

Original article

Analysis of epsilon germline transcripts and IL-4 mRNA expression in the adenoids suggests local IgE switching

Background: We have previously observed more frequent occurrence of IgE⁺ and FcεRI⁺ cells in adenoids of atopic than nonatopic children. To investigate the hypothesis that the adenoids are involved in IgE production, we analyzed the levels of ε germline (εGL), IL-4, and IFN-γ transcripts in the adenoids in relation to atopy and presence of ear disease.

Methods: Adenoidectomy was performed on 19 atopic and 18 nonatopic children (median age 5 years, range 2–12 years) suffering from otitis media with effusion (OME) (*n* = 16) or obstructive adenoids hyperplasia (AH) (*n* = 21). The levels of εGL transcripts, IL-4, and IFN-γ mRNA were analyzed by competitive reverse transcriptase-PCR.

Results: εGL transcript levels in the adenoids were found to be dependent on IL-4 mRNA expression (*P* < 0.01) and serum IgE levels (*P* < 0.05) (*R*² = 0.32, *n* = 37). IL-4 mRNA expression was associated with εGL transcript levels (*r*_s = 0.32, *P* = 0.05, *n* = 37), especially among patients with AH (*r*_s = 0.53, *P* = 0.01, *n* = 21). No significant differences in IL-4 and IFN-γ mRNA levels were observed between the groups.

Conclusions: This study supports an IL-4-induced class switch to IgE production in the adenoids that might be of importance for inflammatory reactions in the upper respiratory tract.

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The adenoids, or pharyngeal tonsils, are part of Waldeyer's throat ring, consisting of lymphoid tissue. Their location at the vault of the nasopharynx makes the adenoids, together with lymphatic tissue associated with the nasal mucosa, the first point of contact of airborne allergens with the immune system. The adenoids have been implicated in the amplification of the IgE response to allergens (1, 2). Adenoidectomy has a significant effect on the resolution of chronic otitis media with effusion (OME) (3, 4), indicating an important role of the adenoids in the pathophysiology of OME. Atopic children are more common among

OME patients (5–7), suggesting that atopy is a predisposing factor for OME.

Our previous study found an increased occurrence of IgE⁺ and FcεRI⁺ cells in the adenoids of atopic as compared to nonatopic children independently of the presence of OME (8). Although there are also some other studies suggesting local IgE production in the adenoids, it has been difficult to provide evidence that the IgE detected is produced locally, and is not merely due to invasion of cells having IgE on their surface. An increase in ε germline (εGL) transcription is considered to be an indication of local IgE production (9–11). A

good correlation between the expression of ϵ GL and mature ϵ transcripts has been observed by *in situ* hybridization, supporting the notion of local IgE production in the nasal mucosa (10, 11).

Further evidence for local IgE production could be obtained by ascertaining an increased ϵ GL expression in the adenoids of atopic subjects and relating such findings to other immunologic parameters. IgE class switching is always preceded by synthesis of ϵ GL transcripts. Transcription of the I ϵ region, occurring under the influence of the cytokines IL-4 and IL-13 that activate specific transcription factors, is necessary for access to the switch sequences located between I ϵ and C ϵ (12) (Fig. 1). The second signal required for induction of IgE synthesis is provided by engagement of CD40 on the B cells involved. During heavy-chain DNA switch recombination, which requires CD40 ligation, the I ϵ sequence is deleted, and in mature ϵ transcripts the C ϵ gene is positioned directly downstream of the VDJ genes (12) (Fig. 1).

To study the hypothesis that the adenoids are involved in IgE production, we used competitive PCR to compare the levels of ϵ GL, IL-4, and IFN- γ transcripts in adenoids from atopic and nonatopic children suffering from OME or adenoid hyperplasia (AH).

