

Differences in cellular infiltrates in the adenoid of allergic children compared with age- and gender-matched controls

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Summary

Background Allergic sensitization of the airways occurs in the mucosa of the shock organ, or in the lymphatic stations draining these structures. The lymphatic structure closest to the nasal mucosa is the adenoid.

Objectives The objective of this study was to find evidence for our hypothesis that allergic sensitization can occur in the adenoid. Of special interest, in this context are cell types involved in antigen-allergen presentation (e.g. Langerhans cells) and effector cells of allergic disease.

Methods In this study cellular infiltrates in adenoids of 16 allergic patients and 16 age- and gender-matched controls were evaluated. The number of cells positive for CD1a, CD4, CD8, CD-68, chymase, tryptase, IgE, MBP and cells positive for interleukin (IL)-4 were determined using immunohistochemical staining techniques. The epithelium, follicles and the interfollicular spaces were evaluated separately.

Results When comparing the two groups a significant increase in cells positive for CD1a was found in interfollicular spaces of the allergic group ($P = 0.008$). CD1a⁺ cells in the follicular space and eosinophils in the interfollicular space showed a trend to be more numerous in the allergic group ($P = 0.02$ and $P = 0.05$, respectively). The other cell types investigated did not show significant differences between the groups.

Conclusions The results of this study show for the first time that cells involved in allergic sensitization and allergic disease differ in the adenoid of allergic children compared with controls. These findings support our hypothesis that allergic sensitization takes place in the adenoid. Furthermore, this study confirms that CD1a⁺ (Langerhans) cells are involved in allergic disease.

Keywords: allergy, children, lymphoid tissue, adenoid, Langerhans cell, immunohistochemistry

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Introduction

The first years of life appear to be of crucial importance to the development of allergic disease. However, it is not clear which factors control the possible manifestation and seriousness of the disease. One of the factors that appears to control the manifestation of the disease is the allergen sensitization route [1].

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The nose is an important sensitization route in allergic airway diseases. CD1a⁺ Langerhans cells seem to be the most important antigen presenting cells in the nasal mucosa [2,3]. These cells are capable of antigen/allergen presentation to T lymphocytes. This is analogous to results obtained in studies on Langerhans cells in the skin [4].

Research on adult nasal mucosa showed the antigen-presentation process to be located among others in the lamina propria, suggesting a local immune response to allergen provocation [5,6]. When looking at APC/T-cell interaction in other epithelial structures, such as skin and

intestinal mucosa, it is more likely for the majority of these interactions to take place in regional draining lymphoid structures, such as lymph-nodes [7,8].

Regional draining lymphoid structures of the nasal mucosa are the cervical lymph-nodes, and Waldeyer's ring, which consists of palatine-, lingual tonsils and the adenoid. Its anatomical position and cell populations make Waldeyer's ring a prime site for antigen presentation [9]. Moreover, animal experimental studies have shown an important role for the murine nasal-associated-lymphoid-tissue (NALT) (the murine equivalent of Waldeyer's ring) during allergic sensitization [10]. This led us to hypothesize that allergic sensitization to aeroallergens occurs primarily in Waldeyer's ring, particularly in the adenoid.

A first indication of a potential role for the adenoid during allergic sensitization in the airways are differences in cell population between allergic and non-allergic children. In reviewing literature, a few articles were found on human adenoid and tonsil cell populations [11,12]. These studies deal with changes in the cellular infiltrate related to age and infectious diseases. No studies were found comparing lymphoid and non-lymphoid cell populations in the adenoid of allergic and non-allergic children.

Materials and methods

Patients and controls

A group of 81 children participated in this study, composed of 51 boys and 30 girls with a median age of 3.2 years (range 0.9–10), who underwent adeno(tonsillectomy) between January 1994 and April 1994 at the ENT Department of the Sophia Children's Hospital in the Netherlands. The parents or foster parents of the children were asked to complete an extensive questionnaire. Data were collected on symptoms and signs of the children's disease(s), medication, family history with respect to allergy, rhinitis, asthma, and eczema, smoking habits, and contact with pets. A blood sample was taken during the operation to measure total and specific IgE by means of Phadiatop RIA (Pharmacia CAP System, Uppsala, Sweden). Allergens most relevant to infants and children like food allergens (milk protein, egg white, peanut, soya) and inhalation allergens (house dust mite, grass pollen, cat- and dog epithelium) were tested.

