay fever been this week?" by placing a mark on a line of 10 cm in length. Measurement of the distance from 0 cm to the mark was used as an indicator of the degree of symptom improvement.¹⁹ Nasal mucosal biopsy specimens were obtained before treatment as well as during peak pollen season from the inferior turbinate with the use of a Gerritsma forceps after local anesthesia was achieved (3% cocaine and 0.025% adrenaline). Biopsy specimens were sectioned, one half was placed directly in phosphate-buffered saline, blocked in OCT medium, and stored at -80° C until use. Cryostat sections (6 μ m) were fixed

in a 60:40 acetone/methanol solution for 7 minutes and left to dry at room temperature. The other half of each biopsy specimen was fixed in 4% paraformaldehyde for 2 hours, washed in two changes of 15% sucrose/phosphate-buffered saline, left overnight, blocked in OCT medium, and stored at -80° C.

Probe preparation

Radiolabeled complementary RNA probes coding for IL-4, C γ , C ϵ , and I ϵ RNA were prepared from cDNA as described previously.¹⁸ In brief, cDNA was inserted into pGEM vectors, linearized, and transcribed in vitro in the presence of ³⁵S-uridine triphosphate and either SP6 or T7 polymerases. Antisense (complementary to RNA) and sense probes (identical to RNA) were prepared. The percentage of incorporation of the radioisotopes was approximately 40% to 50%.

In situ hybridization

In situ hybridization was performed as previously described.²⁰ Briefly, 10 μ m cryostat sections from biopsy specimens were permeabilized with Triton X-100 and proteinase K solution (1 μ g/ml) in 0.1 mol/L Tris containing 50 mmol/L ethylenediamine tetraacetic acid for 20 minutes at 37 $^{\circ}$ C. The samples were subsequently incubated with 0.1 mol/L triethanolamine and 0.5% acetic anhydride for 10 minutes and then with *N*-ethylaldehyde (1.25 μ g/ml) and iodoacetamide (1.85 μ g/ml) for 20 minutes at 37 $^{\circ}$ C to prevent the nonspecific binding of the ³⁵S-labeled complementary RNA probes. Prehybridization of the samples was carried out in 50% formamide in 2 $\times$ standard sodium citrate for 15 minutes at 37 $^{\circ}$ C. Sections underwent hybridization with a hybridization mixture containing the appropriate sense or antisense probes (0.75 $\times$ 10⁶ cpm/slide). Posthybridization involved high stringency washing of the samples in decreasing concentrations of standard sodium citrate at 42 $^{\circ}$ C. To remove any unbound RNA probe, samples were washed with an RNase solution (20 μ g/ml) for 20 minutes at 42 $^{\circ}$ C. The samples were then dehydrated with increasing concentrations of ethanol and left to air dry. After this, samples were dipped in Amersham LM-2 emulsion (Amersham Canada Ltd., Oakville, Ontario, Canada) and exposed for a period of 13 days. The autoradiographs were developed in Kodak D-19 developer, fixed, and counterstained in Mayer's hematoxylin for 60 seconds. The samples were mounted with a coverslip and examined under a graduated microscope for positive signals. Negative control experiments with sense probes or RNase pretreatment of sections before antisense probe application were performed to confirm probe specificity.

Immunocytochemistry

The identification of B cells within nasal biopsy specimens was performed with the alkaline phosphatase anti-alkaline phosphatase technique as previously described.¹ A specific monoclonal antibody detecting B cells (CD20, DAKO) was used. For negative control preparations, the primary antibody was replaced by either nonspecific mouse immunoglobulin or Tris-buffered saline.

Quantification

Sections were coded and counted in a blinded, random fashion with an Olympus light microscope (Olympus America Inc., Melville, N.Y.) with eyepiece graticule at $\times$ 200 magnification. Two sections were immunostained or hybridized from which six to eight fields were evaluated. Positive hybridization signals and immunostaining were expressed as the mean count of cells per high-power field (0.202 mm²).