Material and methods

Subjects

Adenoids were obtained from 37 children (17 girls and 20 boys; median age 5 years, range 2–12 years) after adenoidectomy at the Division of Otorhinolaryngology, Karolinska Hospital (Sweden), as previously described (8). The study was approved by the local ethics committee. A questionnaire about the child's birth,

family, and medical history was filled out by the parents, and all children underwent routine ear, nose, and throat examination. The children were required to have normal, undiseased tonsils by history and physical examination. Patients with hyperplastic tonsils (occupying more than one-third of the oropharynx) were not included in the study. Other exclusion criteria were cleft palate, known immune deficiency, or a severe underlying disease or treatment with antibiotics within 1 month before surgery. The adenoids from 30 of the 37 patients had previously been analyzed immunohistochemically (8).

The children enrolled in the study are characterized with respect to age, sex, atopy, and serum IgE in Table 1. They were divided into two groups according to clinical manifestations. The OME group ($n=16$) consisted of patients with long-standing OME of at least 3-month duration who were scheduled for tympanostomy tubes. The diagnosis of OME was based on pneumatic otomicroscopy and a flat, Jerger type B tympanogram. The AH group ($n=21$) consisted of children with obstructive symptoms but no history of middle-ear disease. The diagnosis of AH was based on visible obstruction of the nasopharynx, during clinical examination with a mirror, and a history of obstructive symptoms. The nasopharyngeal obstruction was confirmed during surgery.

Serum IgE analysis

Blood samples were taken under anesthesia and analyzed for total serum IgE (Pharmacia CAP System IgE FEIA, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Screening for allergen-specific IgE to inhalant allergens (Phadiatop) and to relevant food allergens (fx5: egg, milk, peanut, soybean, fish, and wheat) was performed with Pharmacia CAP System

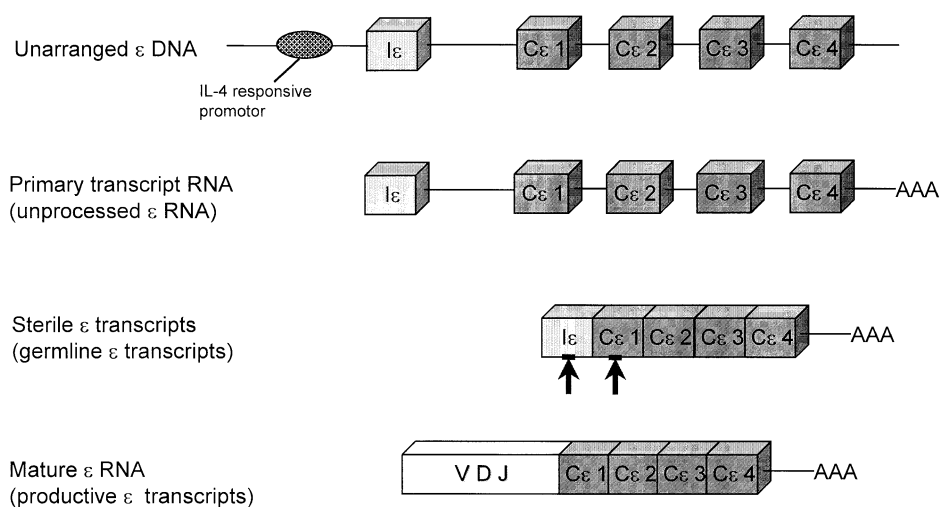


Figure 1. Schematic presentation of unarranged ϵ DNA and ϵ RNA transcripts. $\uparrow$: Localization of primer sequences for ϵ GL transcripts.

RAST FEIA. The children were classified as atopic if they scored positive in either test for allergen-specific IgE (Table 1).

Tissue handling

Adenoid specimens removed during surgery were transported in ice-cold 0.9% NaCl solution, and within less than 1 h were snap-frozen (after removal of unrelated tissue) in chilled isopentane, and stored at -70°C .

Immunohistochemistry

A monoclonal antibody alkaline phosphatase-antialkaline phosphatase (APAAP) staining technique was applied on 6-μm-thick, acetone-fixed cryostat sections with mouse monoclonal anti-IgE (hybridoma B3102E8, Southern Biotechnology Associates, Birmingham, AL, USA), as previously described (8). The sections were evaluated under coded conditions at magnifications of $\times 100$ and $\times 200$. The localization of IgE⁺ cells was related to three different compartments in the adenoid tissue: epithelium, extrafollicular area, and lymphoid follicles (Table 1).