Children were considered allergic when they fulfilled one of the following inclusion criteria:

- signs and symptoms of rhinitis and/or asthma and specific IgE to aero-allergens, or
- rhinitis and/or asthma, a sufficiently high total IgE for age (>10 IU/year of age) and a positive family history (defined as having at least one first-degree relative with a proven allergy to aero-allergens), or

- rhinitis and/or asthma, a sufficiently high total IgE for age and specific IgE to food-allergens.

Children were classified as controls, if there were no allergic symptoms, a low total IgE for age, a negative Phadiatop and a negative family history.

Out of the 81 children studied, 16 children (20%) met the inclusion criteria for allergy and 32 (40%) were classified as controls. For 33 children (40%), classification remained unclear. The group of 16 allergic patients consisted of 10 boys and six girls, the median age being 2.2 years (range 0.9–10) and the median total IgE value 118 IE/mL (range 10 – >2000). Five out of the 16 patients had rhinitis and/or asthma in combination with specific IgE to aero-allergens. Three out of the 16 patients had rhinitis and/or asthma and a high total IgE, combined with specific IgE to food-allergens (Table 1). Eight out of the 16 patients had rhinitis and/or asthma and a high total IgE, combined with a positive family history. Eight out of the 16 allergic children suffered from medication dependent asthma. The 16 allergic patients were matched with 16 controls for age and gender. The control group consisted of eight boys and eight girls, the median age being 3.4 years (range 1.1–9) and the median total IgE value was 8 IE/mL (range <2–45).

Tissue collection

The adenoids of the patients were removed with an adenotome, snap-frozen in liquid nitrogen immediately after surgery, and stored at –80 °C. From each adenoid, 6 µm-thick serial sections were cut on a Reichert-Jung 2800 Frigocut cryostat and transferred to poly L-lysine-coated microscope slides. After transfer, tissue sections were air-dried and kept at –80°C until immuno-histochemical staining.

Table 1. Phadiatop results of eight patients which were included in the allergic group because of specific IgE to aero- and or food allergens. Phadiatop test results are given in class ranging from 0 (no detectable IgE) to 6 (very high levels of specific IgE)

	Age (months)	HDM	Grass	Cat	Milk	Egg white
patient 1	11			2	2	
patient 2	17				2	2
patient 3	19				2	3
patient 4	43					2
patient 5	49	5	2	2		
patient 6	53	6			2	2
patient 7	86	4				
patient 8	120	3		2		

HDM, House dust mite.

Single staining

The sections were stained using the immuno-alkaline phosphatase antialkaline phosphatase (APAAP) method. The sections were fixed in acetone for 10 min, rinsed in phosphate-buffered saline (PBS), pH 7.4 for 5 min, rinsed in 10% normal rabbit serum for 5 min and incubated with a monoclonal antibody (see Table 2) for 30 min at 20°C. Sections were rinsed in phosphate buffered saline (PBS) for 5 min and incubated for 15 min with unconjugated rabbit anti-mouse Ig serum, diluted 1:20 in PBS, supplemented with 10% normal human serum (to saturate Fc receptors and to reduce background staining), rinsed in PBS for 5 min again, followed by incubation with the AP anti-AP complex, diluted 1:40. Subsequently, the sections were rinsed in PBS followed by TRIS buffer, Ph 8.0, and incubated with New Fuchsin staining mixture in the dark at room temperature. After staining the sections were rinsed with distilled water, counterstained with Mayer's haematoxylin, washed in running tap water and mounted in glycerin gelatin. Control staining was performed by substitution with PBS and incubation with non-relevant IgG.

Double staining

Frozen sections were fixed in acetone for 10 min and rinsed in phosphate-buffered saline (PBS pH 7.4). Endogenous peroxidase was blocked with 1% sodium azide and 0.01% hydrogen peroxide in PBS for 30 min. Sections were then rinsed in PBS for 10 min, incubated with 10% normal goat serum and 10% normal rabbit serum. Subsequently sections were incubated with the antibodies to CD1a (see Table 2). After 1 h they were rinsed with PBS for 5 min, incubated with Goat α Mouse AP (Immunotech, France) for 30 min and rinsed again with PBS for 5 min. Following this, sections were incubated with mouse α alkaline phosphatase

