

Increased Levels of IL-2 and IL-4 in Stimulated Adenoidal Lymphocytes of Atopic Children

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Key Words

Adenoid · Allergy · Flow cytometry · Interleukin-2 · Interleukin-4 · Lymphocyte · B cells · 4β-Phorbol 12-myristate 13-acetate · Ionomycin

Abstract

Background: The adenoid is involved in the defence against airway pathogens and its surface is also exposed to airborne allergens. The knowledge about reactions taking place in the lymphatic tissue of this organ is, however, limited. To elucidate the influence of atopy we investigated the cellular and cytokine profile of in vitro-stimulated adenoid lymphocytes. **Methods:** Adenoid tissue cells from 13 atopic and 8 non-atopic children were cultured and stimulated with ionomycin and 4β-phorbol 12-myristate 13-acetate. Supernatants were collected after 4 and 20 h of stimulation and interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) were analysed by ELISA. Flow cytometry was used to quantify the leukocyte markers CD3, CD19, CD25 and HLA-DR. **Results:** Increased levels of IL-2 and IL-4 but not IFN-γ were detected in the supernatants of adenoid cell cultures from atopic children after 20 h of stimulation ($p < 0.05$) and a significant correlation with a positive regression between IL-2 and IL-4 was found. Atopy was also associated with a greater increase

in the percentage of CD19-positive B cells after stimulation ($p < 0.05$). **Conclusions:** A difference in the reactivity of adenoidal lymphoid cells in children was observed between atopic and non-atopic subjects. Atopy was associated with an increased production of IL-2 and IL-4 as well as a more pronounced increase of B cells.

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Introduction

As part of the mucosa-associated lymphoid tissue the adenoid, or nasopharyngeal tonsil, is involved in the defence against viral and bacterial pathogens. This organ, predominantly rich in B cells, contains all the immunocompetent cells necessary for an interaction with T cells, mediated through the secretion of cytokines and resulting in the production of immunoglobulins and the formation of memory cells [1]. Adenoids together with the palatine tonsils function as a local immune system and are able to generate an immunological memory [2]. Growing evidence implies that the lymphatic tissue of Waldeyer's ring, of which the adenoid is part, plays an important role as a source of B cells for the other areas of the upper respiratory tract [3]. It has also been shown that adenoids and tonsils act as sites for the induction of local and distant

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antibody responses against airway pathogens [4]. This function is exerted despite the lack of supplying lymphatic vessels [5], with the surface epithelium playing a central role in antigen presentation. The knowledge about interactions between the lymphoid cells of this organ and inhaled allergens is, however, limited.

We have previously reported an increase of IgE-containing cells in adenoids from atopic children [6] and an interleukin-4 (IL-4)-induced class switch to IgE production in adenoids [7]. Whether these findings indicate that the lymphatic tissue of adenoids is involved in IgE-mediated sensitisation due to local antigen stimulation or just mirror immune responses initiated in other parts of the immune system is not known. Few other studies have explored the relationship between atopy and immunological reactions in adenoids and the results have been based solely on findings from immunohistochemical examinations [8–10]. Stimulation of the lymphoid cell population in adenoids would give a more dynamic picture of the immunological reactions taking place there.

In this study we therefore investigated the cytokine profile of in vitro-stimulated lymphoid cells from adenoids of atopic and non-atopic children. By this we wanted to elucidate the influence of atopy and to obtain more information on the role of adenoidal lymphocytes in IgE-mediated reactions. The secretion of the cytokines IL-2, IL-4 and IFN- γ by adenoidal lymphocytes was of special interest. Furthermore the lymphocyte cell surface markers CD3, CD19, CD25 and HLA-DR were analysed.

Material and Methods

Patients

Adenoids were obtained from 21 children, 15 boys and 6 girls. Participation was by parental informed consent and the Karolinska Hospital Ethics Committee approved the study.