RNA extraction and cDNA synthesis

Total RNA was extracted from cryostat sections, 10 μm thick, corresponding to a total volume of approximately 5 mm³ from the central part of the adenoids. For this purpose, the sections were suspended in 400 μl of ice-cold RNazol B[®] (Biotec^x Laboratories; Houston, TX, USA), and total RNA was extracted according to the manufacturer's instructions. The RNA pellet was air-dried and resuspended in 40 μl of diethyl-pyrocabonate-treated water. A volume of 5 μl was used for spectrophotometric determination of the RNA concentration at 260 nm, and another 5 μl for verifying the

RNA quality by electrophoresis on a 1% agarose gel (BRL, Gaithersburg, MD, USA). The remaining 30 μl was stored at -70°C .

First-strand cDNA synthesis of total RNA from the adenoid specimens (3 μg total RNA in a total volume of 60 μl) was performed essentially as previously described (13), except that 5 μM pd(T)16 (Pharmacia & Upjohn) was used for priming instead of random hexamers.

Oligonucleotide primers

The following primers synthesized by CyberGene AB (Huddinge, Sweden) were used to study εGL expression: sense 5'-AGCTGTCCAGGAACCCGACA-3' (pIε) and antisense 5'-GAGACGGTCAGCAAGCTGAT-3' (pCε1) (14). To obtain an internal standard for εGL transcripts, a composite sense primer (pCompe) was synthesized, comprising the sequence of the entire sense primer pIε and a 3'-10-bp extension representing a downstream sequence in Cε1: 5'-AGCTGTCCAGGAACCCGACATTCCCTCCAA-3' (Fig. 2A). Specific oligonucleotide primers for IL-4 and IFN-γ, and the "house-keeping" gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontech (Palo Alto, CA, USA) or synthesized by CyberGene AB.

RT-PCR

A schematic presentation of unarranged ε gene segments, and sterile and mature ε RNA transcripts is shown in Fig. 1. The PCR primers for amplification of εGL transcripts were chosen in such a way as to avoid interference from genomic DNA that might contaminate the RNA preparation. The PCR reaction mixture (25 μl) for ε gene expression contained 0.6 U *Taq* DNA polymerase (BRL), 2.5 μl cDNA in appropriate dilution, 1.5 mM MgCl₂, 0.2 mM dNTP, 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl (pH 9.0), and 0.01% Tween 20, sense and antisense primers (0.5 μM each). PCR was conducted for 32–35 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension time of 5 min in a DNA Thermocycler 480 (Perkin Elmer, Norwalk, CT, USA).

The internal standard for quantification of εGL transcripts was obtained by performing PCR with the primers pCompe and pCε1 by using purified cDNA from a first PCR reaction with primers pIε and pCε1 (Fig. 2A). The conditions were denaturation at 94°C for 1 min, annealing at 55°C for 1 min in the five first cycles and at 60°C for 1 min in the 25 following cycles, extension at 72°C for 1 min, and a final extension for 5 min. In this way, a 109-bp shorter PCR product (184 bp) was generated that after purification and quantification (Low DNA Mass Ladder, BRL) was used as an internal standard in competitive reverse

Table 1. Characterization of patients included in study according to age, sex, and total serum IgE, and according to IgE⁺ cells in adenoid

Subjects	Age (months)*	Sex (M/F)	Total IgE†* (kU/l)*	IgE ⁺ cells‡*		
				Epithelium	Extra-follicularly	Follicles
Atopic	86 (39–154)	14/5	255 (33–2200)	2 (1–2)	3 (1–3)	1 (0–1)
OME	63 (46–114)	4/2	250 (33–530)‡	1 (1–2)	3 (1–3)	1 (0–1)
AH	87 (39–154)	10/3	260 (55–2200)	2 (1–2)	3 (2–3)	1 (0–1)
Nonatopic	60 (34–151)	6/12	11 (2–170)	1 (0–3)	1 (0–3)	0 (0–1)
OME	61 (39–116)	2/8	7 (2–95)	1 (0–2)	1 (0–2)	0 (0–1)
AH	58 (34–151)	4/4	14 (5–170)	1 (1–2)	1 (0–3)	0 (0–1)

*Median and range.