conjugated to alkaline phosphatase (APAAP, Dako, Denmark) and rinsed with PBS for 5 min. This first step in the double staining procedure was followed by a second step aimed at CD3. Sections were incubated with 10% normal mouse serum and incubated with FITC conjugated monoclonal antibodies to CD3 for 1 h (see Table 2). They were rinsed with PBS for 5 min and incubated with peroxidase-conjugated Rabbit α FITC (Dako, Denmark) for 30 min. Samples were then rinsed with PBS for 5 min and with TRIS buffer [0.1 M pH 8.5] for 5 min. This was followed by an incubation for 30 min in Fast Blue substrate. Finally, samples were rinsed with NaAc 0.1 M pH 4.6 for 5 min, and incubated with AEC substrate for 30 min.

Light microscopic evaluation

The number of positive cells was determined by superimposing a grid of five by five squares, covering a surface area of 0.025 mm² on the section with an eyepiece graticule at a magnification of $\times 200$. T cells were counted at a magnification of $\times 400$. All intact epithelium present on the section was counted. A minimum of 0.25 mm² of epithelium was evaluated. In most sections 0.5 mm² or more was evaluated. Three follicles covering a total surface area of 0.5 mm² or more were counted and at least 0.5 mm² of interfollicular space was counted as well. Representative parts of all compartments were selected for evaluation. Subsequently, the number of positive cells per mm² was calculated.

Statistical analysis

As sections were counted blindly by two independent investigators, inter observer reliability was determined. This was done by calculating the Spearman rank correlation. A strong correlation was found for each cell type ($R > 0.85$).

Table 2. Monoclonal antibodies used in studying cellular infiltrates in the adenoid of allergic patients and controls

Antibody	Concentration	Specificity	Source
Leu 3	1 μ g/mL	CD 4	Becton Dickinson, Dorset, UK
Leu 2	0.1 μ g/mL	CD 8	Becton Dickinson, Dorset, UK
BMK13	2 μ g/mL	MBP	Sambio, Uden, The Netherlands
OKT 6	0.1 μ g/mL	CD1a	Department of Immunology, Erasmus University, The Netherlands
α -IgE	2 μ g/mL	IgE	CLB, Amsterdam, The Netherlands
B7	1 μ g/mL	Chymase	Chemicon, Temecula, California, USA
G3	0.5 μ g/mL	Tryptase	Chemicon, Temecula, California, USA
KI-M6	1 μ g/mL	CD-68	Behring, Marburg, Germany
α -IL-4	20 μ g/mL	IL-4	Genzyme, Cambridge, USA
Leu 4 Fitc $\diamond$	1 μ g/mL	CD 3	Becton Dickinson, Dorset, UK

$\diamond$, used as second-step antibody in double staining.

Statistical analysis of cell numbers was performed with SPSS (version 6.0 for Windows). As the number of cells per mm² did not show a normal distribution, the non-parametric Mann–Whitney *U*-test was used. Because of multiple comparisons performed in this study the *P*-value indicating a significant difference between groups was set at ≤ 0.01 .

Results

General description

The total surface area of the adenoid sections ranged from 3 mm²–100 mm². Considerable immunological activity was seen to occur in the adenoid as shown by the many follicles. The follicles mostly had a dark-staining corona or mantle zone and a pale-staining germinal centre (See Figs. 1 and 2). No differences were found in the diameter of these secondary follicles between allergic children and controls (data not shown). The outermost border of the follicles was not always sharply defined. The cellular density of the interfollicular space varied between the adenoids.

The epithelial lining of the sections varied. The total thickness ranged from one cell layer to six layers or more.