Adenoid tissue was surgically removed (adenoidectomy) for either adenoidal hyperplasia with secondary nasal obstruction or otitis media with effusion of at least 3 months' duration. The diagnosis of adenoidal hyperplasia was based on visible obstruction of the nasopharynx during clinical examination with a mirror or a fiberoptic nasolaryngoscope and a history of obstructive symptoms (mouth breathing when awake and snoring during sleep). The diagnosis of otitis media with effusion was based on pneumatic otomicroscopy and tympanometry showing a flat, Jerger-type B tympanogram. Patients with cleft palate, known immunological deficiency, a severe underlying disease or who had been treated with antibiotics within 1 month prior to surgery were excluded. All children were required to have normal, non-diseased tonsils as shown by history and physical examination. Patients with hyperplastic tonsils were not included in the study.

The parent(s) completed a questionnaire emphasising the children's medical history including allergic symptoms and medication and the family history of atopic diseases [asthma, rhinoconjunctivitis and atopic eczema dermatitis syndrome (AEDS)] [11].

Before surgery all children underwent routine ear, nose and throat examination. A blood sample was obtained during the operation and analysed for total IgE (Pharmacia CAP System IgE FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden) and screened with Phadiatop[®], a mixture of inhalant allergens (including birch pollen, mugwort, timothy grass, *Dermatophagoides pteronyssinus*, *Cladosporium herbarum*, cat, dog, horse; Pharmacia CAP System Phadiatop FEIA) and a mixture for common food allergens (fx5; egg, milk, peanut, soybean, fish and wheat; Pharmacia CAP System Specific IgE FEIA). The sera that scored positive in Phadiatop (≥ 0.35 kU/l) were further analysed for IgE antibodies against common inhalant allergens (Pharmacia CAP System Specific IgE FEIA).

Children were classified as atopic if they had a positive Phadiatop. Eight of the atopic children were in addition sensitised to food allergens (fx5). The atopic children ($n = 13$) had symptoms of rhinitis ($n = 1$) or rhinoconjunctivitis ($n = 10$) and/or asthma ($n = 7$). Seven of them also had AEDS. The non-atopic children ($n = 8$) who served as controls had no symptoms of allergy and a negative Phadiatop and fx5. Eight of the atopic children and 1 of the nonatopic ones had elevated levels of total IgE related to their age. At the time of surgery 5 of the atopic children were receiving treatment for their allergy; 1 with a nasal steroid, 2 with inhalant short-acting β_2 -agonist, 3 with oral antihistamines and 3 with steroid ointment.

Data on patient characteristics for the different clinical groups are presented in table 1.

Preparation of Adenoid Cell Suspension

Removed adenoids were immediately transferred to 4°C saline, kept on ice, and transported to the laboratory. In order to obtain a homogeneous tissue suspension the adenoids were cut into small pieces and passed through a fine wire mesh that was rinsed with RPMI 1640 (with *L*-glutamine and 25 mM Hepes, Gibco, Invitrogen, Paisley, UK). The cell suspension was then mixed and centrifuged at 200 *g* for <1 min at 4°C. Remaining tissue elements were thus allowed to sediment and the supernatant containing leucocytes was centrifuged at 350 *g* for 10 min at 4°C. Following a second washing with RPMI 1640 (350 *g*, 10 min, 4°C) the cells were finally resuspended in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (Gibco) and gentamycin (50 μ g/ml).

Stimulation of Adenoid Lymphocytes

Adenoid lymphocytes in suspension were cultured at a concentration of 1.75×10^6 cells/ml in RPMI 1640, as described above, at 37°C in a humidified 5% CO₂ atmosphere in 24-well flat-bottomed microtiter plates (Becton Dickinson, N.J., USA). The cells were stimulated for 20 h with 10^{-8} M 4 β -phorbol 12-myristate 13-acetate (PMA, Sigma) and 10^{-7} ionomycin (Sigma) or medium alone (control group). The cells were then washed with RPMI 1640 (350 *g*, 10 min, 4°C) and were finally resuspended in RPMI 1640 and kept on ice. Leucocyte immunostaining (see below) was thereafter done.