†Normal reference range: 2–22 kU/l for patients up to 4 years of age and 2–263 kU/l for 4–20-year-old patients.

‡One serum sample could not be analyzed.

§0) no stained cells; 1) low; 2) moderate; 3) high occurrence of positive cells.

transcriptase-PCR (RT-PCR), as shown in Fig. 2B. The internal standards (MIMICs) used in competitive RT-PCR for G3PDH, IL-4, and IFN- γ were obtained from Clontech.

Competitive RT-PCR for ϵ GL transcripts, IL-4, IFN- γ , and G3PDH was performed in a reaction mixture (20 μ l) containing 2 μ l of target cDNA, 2 μ l of internal standard cDNA, appropriate primers, and other reaction constituents as detailed above. An appropriate dilution of the sample cDNA was chosen after a preliminary PCR experiment. For each sample, three-fold dilution series were run with five dilutions for G3PDH and ϵ GL internal standard, six dilutions for IL-4, and seven dilutions for IFN- γ . Manual hot start (starting at 95°C when adding *Taq* polymerase, followed by 1-min denaturation at 94°C) was used for amplification with primers for IFN- γ -specific sequences. The PCR products were separated on a 1.6% agarose gel (BRL). The ethidium bromide-stained gel was photographed under UV light. ϵ PCR products were photographed and analyzed with the Kodak Digital Science™ Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY, USA). IL-4, IFN- γ , and G3PDH PCR products were photographed on Polaroid Positive/Negative Instant Pack 665 film (Cambridge, MA, USA), and the negatives were analyzed with a microcomputer imaging device (Imaging Research, Inc., St Catharines, Ontario, Canada) coupled to a video camera. The data were imported into Microsoft™ Excel for subtraction of background values and calculation of the $\log_{10}$ of the ratio of the relative optical density for the sample cDNA to that of the internal standard for each point in a dilution series of internal standards. The molar ratio of each specific gene product to the housekeeping gene G3PDH, as well as the ratio of IL-4 to IFN- γ , was determined for each adenoid specimen, as

previously described (15). An example of a competitive PCR dilution series using primers amplifying ϵ GL transcripts is shown in Fig. 2B.

Statistical analysis

The software program STATISTICA (Release 5, StatSoft, Inc., Tulsa, OK, USA) was used for statistical analysis. Nonparametric statistical tests were used in comparative analysis. The Kruskal-Wallis analysis was applied first to the data in all groups. The Mann-Whitney *U*-test was thereafter used for analysis of differences between two groups. Spearman's rank correlation test was used to evaluate correlations between parameters. Multiple regression analysis (using $\log_{10}$ values) was carried out when assessing the simultaneous influence of age, atopy, ear disease, and cytokine mRNA expression on ϵ GL transcript levels. A two-tailed *P* value of ≤ 0.05 was considered significant.

Results

Expression of ϵ GL transcripts

In preliminary PCR experiments, ϵ GL transcripts were detected in the adenoids of all 37 children irrespective of disease. Competitive PCR showed a 300-fold range in expression levels of ϵ GL in the adenoids calculated as the ratio of the levels of ϵ GL to those of G3PDH transcripts (Fig. 3). Although the levels of ϵ GL transcripts were higher among atopics than nonatopics, this difference was statistically significant ($P < 0.01$) only for children with OME (Fig. 3). Moreover, in the OME group, the levels of ϵ GL transcripts correlated significantly with both serum IgE ($r_s = 0.67$; $P < 0.01$, $n = 15$) and the number of IgE⁺ cells in the extrafollicular compartment ($r_s = 0.60$; $P = 0.01$, $n = 16$) (Table 1).

