

Children with recurrent otitis show defective IFN γ -producing cells in adenoids

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Infectious diseases are frequently observed in children and their recurrence represents a demanding challenge for the paediatrician. It has been hypothesized that a defective immune response may occur in these patients. The aim of the present study was to evaluate whether children presenting with recurrent otitis have a defective interferon (IFN) γ production by the lymphocytes of peripheral blood and of adenoid tissue, in comparison with children without recurrent otitis. Our study group was represented by 58 children undergoing adenoidectomy for adenoid hypertrophy. They were subdivided into two groups according to the recurrence of otitis (≥ 3 per year) or not (< 3 per year). Intracellular cytokine profile of lymphocyte subsets in adenoids and peripheral blood was evaluated by flow cytometry analysis. Children with recurrent otitis showed a significantly lower percentage of CD8 $^{+}$ -producing IFN γ cells in adenoids than children with < 3 otitis per year ($p = 0.003$). The reduced capability of the adenoid cells to produce IFN- γ may induce a high susceptibility to the recurrence of otitis in children.

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Tonsils and adenoids, two lymphoid organs, situated in a critical position in the upper respiratory tract, play a basic role in protecting the nasal cavity from bacterial and viral antigens (1–3). Recurrent respiratory infections (RRI), including recurrent otitis media, are very common clinical issues in children (4), which represent a social problem concerning both national health care economics and impact on the patient's family and social milieu.

Interferon (IFN) γ is a crucial cytokine important in recovering from infections (5) and it has been hypothesized that children with RRI may have a specific deficiency in IFN γ production (6, 7). This correlation has never been substantiated; therefore, the aim of the present study was to investigate whether children presenting with repeated episodes of otitis media may concomitantly present a deficiency in IFN γ production in adenoids and in peripheral blood cells.

Patients and methods

Patients

Fifty-eight children (32 males, 26 females), median age 5 yr (range, 4–8 yr), requiring either an adenoidectomy ($n = 21$) or adenotonsillectomy ($n = 37$) were prospectively enrolled in the study. Adenoid tissue was surgically removed for hyperplasia causing secondary upper airway obstruction. The diagnosis of adenoidal hyperplasia was based on a clinical history of chronic upper airway obstruction, and confirmed by fibre-optic nasal endoscopy performed by an ENT specialist.

All children suffered from recurrent upper and lower respiratory infections defined as the presence of at least six episodes (including common cold, tonsillitis, otitis media, rhinosinusitis, pneumonia) during 1 yr prior to surgery.

In order to evaluate presence of atopy, before surgery, skin prick tests for relevant common allergens were performed in all patients. The

allergen panel consisted of house dust mites (*Dermatophagoides farinae* and *pteronyssinus*), cat, dog, a grass mix, *Compositae* mix, *Parietaria judaica*, birch, hazel tree, olive tree, *Alternaria tenuis*, *Cladosporium* and *Aspergilli* mix; the concentration of allergen extracts was 100 IR/ml (Stallergenes, Milan, Italy).

Children were considered atopic if their skin reacted to at least one allergen with a weal diameter of 3 mm greater than the negative control (8). Thirteen children presented with three or more episodes of otitis media during the preceding year. Acute otitis media was diagnosed after a history of acute onset, signs and symptoms such as earache, fever, irritability and hearing loss, associated to the presence of middle ear effusion, and to signs and symptoms of middle-ear inflammation according to recommendations of the American Academy of Pediatrics (9).

Children receiving inhaled or intranasal corticosteroids, or antihistamines or antibiotics during the 6 wk preceding surgery, and those treated with oral corticosteroids in the three preceding months were not enrolled in the study. Children with immunodeficiency, assessed by routine tests, or chronic or acute diseases were also excluded. Blood samples were obtained at admission to the hospital prior to surgery. The study was approved by the local ethics committee and an informed consent was obtained from both parents.

Adenoid cell separation

Adenoids were pressed through a filter in order to eliminate stromal tissue; adenoidal mononuclear cells (AMC) were then isolated by Ficoll-Hypaque density gradient centrifugation and re-suspended in complete medium [RPMI 1640 (Gibco, Life Technologies Ltd, Paisley, UK) supplemented with heat-inactivated 10% foetal calf serum (FCS; Euroclone), 2.5 mmol/l of L-glutamine (Gibco) and 50 µg/ml of gentamycin (Gibco)]. Cells were eventually cryopreserved in liquid nitrogen by a programmable-controlled rate cell freezer. After thawing, cell viability was evaluated.