Monoclonal Antibodies

The following monoclonal antibodies were used: mouse IgG1-FITC negative control (100 mg/l), DAKO A/S, Glostrup, Denmark; mouse anti-human CD3-FITC (100 mg/l), DAKO; mouse anti-human CD19-FITC (100 mg/l), DAKO; mouse IgG1-FITC/mouse

Table 1. Characterisation of patients according to age, sex and total serum IgE

	All children (n = 21)	Atopic children (n = 13)	Non-atopic children (n = 8)
Age, years ^a	7.2 (± 1.0)	8.5 (± 3.0)	5.2 (± 1.1)
Sex (M/F)	15/6	11/2	4/4
Adenoidal hyperplasia	13	9	4
OME	8	4	4
Serum IgE, kU/l ^{a, b}	491	599 (± 509)	50 (± 58)

OME = Otitis media with effusion.

^a Data are given as means (SD).

^b Normal values: <22.3 kU/l 9 months to 5 years of age and <263 kU/l above 5 years of age.

Ig2a-PE, Immunotech, Marseille, France; mouse anti-human CD3-FITC/CD25-PE, Immunotech; mouse anti-human CD3-FITC/HLA-DR-PE, Immunotech.

The concentrations of the antibodies used were according to the manufacturer's instructions, for the antibodies from DAKO 1 µg/5 × 10⁵ cells and for the antibodies from Immunotech 20 µl/5 × 10⁵ cells.

Viability Testing

The viability of the cultured cells was assessed using the fluorescence intensity of the vital dye CFDA (carboxyfluorescein diacetate). Briefly, the cultured adenoid cells were resuspended in 100 µl CFDA solution (10 mg/ml) diluted in Tris-buffered Hanks' solution and incubated for 15 min at room temperature. After a final washing in PBS by centrifugation (300 g, 7 min, 4°C) the cells were resuspended in PBS and immediately analysed by flow cytometry. The viability was >95% in all experimental settings.

Immunostaining and Flow Cytometry

The specimens were examined using an EPICS XL (Coulter, Hialeah, Fla., USA) flow cytometer. Discriminating gates were set around the adenoid cell populations on the basis of forward and side scatter. The lymphocyte cell population was further analysed with a minimum of 1,000 cells counted within the lymphocyte gate.

Results are given as the percentage of positively labelled cells or as mean fluorescence intensity units.

Cytokine Assay

The adenoid lymphocytes were stimulated as previously described with PMA and ionomycin. The supernatants were collected 4 and 20 h after stimulation and stored at -80°C until cytokine analysis. IL-2, IL-4 and IFN-γ were quantified using commercial enzyme-linked immunosorbent assay kits (IL-2 and IFN-γ, R&D Systems, Minneapolis, Minn., USA; IL-4, BioSource International, Camarillo, Calif., USA) according to the manufacturer's protocols. The detection range was 7–2,000 pg/ml for IL-2, 0.27–25 pg/ml for IL-4 and 8–1,000 pg/ml for IFN-γ.

Statistical Methods

Student's *t* test was used to analyse differences regarding cell populations. The Mann-Whitney *U* test was used to analyse differences

regarding the production of the cytokines IL-2, IL-4 and IFN-γ. Significance was determined at a *p* value of <0.05. In correlation analysis Pearson correlation coefficients were computed and linear regression performed.

Results

Cellular Data

When we analysed changes in cell populations over time (0–20 h) significant differences between atopic and non-atopic subjects were observed in the stimulated cultures (table 2). The increase in the percentage of CD19-positive cells was larger among atopic subjects than among non-atopic ones (*p* < 0.05). In the stimulated cultures the percentage of CD3-positive cells decreased over time (0–20 h) and this decrease was more pronounced among atopic subjects (*p* < 0.05).

When comparing the cell composition in the non-stimulated cultures from atopic and non-atopic subjects at 20 h we found significant differences. The percentage of CD19-positive cells was significantly higher among atopic compared to non-atopic subjects and the percentage of CD3-positive cells significantly lower (table 2).