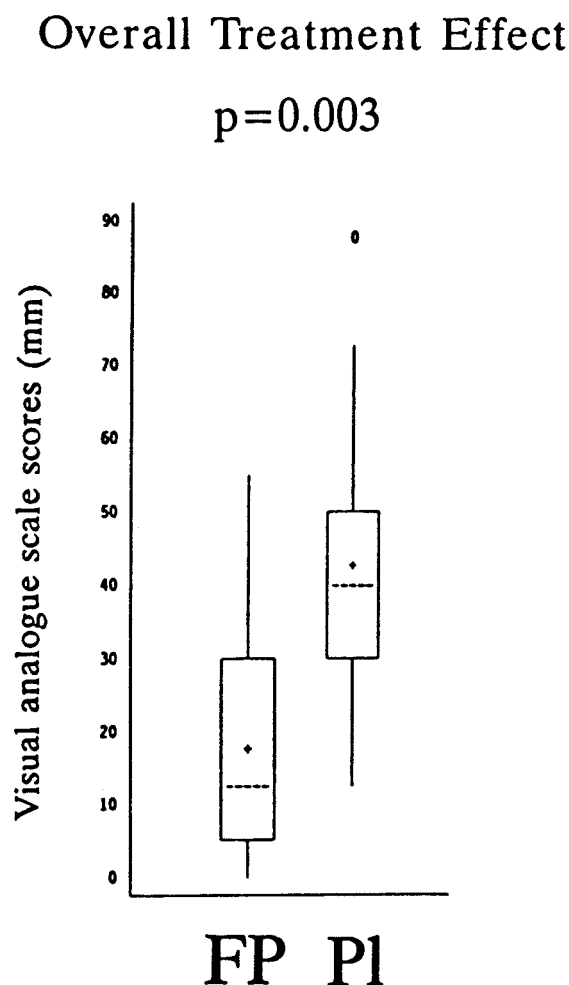


FIG. 1. Visual analogue scores of patients with seasonal allergic rhinitis after a 6-week pretreatment with fluticasone propionate (FP) or placebo (PI) and natural exposure to antigen during pollen season ($*p = 0.003$).

Statistics

The number of cells expressing IL-4, C γ , C ϵ , and I ϵ RNA and cells expressing CD20 immunoreactivity at baseline were compared with those after exposure with the use of a paired Student's *t* test. The mean Δ values for the FP and placebo-treated patients were compared with the use of an unpaired Student's *t* test. Results were considered statistically significant for values of $p < 0.05$.

RESULTS

Patients treated with FP and those receiving the matched placebo were well matched for age, sex, severity of symptoms, and circulating total and grass pollen-specific IgE concentration. Marked reduction in seasonal symptoms, the requirement for rescue medication, and weekly nasal visual analogue scores were seen in FP-treated patients ($p < 0.05$) as compared with those administered the placebo (Fig. 1).

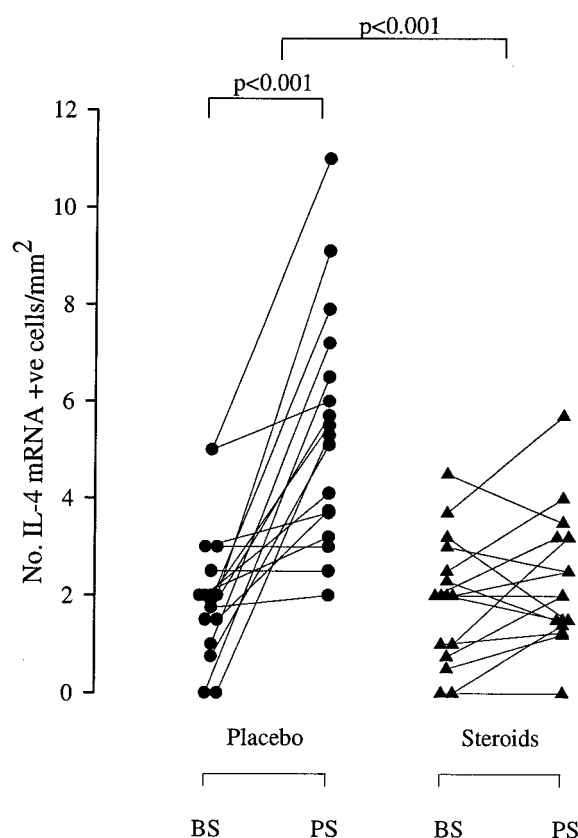


FIG. 2. Numbers of cells expressing IL-4 mRNA detected before season (BS) and during peak season (PS) in patients with seasonal allergic rhinitis and those who received a 6-week pretreatment with FP or placebo ($p < 0.001$ by Student's *t* test).