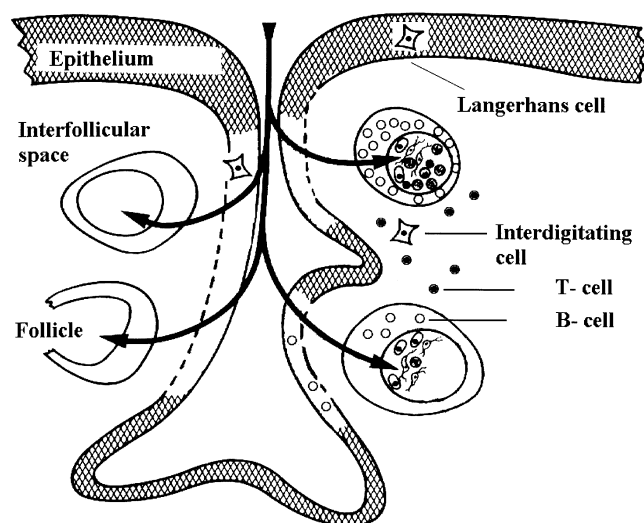


Fig. 1. Schematic representation of the different regions within the adenoid tissue. The surface epithelium folds towards the centre of the adenoid to form 'pseudo crypts'. On either side of these 'cryptal' structures follicles are located consisting of a germinal centre and a (semi)circular mantle zone. The latter is usually wider at the side directed towards the epithelium. The interfollicular spaces, which are predominately T-cell areas, are situated between the follicles and epithelium. Modification of original schematic representation by Professor P. Brandtzaeg, Institute of Pathology, University of Oslo, The National Hospital, Oslo, Norway [11]. Reproduced with kind permission of the author.

The epithelium was columnar and/or partially stratified cuboidal with or without goblet cells. Not all epithelium could be evaluated, because it had been partially damaged in the operational procedure.

Between 0.25 mm² and 0.5 mm² was counted in 13 sections, while over 0.5 mm² was evaluated in 19 sections. In all sections more than 0.5 mm² was counted of the interfollicular space and the follicles.

T cells

CD4⁺ cells were seen in all compartments. Intra-mucosal lymphocytes were observed, but no differences were found between surface epithelium and crypt epithelium. The follicles showed some staining in the mantle zone, although staining in the germinal centre was more pronounced. The interfollicular space contained the highest number of cells positive for CD4. No statistical differences were found for these compartments.

CD8⁺ cells were observed intra-epithelially and no differences were found between surface epithelium and crypt epithelium. Cells staining positive were mainly seen in the interfollicular spaces. The staining pattern for the secondary follicles showed no differences compared with that of CD4⁺ cells. Hardly any positive cells were seen in the mantle zone in comparison with the germinal centre. No statistical differences were found between the compartments of allergic patients and controls.

Mast cells

Tryptase⁺ cells were seen predominantly in the epithelium and the interfollicular space. No tryptase⁺ cells were found in the follicles. No significant differences were found between the groups.

Similarly, chymase⁺ cells were predominantly found in the epithelium and interfollicular space. No chymase⁺ cells were found in the follicles. The number of chymase⁺ cells and the tryptase⁺ cells roughly corresponded, although on the whole there were fewer chymase⁺ cells. The number of chymase⁺ cells in the epithelium and interfollicular space did not differ significantly between the groups.

IgE⁺ cells

IgE⁺ cells were seen in the epithelium, the interfollicular space and in the follicles. Significant differences in IgE⁺ cells between the groups were not found for any of these compartments.

Macrophages

Macrophages were found in the epithelium, follicles and interfollicular space of the adenoid. No significant