The HLA-DR expression decreased in non-stimulated cultures and increased slightly in stimulated cultures. The decrease in the non-stimulated cultures was larger among non-atopic subjects (*p* < 0.05). The percentage of CD25-positive cells increased marginally in non-stimulated cultures but substantially (10-fold) in the stimulated cultures without any differences between atopic and non-atopic subjects.

No age-related differences were found regarding the cell markers CD3, CD19, CD25 and HLA-DR (data not shown).

Table 2. Cellular data for the different patient groups before and after 20 hours of stimulation with ionomycin and PMA

	0 h			Non-stimulated cultures at 20 h			Stimulated cultures at 20 h			Stimulation effect ^a	
	all	A	NA	all	A	NA	all	A	NA	A	NA
CD3, %	44.4 (5.8)	45.7 (6.1)	41.4 (4.0)	54.3 (7.4)	51.9 (7.2)	59.4 (5.0) ^b	37.1 (6.0)	36.2 (6.4)	39.2 (4.9)	-9.5 (5.3)	-2.2 (5.1) ^b
CD19, %	56.0 (6.1)	55.0 (6.6)	58.3 (4.3)	44.5 (7.2)	46.7 (7.4)	39.8 (3.8) ^b	62.8 (5.8)	63.6 (6.2)	61.1 (5.0)	+8.6 (5.3)	+2.8 (5.2) ^b
CD25, MFI	2.3 (0.6)	2.3 (0.7)	2.4 (0.5)	2.7 (0.7)	2.7 (0.7)	2.6 (0.8)	4.5 (1.0)	4.5 (1.2)	4.5 (0.9)	+2.2 (1.0)	+2.1 (1.1)
CD25, %	4.2 (2.0)	4.2 (2.2)	4.2 (1.7)	7.6 (2.7)	8.4 (2.7)	6.4 (2.3)	39.4 (9.9)	39.0 (11.4)	40.0 (7.6)	+34.8 (9.7)	+35.8 (7.3)
HLA-DR, MFI	24.5 (6.5)	25.8 (6.5)	22.4 (6.4)	20.9 (6.1)	23.8 (4.6)	16.4 (5.6) ^c	24.8 (7.1)	26.5 (6.5)	22.0 (7.5)	+0.7 (7.2)	-0.4 (2.2)
HLA-DR, %	67.3 (6.7)	66.1 (7.0)	69.2 (6.0)	61.6 (9.3)	62.9 (9.3)	59.4 (9.6)	79.4 (6.7)	78.5 (7.3)	80.9 (5.7)	+12.4 (5.5)	+11.7 (5.7)

Data are given as means (SD). all = All patients; A = atopic children; NA = non-atopic children; MFI = mean fluorescence intensity units.

^a Values obtained after 20 h of stimulation subtracted by values for the same group at 0 h.

^b $p < 0.05$, when comparing atopic and non-atopic children.

^c $p < 0.01$, when comparing atopic and non-atopic children.

Cytokine Levels and Atopy

After 20 h of stimulation the adenoid cells in cultures from atopic subjects were found to have significantly higher levels of IL-2 and IL-4 compared to cultures from non-atopic subjects (fig. 1a, b, table 3). The IL-2 levels were higher among atopic subjects already before stimulation (mean values in pg/ml: atopic: 12.9, non-atopic: 8.5, $p < 0.01$) as well as after 4 h of stimulation (mean values in pg/ml: atopic: 60.8, non-atopic: 45.7, $p < 0.05$). In contrast, the IL-4 levels were not detectable before stimulation and at 4 h there were no differences between atopic and non-atopic subjects. Regarding IFN- γ no differences were observed at any time point between atopic and non-atopic subjects.

Cytokine Kinetics

After 20 h of stimulation IFN- γ , IL-2 and IL-4 were detectable in all adenoid cell cultures (fig. 1, table 3). IL-2 was present in all cell cultures already before stimulation and the levels increased approximately 5-fold after 4 h of stimulation and 50-fold after 20 h of stimulation (fig. 1a). In the non-stimulated cultures the levels of IL-2 were unchanged.