In multiple regression analysis, ϵ GL transcript levels

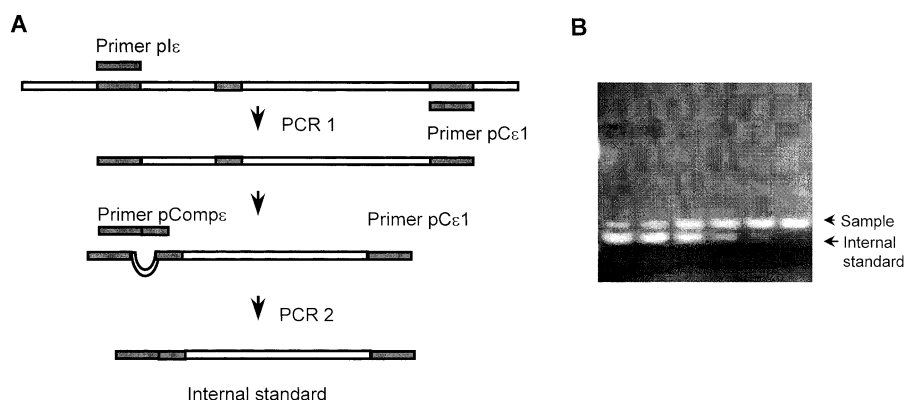


Figure 2. A) Strategy for construction of internal standard used in competitive PCR reactions. First PCR reaction was performed with primers pI ϵ and pC ϵ 1. cDNA was purified and internal standard for quantification of ϵ GL transcripts was obtained by performing PCR with primers pComp ϵ and pC ϵ 1. B) Example of competitive PCR of one patient sample with six dilutions of internal standard using primers amplifying ϵ GL transcripts.

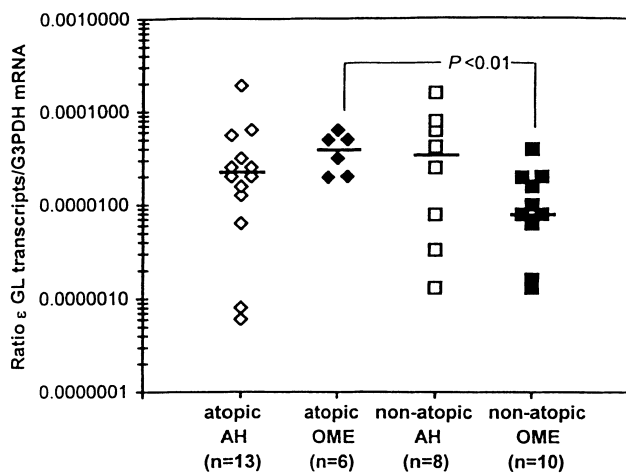


Figure 3. Competitive RT-PCR for εGL transcripts of adenoid specimens. RNA from adenoid samples from atopic and nonatopic AH and OME patients was extracted, reverse transcribed to cDNA, and amplified with specific primers in presence of threefold serial dilutions of corresponding internal standard, as described in "Material and methods". Log₁₀ of ratio of internal standard and sample was plotted against log₁₀ concentration of internal standard for each point of dilution series of internal standard. Molar ratio of εGL transcripts and G3PDH was determined for each sample. Median ratios for each group are indicated.

were found to be dependent on IL-4 mRNA expression ($P < 0.01$) and serum IgE levels ($P < 0.05$) ($R^2 = 0.32$, $n = 37$), but not on IFN-γ mRNA expression, age, or ear disease. A weak correlation between εGL transcript and IL-4 mRNA expression was found when all the children were included in the analysis ($r_s = 0.32$, $P = 0.05$, $n = 37$) (Fig. 4). This relation was not significant for the OME group but became stronger for the AH group ($r_s = 0.53$, $P = 0.01$, $n = 21$).

IL-4 and IFN-γ expression

Both IL-4 and IFN-γ mRNA expression was easily detected in all adenoid samples. The results with competitive PCR showed that the IL-4 and IFN-γ gene expression varied 150- and 300-fold, respectively (Fig. 5). However, the levels of the two cytokines as well as their ratio did not differ significantly between atopics and nonatopics, nor between OME and AH patients.