Expression of IL-4, C γ , C ϵ , and I ϵ RNA in allergic rhinitis during natural allergen exposure

Positive in situ hybridization signals for RNA were observed as dense collections of silver grains overlying cells on autoradiographs when the antisense probes for IL-4, C γ , C ϵ , and I ϵ were used. No signals were observed with the use of the sense probes or from preparations pretreated with RNase. In patients receiving the placebo medication, we observed a significant increase in the number of cells expressing IL-4 mRNA in biopsy specimens taken during the pollen season compared with those taken at baseline (Fig. 2; $p < 0.001$). In these individuals, there were also significant increases in the number of cells positive for C ϵ (Fig. 3; $p < 0.001$) and I ϵ (Fig. 4; $p < 0.001$) RNA during the pollen season compared with before seasonal exposure. Although the numbers of C ϵ ⁺ cells were very small at baseline, statistical analysis indicated that in both groups, steroid-treated and placebo-treated, there was a significant difference between the number of C ϵ and I ϵ ⁺ cells ($p < 0.001$). No change in C γ RNA expression was observed after seasonal exposure ($p > 0.05$).

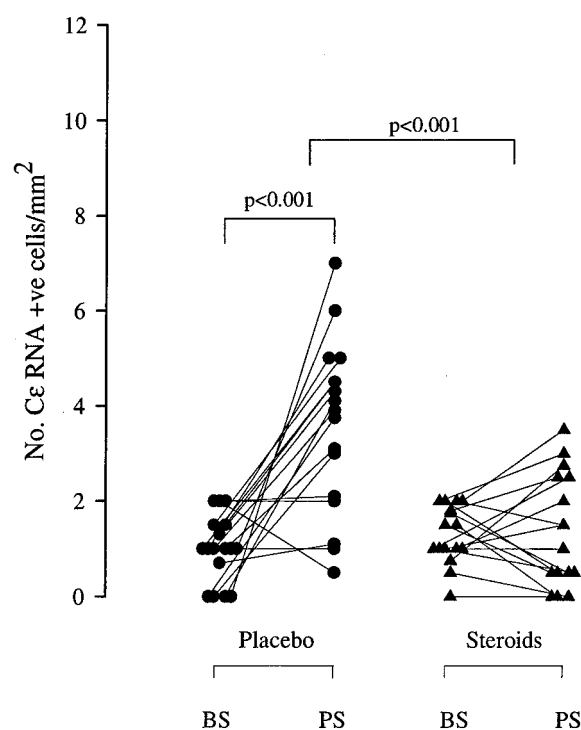


FIG. 3. Cells expressing Cε RNA detected before season (BS) and during peak season (PS) after exposure to antigen in patients with seasonal allergic rhinitis receiving a 6-week pretreatment with either FP or placebo ($p < 0.001$ by Student's *t* test).

Effect of corticosteroid therapy on the expression of IL-4, Cε, and Iε RNA during natural allergen exposure

There was no significant difference at baseline in the numbers of IL-4 mRNA and Cε and Iε RNA-positive cells between patients subsequently treated with either FP or placebo (Figs. 2 through 4; $p > 0.05$). However, during the pollen season, the increase in cells expressing mRNA for IL-4 and RNA for Cε and Iε in the placebo-treated group was not observed in patients receiving FP ($p > 0.05$). Comparison of the mean differences for the two groups indicated that the placebo-treated patients had significantly more IL-4, Cε, and Iε RNA-positive cells when compared with the steroid-treated group (Figs. 2 through 4; $p < 0.001$). The number of Cγ RNA⁺ cells was not changed by steroid treatment ($p < 0.05$).

Immunocytochemistry for CD20 during natural allergen exposure and effects of steroids

Immunocytochemistry with a specific antibody recognizing CD20-immunoreactive cells (B cells) demonstrated the presence of B cells in the nasal mucosa before and during the pollen season. There was no significant increase in the number of cells expressing CD20 immunoreactivity during the pollen season compared with preseason values in patients treated with placebo or steroids ($p > 0.05$). During natural exposure, the CD20-positive cells were predominantly within the