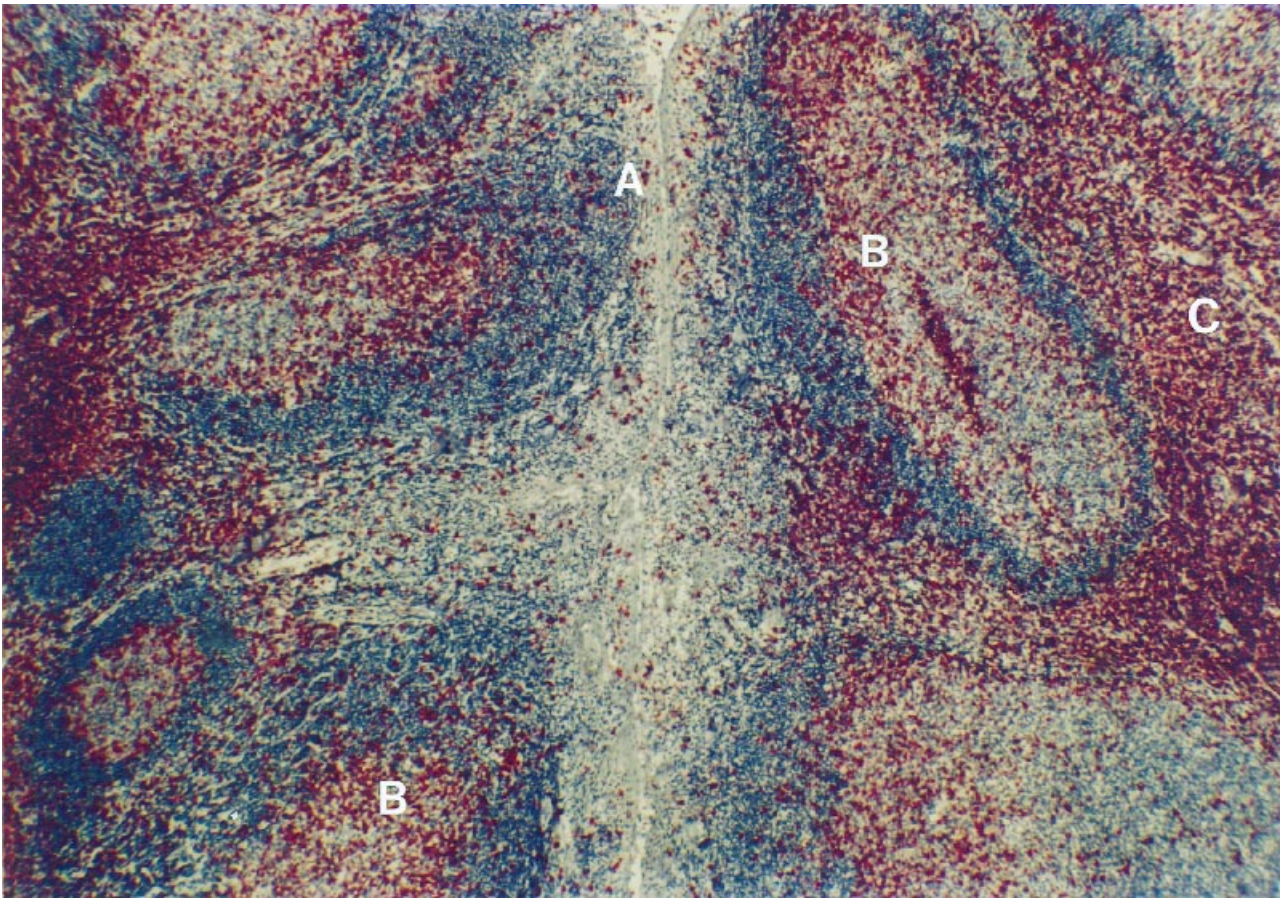


Fig. 2. Section of typical adenoid tissue after APAAP staining for CD3. The cryptal epithelium (A), the follicles (B) and the interfollicular spaces (C) are clearly seen (40 $\times$).

differences between the groups were found in any of these compartments.

CD1a⁺ cells

CD1a⁺ cells were observed in all three compartments. CD1a⁺ cells in the epithelium were dendritic and probably represent Langerhans cells (see Fig. 3). The number of CD1a⁺ cells in the epithelium showed a tendency to be more numerous in the allergic group ($P=0.07$).

In the follicles the median number of CD1a⁺ cells was 2 cells/mm² (range 0–41) for the allergic group and 0 cells/mm² (range 0–4) for the control group. This difference showed a strong trend to be more numerous in the allergic group ($P=0.02$) (Table 3). Apart from dendritic CD1a⁺ cells, mononuclear CD1a⁺ cells were also found in the follicles.

The median number of CD1a⁺ cells in the interfollicular space in the allergic group was significantly ($P=0.008$) higher than in the control group (Table 3 and Fig. 4).

Eosinophils

Eosinophils were found mainly in the epithelium and in the interfollicular space. No eosinophils were seen in the follicles (Fig. 5). The number of eosinophils in the epithelium and interfollicular space showed a tendency to be higher in number in the allergic group ($P=0.06$ and $P=0.05$, respectively; Table 3). Comparison of serial sections stained against mast cells (anti-tryptase, anti chymase) and eosinophils (anti-MBP) showed a concentration of mast cells and eosinophils in the same location.