Discussion

In this study, we detected IL-4, IFN-γ, and εGL transcripts by competitive PCR in adenoid specimens of all children investigated irrespective of whether they had atopy or ear disease. We observed higher εGL transcript levels in the adenoids among atopic children, but this reached significance only in the OME group. Furthermore, significant associations between the levels of IL-4 and εGL transcripts were noted, particularly among children with AH. No such differences were

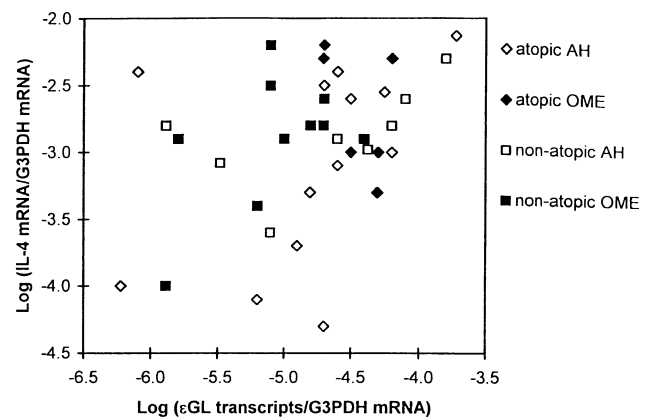


Figure 4. Relationship between εGL and IL-4 mRNA levels in adenoid specimens. Note significant but weak correlation between εGL and IL-4 mRNA levels for all patients ($r_s = 0.32$, $P = 0.05$, $n = 37$). This relation was not significant for OME group but became stronger for AH group ($r_s = 0.53$, $P = 0.01$, $n = 21$).

observed for IFN-γ transcripts or in the ratio of IL-4 to IFN-γ transcripts. These results suggest a local IL-4-induced IgE class switch among B cells in the adenoids.

Initial antigen priming of B cells for local production of IgA, IgM, and IgG is believed to take place mainly in mucosa-associated lymphoid tissue (MALT), such as the adenoids (16). Thus, it is conceivable that B cells are primed for local IgE production in the adenoids and/or such cells may home to different mucosal effector sites through lymph and peripheral blood. We have previously found an increased number of IgE⁺ cells in adenoids of atopic children by immunohistochemistry (8). However, only a few of these cells stained positive for plasma cell marker PCA-1, pointing to the difficulties involved in proving local IgE production. As εGL transcripts precede IgE synthesis (9, 12), they are considered alternative markers for locally induced IgE synthesis. Such transcripts have previously been demonstrated in PBMC, nose, tonsillar, and adenoid tissue (10–12, 16). Moreover, a good correlation between the expression of GL and mature ε transcripts has been shown with *in situ* hybridization, providing evidence for local IgE production in the nasal mucosa (11, 12). With competitive PCR, we have shown here detectable levels of εGL, IL-4, and IFN-γ mRNA in all adenoid specimens investigated, allowing quantitative comparisons. Our data show a positive correlation between IL-4 and εGL transcript levels, a finding which indicates a local induction of IgE in the adenoids. Moreover, the εGL transcript levels correlated with the number of IgE⁺ cells in the extrafollicular compartment among children with OME. However, we cannot rule out the possibility that some of the B cells were of peripheral origin. Interestingly, we found that the levels of εGL transcripts were higher among atopic than nonatopic OME children, a finding which may relate to the higher prevalence of OME observed among atopics