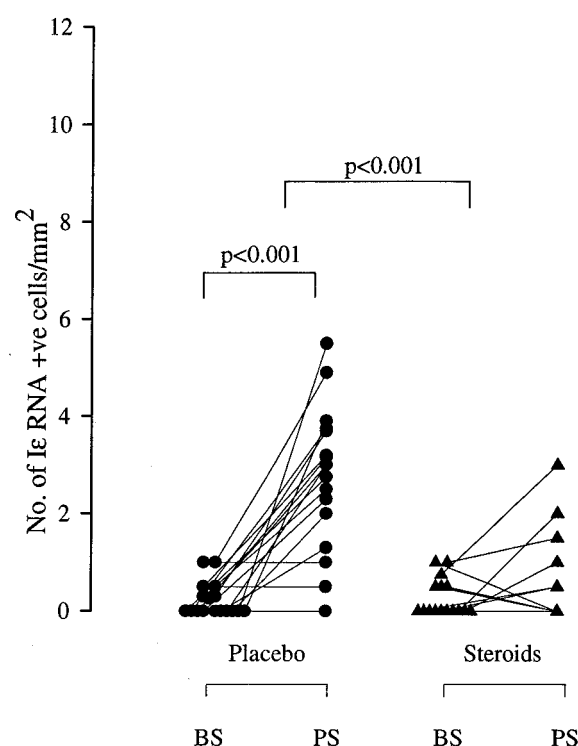


FIG. 4. Numbers of cells expressing Iε RNA detected before season (BS) and during peak season (PS) after exposure to antigen in patients with seasonal allergic rhinitis receiving a 6-week pretreatment with either FP or placebo ($p < 0.001$ by Student's *t* test).

submucosa, with some positive signals being detected in the epithelial cell layer.

DISCUSSION

We have recently demonstrated the presence of Cε- and Iε-positive B cells, as well as IL-4 mRNA⁺ cells, in the nasal mucosa of patients who received specific antigen challenge.¹⁵ In addition, we have shown that pretreatment of the nasal mucosa with topical corticosteroids suppresses this increase in gene transcription and the associated clinical symptoms.¹⁸ Although it has been shown that IgE is increased in the serum²¹ and nasal secretions²² of patients with seasonal allergic rhinitis, it was unclear whether this IgE originated from the influx of IgE-secreting B cells, IgE-bound inflammatory cells, or IgE production by B cells resident to the tissue. Further studies by Naclerio et al.²³ illustrate that intranasal steroids inhibit the seasonal increase of antigen-specific IgE in serum and nasal secretions; however, they do not investigate whether steroid action is through an effect on B cells within the nasal mucosa. In this study we have examined biopsy specimens taken both before and during the pollen season to determine whether the expression of IL-4, Cε, and Iε RNA transcripts is increased after natural exposure to antigen. Furthermore, we assessed whether such effects could be inhibited by pretreatment with topical glucocorticosteroids. Our re-

sults demonstrate that seasonal exposure to allergen increases the expression of IL-4, C ϵ , and I ϵ RNA within the nasal mucosa of patients with allergic rhinitis and that these effects are abrogated by topical steroid treatment.

Although increased numbers of cells expressing IL-4 have been reported after acute antigen provocation in subjects with allergic rhinitis, the finding of an increased expression of IL-4 mRNA⁺ cells as a result of natural allergen exposure is novel and may be partly responsible for the eosinophil accumulation²⁴ and rise in serum IgE levels observed at this time.²⁵ In vitro, IL-4 has been shown to promote B-cell immunoglobulin isotype switching to IgE by stimulating transcription through I ϵ . Consistent with an in vivo role for IL-4 in the induction of IgE switching, we observed an increased expression of I ϵ after seasonal allergen exposure.

By using in situ hybridization with antisense riboprobes complementary to I ϵ RNA, we have provided support for the hypothesis that local immunoglobulin class switching to IgE may occur. Prior studies have shown that transcription through the I ϵ region is necessary for the cellular recombinase machinery to gain access to the switch sequences located between I ϵ and C ϵ .⁸⁻¹¹ During heavy chain DNA recombination, the I ϵ sequence is looped out and the C ϵ gene is positioned directly downstream of the VDJ (genes which encode for antigen specificity). Therefore cells that have already switched to IgE production do not express I ϵ transcripts. In contrast, C ϵ RNA is present in both germline and mature ϵ transcripts. The difference between the number of cells detected with either probe indicates the number of cells expressing the mature ϵ transcript.