IFN- γ and IL-4 levels showed a different pattern (fig. 1b, c). Four cell cultures had detectable levels of IFN- γ before stimulation and in only one cell culture could IL-4 be detected before stimulation. Most of the stimulated cell cultures had initiated an IFN- γ production after 4 h (18 of 21) in comparison with IL-4 that could be detected at low levels in 12 of 21 cultures. However, after 20 h both cytokines were detectable in all stimulated adenoid cell cultures in contrast to non-stimulated cultures at the same time point, when only a few adenoid cell cultures

had detectable levels (six adenoids for IFN- γ and four for IL-4).

When cytokine levels after 20 h of stimulation were put in relation to the presence of CD25 and HLA-DR, a statistically significant correlation with negative regression was found between the percentage of CD25-positive cells and IL-2 ($p < 0.05$, Pearson correlation coefficient 0.45), whereas no such correlation was found for the other cytokines or to HLA-DR. There was also a statistically significant correlation with positive regression between IL-2 and IL-4 ($p < 0.05$, Pearson correlation coefficient 0.54), but not for either of these cytokines related to IFN- γ .

Discussion

In the current study we present data showing an association between atopy and an increased production of IL-2 and IL-4 but not IFN- γ in adenoidal cell cultures, stimulated for 20 h with ionomycin and PMA. Moreover, stimulation resulted in a greater increase in the percentage of B cells in the cultures from atopic than from non-atopic children. These data support the concept that the immune reactivity of adenoid tissue differs between atopic and non-atopic individuals, and are in agreement with the findings in our previous studies on adenoids [6, 7, 12].

Several studies have investigated lymphocyte subpopulations in adenoids in an effort to elucidate the role of this organ in the pathogenesis of otitis media with effusion [12–16]. In contrast, few studies have examined cytokine production by adenoidal lymphocytes [17–19]. In one of these studies by Bernstein et al. [18] there was a comparison between atopic and non-atopic children. The