Peripheral blood mononuclear cell separation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation and re-suspended in complete medium. Cells were cryopreserved in liquid nitrogen. After thawing, cell viability was evaluated. For each patient, AMC and PBMC were evaluated at this time.

Intracellular cytokine analysis

The detection of intracellular cytokines was performed following the slightly modified method of Jung et al. (10). After thawing and cell viability evaluation, 2×10^6 cells were incubated in complete medium in 24-well plates overnight at 37°C in 5% CO₂. After harvest, cells were stimulated with phorbol myristate acetate (PMA; Sigma-Aldrich Corporation, St Louis, MO, USA) (25 ng/ml) and ionomycin (Sigma) (1 µg/ml) for 4 h at 37°C in 5% CO₂. Simultaneously, Brefeldin-A (BFA; Sigma) (10 µg/ml) was added to disrupt intracellular Golgi-mediated transport and to allow cytokine accumulation.

After stimulation, different aliquots of the cell suspension were incubated with anti-CD3PerCP (Becton Dickinson) at 4°C for 30 min. Anti-CD8Fic (Becton Dickinson) and anti-CD4Pe (Becton Dickinson) were used to identify respectively the total number of T cytotoxic-suppressor and the total number of T-helper cells. After surface staining, the cells were treated with a fixing medium (Caltag, Carlsbad, CA, USA) for 15 min at room temperature, then washed with phosphate-buffered saline (PBS) and resuspended in a permeabilization medium (Caltag). At this stage, fluorescence-conjugated monoclonal antibodies specific for interleukin (IL)2, IL4 or IFN γ were added for 15 min at room temperature. The cells were then washed and resuspended in sheath fluid for flow cytometry analysis.

Flow cytometry analysis

Phenotypic analyses of the fluorescently stained cells were performed by three-colour flow cytometry using a Becton Dickinson FACScan flow cytometer. A minimum of 30,000 CD3+ lymphocyte-gated events was acquired in list mode and analysed with Cell Quest software (Becton Dickinson). Results are expressed as the percentage of CD4+ and CD8+ cells producing IL2, IL4 or IFN γ .

Statistical analysis

Descriptive statistics were computed as the mean and s.d. for continuous variables and as the absolute frequency and percentage for categorical variables. The percentage of cytokine-producing cells was compared by the Mann Whitney U-test. Two multiple regression models were used considering CD4+ and CD8+ cells producing IFN γ , as dependent variables. The presence of more than three episodes of otitis in a year, sex, age, number of respiratory infections, positivity

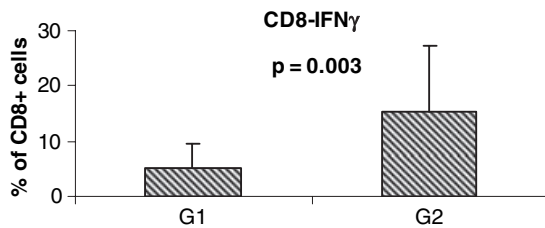


Fig. 1. Percentage of CD8+ cells producing interferon- γ in adenoid of G1 (≥ 3 otitis media per year) and G2 (< 3 otitis media per year) children.

for skin prick test results have been used as explanatory variables. A p -value < 0.05 was considered statistically significant; all analyses were performed with STATA v8.0.

Results

Patients were divided into two groups: G1 = 13 children suffering from three or more episodes of otitis media per year and G2 = 45 children without or presenting with fewer than three episodes of otitis media per year.

We found a lower frequency of CD3+ cells producing IFN γ in G1 children (mean: 3.38; s.d.: 2.76%) than in the G2 patients ($8.11 \pm 6.12\%$) ($p = 0.01$). In particular, the percentage of CD8+ cells able to produce IFN γ in adenoids was significantly lower in G1 ($5.00 \pm 4.43\%$) than G2 children ($15.19 \pm 12.00\%$) ($p = 0.003$) (Fig. 1).

In our study group, 37 children resulted as allergic. Even taking into account multiple variables, such as sex, age, number of infections, positivity to the skin prick test, we found that children with three or more episodes of otitis were associated with a decreased capacity of CD3+ cells ($p = 0.012$) and especially of CD8+ cells ($p = 0.005$) to secrete IFN γ . The adjusted difference of CD3+ cells producing IFN γ between children with ≥ 3 otitis and children with < 3 or without otitis events per year was -4.7 [95% confidence interval (CI), -8.3 to -1.0]; moreover, the adjusted difference of CD8+ cells producing IFN γ between the same groups was -10.1 (95% CI, -16.9 to -3.2).