Interleukin-4⁺ cells

Most positive staining cells were located in the interfollicular spaces and in the epithelium. The preferred location in the interfollicular spaces is the loose connective tissue around the blood vessels. No differences were found between allergic children and controls. The

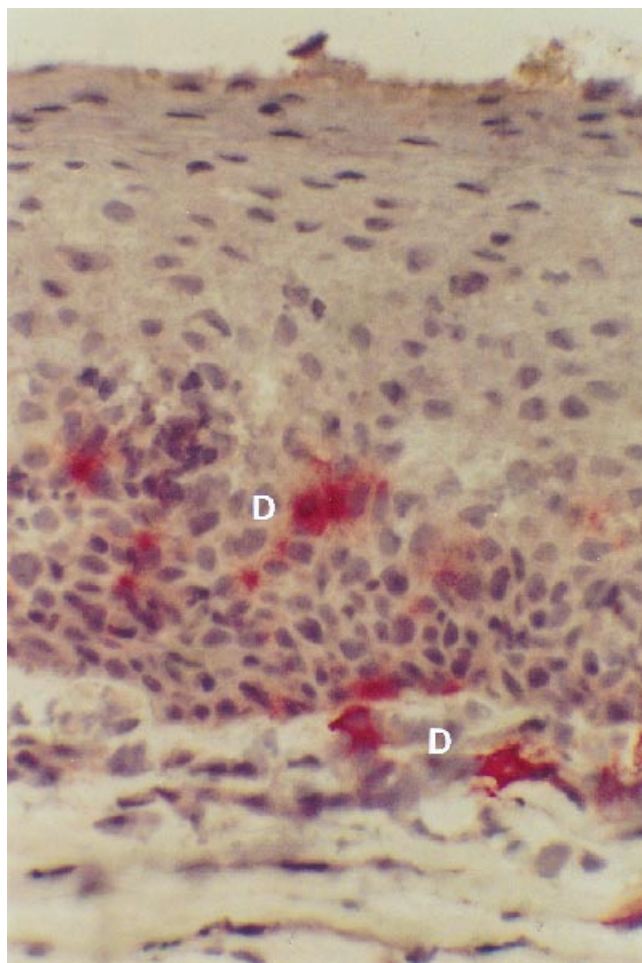


Fig. 3. Stratified epithelial and subepithelial region showing the typical CD1a Langerhans cell staining pattern (D) (400 $\times$).

follicular staining pattern was dominated by the presence of a small number of positive cells in the mantle zone. No positive cells were seen in the germinal centre. Again, no statistically significant difference were found between the groups.

In the epithelium a significant difference was found between the number of positive cells in the surface (median = 77, range 6–691) and the crypt epithelium (median = 45, range 0–168). The surface epithelium contained more IL-4⁺ cells compared with the crypt epithelium in both groups ($P=0.001$, data not shown). No significant differences were found when comparing epithelial counts in the allergic and the control group.

Double staining

To characterize the mononuclear cells in the interfollicular and follicular spaces double staining against CD1a and CD3 was performed. Evaluation of double positive cell numbers in the interfollicular space proved difficult and not reliable enough to be used in a numerical comparison of cell numbers between the two groups. Dendritic CD1a⁺ cells are usually found in close proximity to T cells. As a result of this, substrates of adjacent cells can mingle. Consequently, it is not always possible to definitely distinguish a double staining cell from two mono-staining cells lying closely together. However, the majority of CD1a⁺ cells were mono-staining cells. In the follicle CD1a/CD3 double staining cells were found sporadically.

Discussion

The aim of this study was to investigate the possible involvement of the adenoid in the development of allergic disease by evaluating differences in cellular infiltrates of the adenoids of allergic children compared with controls. The role of allergy in adenoid and tonsillar disease is not clear. However, no differences in prevalence of allergy were found between children with adeno-tonsillitis and age matched-controls [13]. When studying cellular differences in lymphatic organs, one must bear in mind that recurrent

Table 3. Median and range of positive cells per mm² found in the adenoid of allergic children and non-allergic age- and gender matched controls. Only cell types showing significant differences are shown.

Antibody against	Allergic children			Controls			<i>P</i> -value
	Epithelium	Follicular	Interfollicular	Epithelium	Follicular	Interfollicular	
BMK13	13 (0–20)	0	19 (0–77)	1 (0–4)	0	10 (3–22)	epith. $P=0.06$ interfoll. $P=0.05$
CD1a	31 (0–146)	2 (0–41)	36 (6–647)	4 (0–23)	0 (0–4)	6 (0–39)	epith. $P=0.07$ follicular $P=0.02$ interfoll. $P=0.008$

epith., epithelium; interfoll., interfollicular.