The increase in the number of C ϵ RNA-positive cells after seasonal allergen exposure may be partly attributed to the enhanced expression of germline ϵ -transcripts. In addition, C ϵ RNA-positive cells could represent resident B lymphocytes already switched to IgE production or those recruited as a result of IgE-mediated inflammatory events during allergen exposure. However, the increased numbers of cells expressing I ϵ are suggestive of local immunoglobulin synthesis. These data support previous studies demonstrating IgE in nasal secretions^{13, 14} and the recent identification of IgE-secreting preplasma cells within germinal centers of lymphoid tissue in a murine model of allergic pulmonary inflammation.²⁶ Furthermore, although we cannot rule out the recruitment of B cells into the nasal mucosa during allergen exposure, we did not observe an increase in the numbers of CD20⁺ cells at this time. Taken together, these observations support the view that immunoglobulin isotype switching to IgE might occur locally within the nasal mucosa in the pathogenesis of seasonal allergic rhinitis. However, confirmation will require demonstration of ϵ -gene recombination with the use of reverse-transcriptase polymerase chain reaction with primers encoding C ϵ and the J region with nucleotide sequencing or alternatively, demonstration of IgE probes within B cells. These studies are currently ongoing. To address specificity of the seasonal

increase in C ϵ and I ϵ transcripts, we have examined the expression of C γ RNA after seasonal exposure. Consistent with previous work on antigen-challenged patients,¹⁸ we observed no difference in the number of C γ mRNA⁺ cells when biopsy specimens taken before and during pollen season in both placebo- and steroid-treated patients were analyzed.

Pretreatment with FP abrogates the increase in IL-4 and I ϵ RNA expression observed as a result of natural allergen exposure in the placebo-treated subjects. Although C ϵ RNA is decreased, it is equivalent to the decrease seen in I ϵ and most likely is due to the inhibition of germline transcripts (I ϵ -C ϵ ⁺). These data are consistent with previous studies that have demonstrated similar findings after acute allergen provocation in subjects with allergic rhinitis.¹⁶⁻¹⁸ Any such action is likely to be locally mediated because FP is known to have no systemic side effects at the dose used (200 μ g/day). Numerous proposals for the primary mechanism of steroid action have been hypothesized. Because of the inhibitory effects of interferon- γ on IL-4 transcription, it is conceivable that steroids may act through increased expression of this cytokine. This has been demonstrated in asthmatic subjects after administration of oral steroids.²⁷ However, previous examination of nasal mucosal biopsy specimens from antigen-challenged patients pretreated with topical steroids illustrated no significant increase in the number of interferon- γ mRNA⁺ cells.¹⁷ In addition to this, we have previously shown that T-cell numbers do not increase during the season and have not been inhibited by steroid pretreatment.²⁸ These results suggest that steroid action may, at least in part, be through direct inhibition of IL-4 synthesis, thus limiting IgE production and the expression of C ϵ and I ϵ RNA. In contrast to these observations of decreased IL-4, C ϵ , and I ϵ RNA in response to steroids, clinically the use of these agents has been associated with an increase in serum IgE levels. In addition to its influence in attenuating IL-4 mRNA expression, steroids appear to enhance total non-allergen-specific IgE production through a direct action on B cells.^{29, 30} Whether the clinical response to steroids can be attributed to the competition of polyclonal IgE with antigen-specific IgE for Fc ϵ -binding sites, the downregulation of cytokine mRNA expression or both remains uncertain.

The possibility that IgE production occurs locally within the nasal mucosa might prove to be an influential determinant of therapeutic investigation, and the role of IL-4 in B-cell isotype switching could be an important avenue of exploitation. Anti-IL-4 antibodies,³¹ mutant IL-4 protein antagonists that block the IL-4 receptor,³² and even soluble IL-4 receptors³³ have been suggested as interrupting the action of IL-4 on B cells. Although IL-4 induces transcription of the I ϵ region,⁴⁻⁶ the CD40 molecule must also be activated through ligand (gp39) binding for proper switch recombination and IgE expression.³⁴ Blocking this interaction with soluble CD40³⁵ may also provide an effective method for local preven-

tion or downregulation of IgE production within the nasal mucosa. This observation may also be applicable to local strategies for the treatment of allergic asthma, food allergies, or both while reducing the potential for systemic side effects.

In summary, we have shown that natural allergen exposure in patients with seasonal allergic rhinitis results in an increase in the number of cells expressing IL-4, C ϵ , and I ϵ RNA and that these changes were abrogated by 6 weeks of treatment with topical FP (200 μ g/day). In addition, we observed no difference in the number of C γ RNA⁺ cells in either placebo-treated or steroid-treated patients when biopsy specimens taken before and during season were compared. Local expression of IL-4, C ϵ , and I ϵ RNA in the absence of significant increases in B cells in response to natural allergen exposure suggests that IgE synthesis might occur within the nasal mucosa. Further studies with an isolated model of allergic inflammation are necessary to confirm the local production of IgE.

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