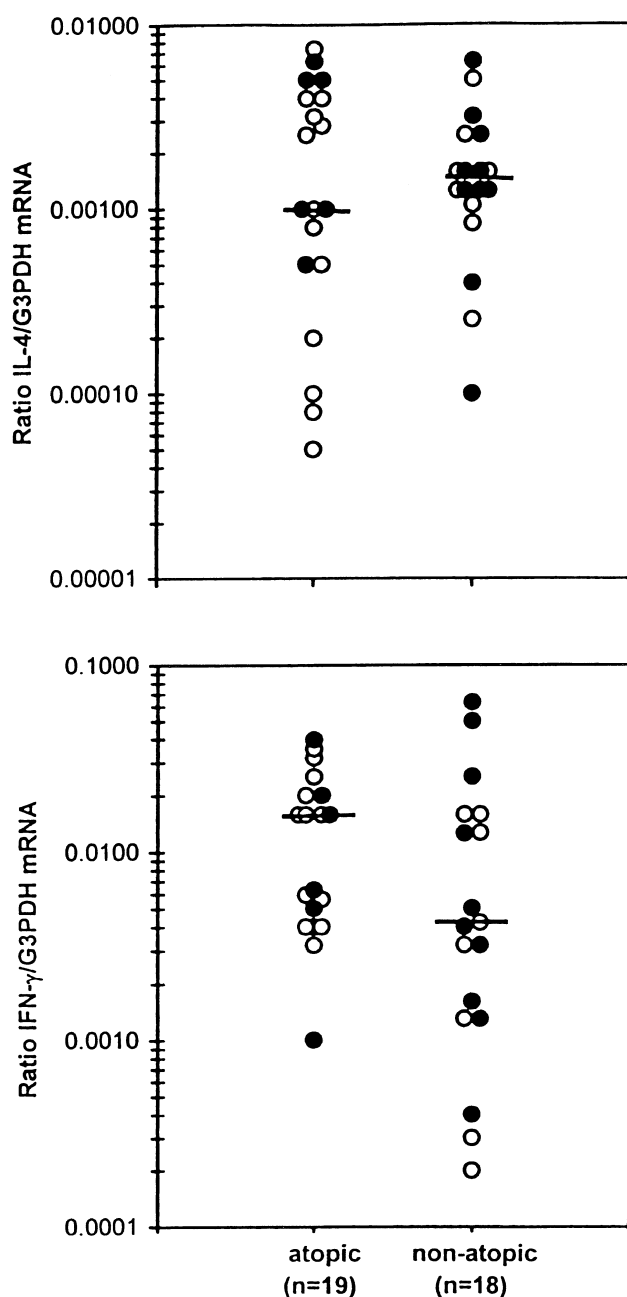


Figure 5. Competitive RT-PCR for IL-4 and IFN- γ mRNA of adenoid specimens from atopic and nonatopic AH (○) and OME (●) patients. RNA extraction, cDNA synthesis, competitive PCR, and gel analysis were performed as described in Fig. 3.

(3, 4). IgE production in the adenoids might influence local inflammation in the eustachian tube and middle-ear mucosa, thereby contributing to OME. The lack of a similar relation for AH children may be related to differences in the pathogenesis of the diseases and/or the effector organs involved, both with respect to the atopic condition as the origin of inflammatory activity.

Interestingly, the correlation between ϵ GL transcripts and IL-4 mRNA expression was strongest among AH patients.

There is a large amount of evidence indicating a reciprocal role of Th1- and Th2-related cytokines in IgE-mediated disease (17–19). IL-4 selectively promotes outgrowth of Th2 cells, while IFN- γ favors the development of Th1 cells (20, 21). However, no skewing toward a Th2-like cytokine response with increased IL-4 mRNA production and/or decreased IFN- γ mRNA production was observed in the adenoids of our atopic patients. Nor did Bernstein et al. (22) find any significant differences in the cytokine profiles, including IL-4 and IFN- γ , between otitis-prone and non-otitis-prone children nor in those of allergic and nonallergic children after *in vitro* stimulation of adenoidal lymphocytes with mitogen. There are several possible explanations for these findings. The adenoids, like other peripheral lymphoid tissues, are a highly compartmentalized organ with many distinct germinal centers where B cells undergo intense proliferation after encountering their specific antigen and helper T cells. It is likely that in some B-cell follicles the IL-4-induced switch is counteracted by IFN- γ (21). IFN- γ has several stimulatory effects on the immune response (23), and IL-4, like IL-13, also has anti-inflammatory properties (24, 25). Although we studied adenoids from children operated on during clinically infection-free periods, subclinical infections cannot be excluded, and one can assume, as in the tonsils, a continuous ongoing immune regulation with the production of numerous cytokines (26). Moreover, it can be assumed that cytokine expression and induction of IgE synthesis depend on allergen exposure at the time of surgery. Ideally, we would make comparisons with “healthy” controls, but ethical considerations preclude such studies.

In conclusion, this study suggests an IL-4-induced class switch to IgE production in the adenoids that may contribute to inflammatory reactions in the mucosa of the upper respiratory tract.

Acknowledgments

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