Table 4. Median and range of positive cells per mm² found in the adenoid of allergic children and non-allergic age- and gender matched controls. Only cell types not showing significant differences are shown

Antibody against	Allergic children			Controls			<i>P</i> -value
	Epithelium	Follicular	Interfollicular	Epithelium	Follicular	Interfollicular	
Tryptase	14 (0–59)	0	112 (28–207)	15 (4–51)	0	130 (59–208)	NS
Chymase	0 (0–13)	0	68 (2–164)	2 (0–8)	0	42 (28–267)	NS
IgE	17 (0–217)	102 (30–206)	61 (9–179)	31 (0–100)	94 (27–192)	42 (8–164)	NS
CD 4	468 (224–977)	1940 (912–2604)	3554 (408–4675)	642 (307–1528)	1846 (939–2617)	4421 (2271–8595)	NS
CD 8	332 (0–808)	298 (103–608)	2626 (1162–2978)	319 (88–588)	236 (137–589)	2004 (831–2573)	NS
CD 68	13 (3–230)	100 (25–390)	94 (25–294)	17 (7–59)	100 (60–276)	108 (25–390)	NS
IL-4	59 (5–306)	9 (1–19)	38 (10–102)	56 (5–110)	7 (1–37)	51 (15–100)	NS

NS, not significant.

disease and age can change the immunological cytoarchitecture of these organs [11,12]. The adenoids evaluated were all removed because of symptoms associated with inflammation and/or hypertrophy. These diseased adenoids are thought to have a different cell population from healthy adenoids [14]. In this study the adenoids of allergic children as well as controls were subject to inflammatory processes, therefore the differences to be found between the groups are

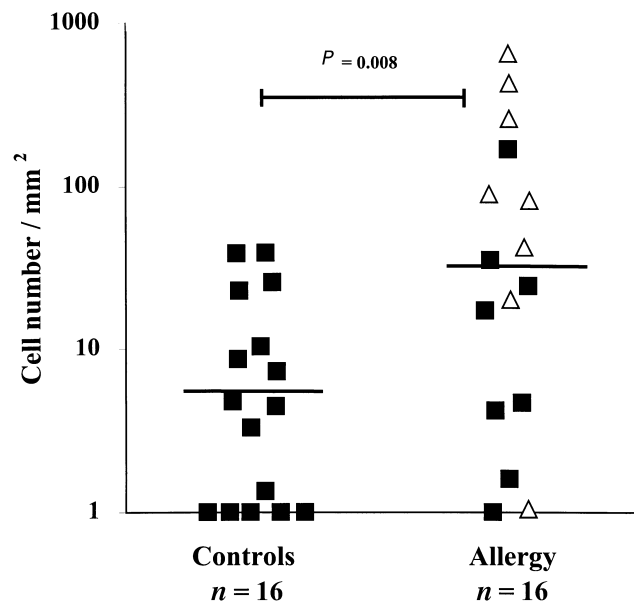


Fig. 4. Logarithmic dotplot showing number of CD1a⁺ cells/mm² in the interfollicular spaces of both groups. In the allergic group children with specific IgE against aero allergens are depicted by open triangles. Children without detectable specific serum IgE in both groups are depicted by closed squares. Median cell numbers are represented by horizontal bars.