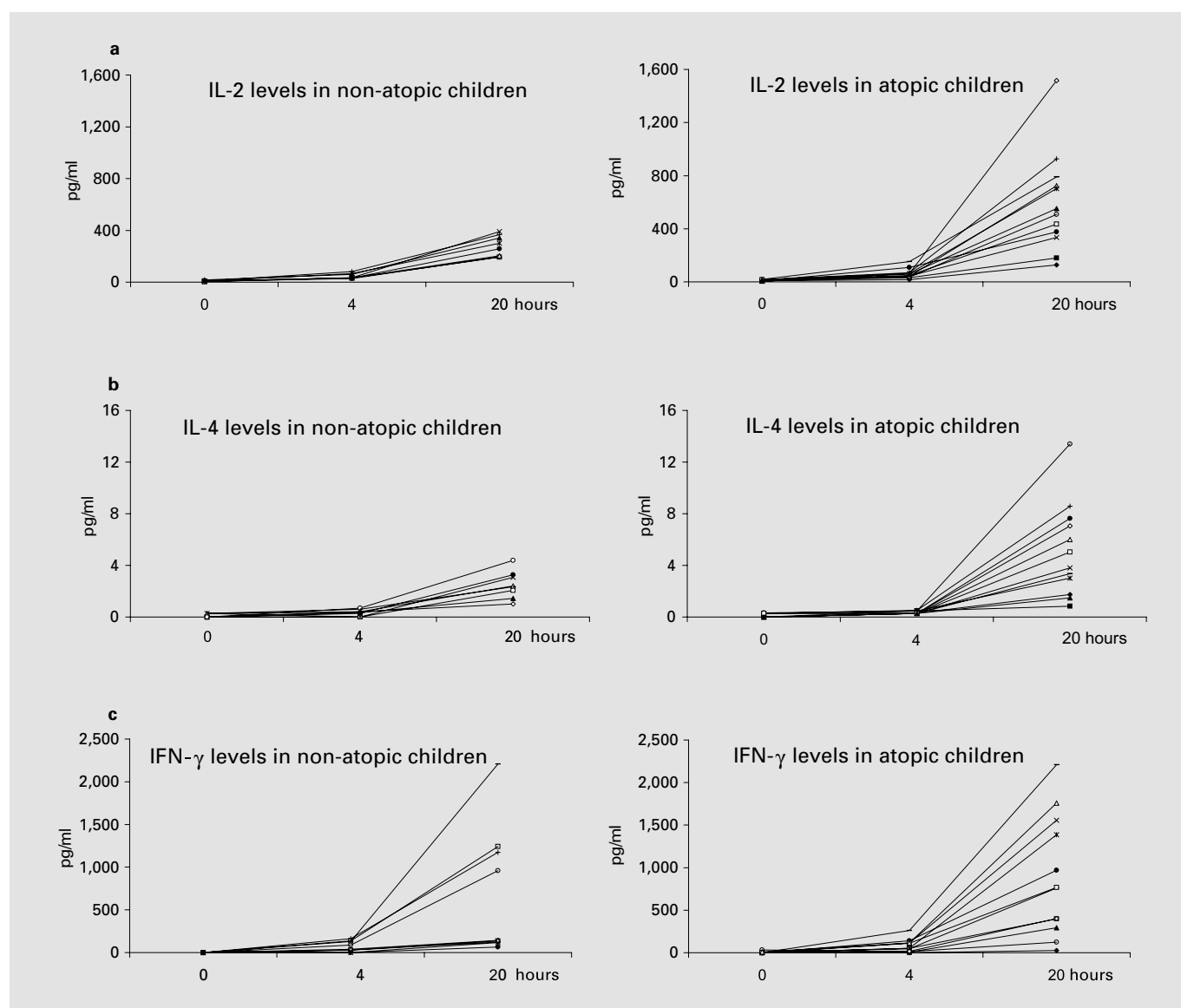


Fig. 1. Comparison of cytokine secretion in supernatants of cultured cells from adenoid tissue of atopic and non-atopic children. Levels after 4 and 20 h of stimulation with ionomycin and PMA. **a** IL-2 secretion, $p < 0.05$, atopic vs. non-atopic children after 20 h of stimulation. **b** IL-4 secretion, $p < 0.05$, atopic vs. non-atopic children after 20 h of stimulation. **c** IFN- γ secretion.

Table 3. Cytokine levels in supernatants of adenoid tissue cultures after 20 h of stimulation with ionomycin and PMA

	All children	Atopic children	Non-atopic children
IL-2, pg/ml	376/505 (360)	553/643 (397)	279/281 (80) ^a
IL-4, pg/ml	3.3/4.3 (3.2)	5.0/5.4 (3.5)	2.4/2.5 (1.1) ^a
IFN- γ , pg/ml	403/703 (649)	760/831 (702)	140/495 (528)

Data are given as median/mean (SD) levels.

^a $p < 0.05$, Mann-Whitney U test, when comparing atopic and non-atopic children.

study showed that lymphatic cells of adenoids produced cytokines of both Th1 and Th2 responses and that, compared with peripheral blood lymphocytes, their secretion of IFN- γ was lower, whereas the secretion of IL-4 was higher. However, they could not demonstrate any differences in cytokine profiles when comparing atopic and non-atopic children [18]. The severity of the allergic symptoms in the patients included could explain this discrepancy. The children in the above-mentioned study were only suffering from allergic rhinitis, whereas over one third of the children in our study, in addition to rhinitis, also had asthmatic symptoms and AEDS, indicating a more severe atopic disease, which could increase the reactivity of the adenoid lymphocytes.

Boyaka et al. [20], who analysed phytohaemagglutinin-stimulated adenoid mononuclear cells, reported secretion of IFN- γ , IL-4, IL-5 and IL-10. Interestingly cultures with a high proliferative response secreted higher levels of the Th2-type cytokines (IL-4, IL-5 and IL-10) compared with IFN- γ and the opposite was noted for the cultures with a low proliferative response. The atopic status of the children included was not investigated, however, which means that the children with the higher levels of Th2-type cytokines could well be atopic.