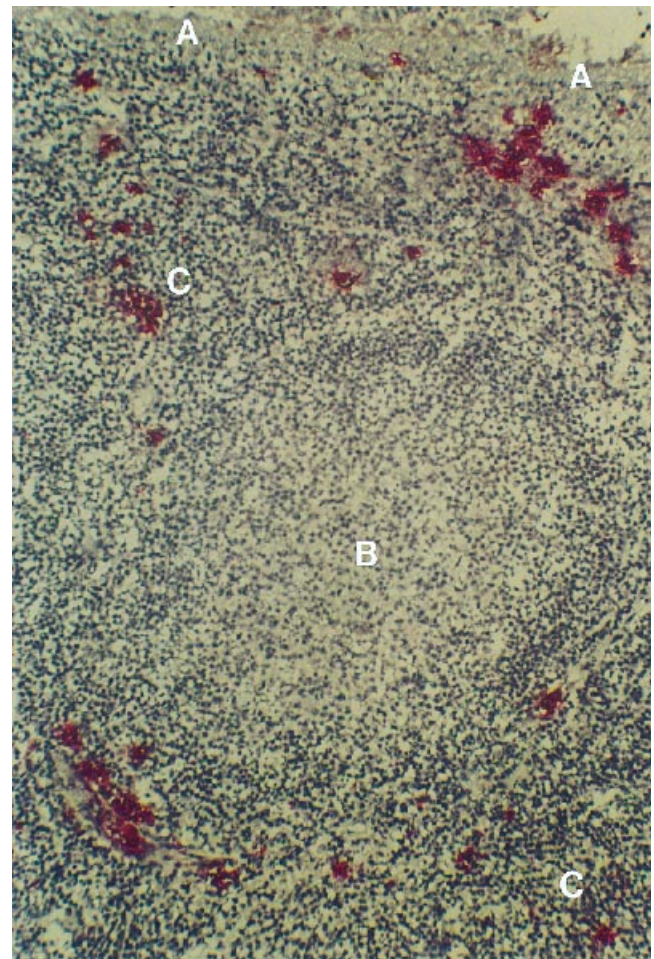


Fig. 5. Section of adenoid tissue of an allergic patient showing the location of BMK 13⁺ eosinophilic cells. Note the absence of eosinophils in follicular areas (B) and their position in the epithelium (A), and the interfollicular region (C) (100x).

to be attributed to allergy. It is also unlikely for the changes found to be the result of ageing, as studies on the effects of age, mainly done on tonsil tissue [11], only show differences after the first decade of life.

One of the main points of interest in this study is the fact that a significantly larger number of CD1a⁺ cells was found in the interfollicular area of allergic adenoids ($P = 0.008$). CD1a⁺ cells in the follicular area showed a strong trend to be more numerous in the same group ($P = 0.02$). CD1a⁺ cells in the epithelium (Langerhans cells = LC) showed a tendency to be more numerous in the allergic adenoids. These findings are in accordance with studies performed on nasal mucosa showing a significantly higher number of CD1a⁺ cells in patients with a grass pollen allergy compared to controls [2] and a significant increase in CD1a⁺ cells in allergic patients after provocation with aero-allergens [15]. LC in the adenoid were first demonstrated by van Nieuwkerk [16]. Forsgren described a large interindividual variation in CD1a⁺ cell numbers in the interfollicular spaces [17]. Patients in these studies were not screened for allergy, which means that the patients with high interfollicular CD1a⁺ cell counts could well be allergic patients.

Interestingly, we found CD1a⁺ mononuclear cells in the follicles in addition to CD1a⁺ dendritic cells. These cells were shown to partly represent young T lymphocytes expressing CD1a (CD1a/CD3 double staining cells). However, the majority of CD1a⁺ cells were mono staining cells. The exact nature of these cells is unknown.

This study supports the hypothesis that CD1a⁺ cells are involved in the process of allergic sensitization [3,18]. A second point of interest in this study is the fact that a trend was found for the eosinophils to be more numerous in the epithelium and the interfollicular space of allergic adenoids compared to controls ($P = 0.06$ and $P = 0.05$, respectively). Eosinophils are considered to be one of the major effector cell types in the process of allergic inflammation [19]. These cells are usually seen in the mucosa lining the shock organs of allergy, such as the nasal mucosa and the bronchi, and are rapidly recruited and activated after allergen stimulation [20–22]. The increase in eosinophils shows that effector cells of allergic disease are present in the atopic adenoid.

Becker *et al.* also demonstrated eosinophilia of the adenoid in 50% of children with a positive skin test [23]. Five out of the 16 allergic children in our study had detectable serum IgE against aeroallergens making the adenoid with its lining epithelium a credible shock organ. Exposure to food allergens could be another route of natural allergen challenge [24,25]. Three out of 16 allergic children had detectable serum IgE against at least two food allergens. Food could potentially be an effective

allergen challenge for these children in the period preceding adenotomy. Consequently, seven out of 16 allergic patients had a high probability of exposure to activating allergens (Table 1). When comparing the eosinophilic cell numbers of this subgroup with those of the patients not yet showing allergic sensitization by RAST or skin test, no statistical differences were found. Either allergic sensitization and/or disease is found earlier in the shock organ than in the skin/serum or these patients were allergic to an allergen which was not tested. Another possibility is that the eosinophilia is an 'intrinsic' factor of the allergic adenoid, although usually eosinophilia is not seen in organs not affected by allergic disease [20,26,27]. Factors inducing eosinophilia, such as IL-5 and RANTES and biopsies of the adjacent nasal mucosa form the subject of further research.

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