The greatest amount of experience in cytokine flow has been gained with PMA and ionomycin, as these conditions yield a reproducible and robust activation and represent the physiologic potential of cellular cytokine production [21]. When peripheral blood mononuclear cells (PBMC) are stimulated with PMA and ionomycin the kinetics of intracytoplasmic expression of IL-2 and IFN- γ show similarities [22] with the increasing levels of IL-2 and IFN- γ we could detect in adenoids up to 20 h. In contrast, while IL-4 levels seem to have a peak 4–6 h after stimulation of PBMC [22, 23], in our study the levels of IL-4 were very low or not detectable at 4 h. An increase in IL-4 could not be measured until after 20 h of stimulation, both in the atopic and non-atopic children, although it was more pronounced in the former group. This discrepancy indicates that the T cell response in PBMC differs from that in adenoids. The few data that are available also show that significantly more IL-4 is produced by adenoidal lymphocytes compared to peripheral blood lymphocytes, measured both in supernatants [18] and at the single cell level [24]. This imbalance in IL-4 production has been hypothesised to be the result of a Th2 response inducing plasma cell formation and synthesis of immunoglobulins necessary for the host defence against potential pathogens [18]. The high bacterial load in adenoids during childhood might thus result in interfering levels of

locally produced cytokines and our findings of increased levels of IL-4 among atopic subjects can therefore only be observed when adenoid cells are stimulated, in contrast to our previous immunohistochemical study [7]. It can be assumed that these differences in environmental stimuli lead to great individual variations in cytokine levels in adenoidal tissue observed in this as well as in other studies [7, 25], and difficulties in identifying effects of a single environmental factor such as atopy.

IL-2 plays a central role in regulating B cell activation, proliferation and differentiation [26] and the activation of T lymphocytes is accompanied by increased surface expression of CD25 (i.e. IL-2 receptors). The negative regression that we report of between the percentage of CD25-positive cells and IL-2 indicates a downregulation of the IL-2 receptor with increasing levels of IL-2, as found by others [27].

The conclusion that the differences in cytokine levels are caused by the presence of atopy is hampered by the age difference between the atopic and non-atopic groups in our study. The question regarding the impact of age on the adenoidal lymphatic tissue is, however, not fully understood due to the limited number of studies that have dealt with this subject. We have previously demonstrated a difference in memory cells of the T helper subset related to age [12]. Epsilon germline transcript levels in adenoids of atopic and non-atopic subjects were, however, not related to age in another study from our laboratory [22]. No age-related differences could either be detected among CD19-positive B cells or T lymphocytes of helper (CD4+) and suppressor (CD8+) type in a recently published study that used flow-cytometric analysis to examine different lymphocyte subpopulations in relation to age [28]. In the present study we did also not find any age differences regarding the cell markers CD3, CD19, CD25 and HLA-DR.

The percentage of cells positive for CD3, CD19 and HLA-DR in the adenoid cell suspensions prior to stimulation are consistent with data reported by others [28–30]. In the stimulated cultures we observed a decrease in the percentage of CD3-positive cells. This is unlikely a consequence of apoptosis and secondary necrosis, since the viability in all experimental conditions was more than 95% and a difference in decrease was noted between atopic and non-atopic children. The CD19-positive B cells were equally distributed in the adenoids of all our patients, as has been described in the nasal mucosa of atopic and non-atopic children [31]. In our study stimulation led to a greater increase of CD19-positive B cells in the adenoids of atopic children. This could be explained by the hypoth-

esis that adenoids are involved in IgE-mediated sensitisation, with a stimulation-induced differentiation of B cells aimed at IgE production. The findings showing a relationship between allergy and memory cells in adenoids [12] also support this hypothesis, as do the increased numbers of antigen-presenting Langerhans' cells and eosinophils in adenoids of allergic patients [32].

In conclusion, this study shows an increased reactivity in adenoidal cells of atopic children. Stimulation with ionomycin and PMA led to an increased production of IL-2 and IL-4 and a more pronounced increase of B cells in atopic compared with non-atopic children.

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