No differences emerged between G1 and G2 either for CD4+ cell percentage producing IFN γ or for data concerning the production of IL2 and IL4 cytokines (Table 1). No differences were observed in the capability of PBMC to produce cytokines between the two groups of patients. The capability of adenoids and blood cells to produce IFN γ was not affected by atopy.

Table 1. Percentages of CD4- and CD8-producing cytokines in adenoid tissue of G1 (> 3 otitis media per year) and G2 (< 3 otitis media per year) patients

	G1* (%) (mean $\pm$ s.d.)	G2† (%) (mean $\pm$ s.d.)	p
CD8-IFN γ	5 $\pm$ 4.43	15.19 $\pm$ 12	0.003
CD4-IFN γ	3.9 $\pm$ 2.76	5.5 $\pm$ 6.8	0.07
CD8-IL2	11.4 $\pm$ 8.3	14.3 $\pm$ 7.6	0.5
CD4-IL2	20.4 $\pm$ 17	31.4 $\pm$ 13.9	0.06
CD8-IL4	1.15 $\pm$ 1.02	0.78 $\pm$ 0.55	0.08
CD4-IL4	5.15 $\pm$ 12.8	2.21 $\pm$ 4.43	0.48

*Children suffering from three or more episodes of otitis media per year ($n = 13$).

†Children without or presenting with fewer than three episodes of otitis media per year ($n = 45$).

IFN, interferon; IL, interleukin.

Discussion

Adenoids and palatine tonsils play a central role in providing a source of immune-competent cells that may either prevent or control infectious diseases at these sites (11). It is well known that IFN γ is a Th1 cytokine and that it is truly important in host defence against viral infections and in killing intracellular pathogens (12, 13).

The localization and function of effector T cells is crucial in order to generate an effective immune response. In particular, CD8+ T lymphocytes can mobilize two main mechanisms: cytotoxicity and production of cytokines, chemokines and microbicidal molecules. For instance, IFN γ plays an important role in the induction of cellular antiviral proteins and macrophage activation.

Moreover, the recruitment of effector T-cell subsets requires molecular signals generated by dendritic cell (DC) subsets that regulate homing-receptor expression by responding lymphocytes, whereas maintenance of effector CD8+ T cells depends on survival factors provided by the local micro-milieu, such as IL7 and IL15, which are in turn influenced by tissue-DC.

Recent studies have highlighted a central role for DC and the tissue environment in generating tissue-tropic effector T cells. Nevertheless, it still needs to be clarified if the lower CD8+ T cell-mediated immunity depends on the lower recruitment of CD8+ T cells in adenoids by chemokines, or on the defective function of CD8+ T cells by local factors.

The most important finding of this study is that children with recurrent otitis show defective IFN γ production in adenoid-derived CD8+ T cells. Our results agree with those published by Bernstein et al. (5), who have

demonstrated that in children presenting with recurrent otitis that there is a relative decrease in the synthesis of Th1 cytokines, IL2 and IFN γ , in comparison with peripheral blood lymphocytes, while Th2 cytokines were equally synthesized in adenoids and peripheral blood (5, 14). In addition, we also propose that it is likely that IFN γ reduction is proportional to the number of otitis events affecting the child per year.

As a consequence of reduced IFN γ production, patients show an increased susceptibility to infectious viral diseases, which often precede upper respiratory tract infections, with a possible consequential increase in replication of pathogenic bacteria in the adenoidal tissue. As the important and most common pathogens responsible for episodes of acute otitis media in children are extracellular bacteria (i.e. *Streptococcus pneumoniae*, *Haemophilus influenzae*, non-typeable *H. influenzae* and *Moraxella catarrhalis*), the presence of a valid and complete immune response for the complete elimination of these potential pathogens is necessary. More importantly the balance between Th1 and Th2 responses is essential (5). In those patients presenting with reduced IFN γ production, the adenoid tissue becomes a reservoir for pathogenic bacteria, and the tissue is no longer able to limit its normal flora (14). A change in the microecology of the local bacterial flora may therefore end up with the recurrence of episodes of acute otitis media (15).

A very recent study has provided evidence that allergic children, who present a Th2 polarization, show a greater incidence of severe RRI as compared with non-allergic children (6). Nevertheless, in our study there was no difference between atopic and non-atopic children regarding adenoid and circulating lymphocytes. A possible explanation for this finding might be that a primary IFN γ defect is present in all children presenting with RRI which may transcend atopy (16).

In the present study, we have clearly demonstrated that there is no significant difference in IL2 and IL4 production between atopic and non-atopic children, but children with recurrent otitis exhibit a defective IFN γ production that may account for this disorder. Considering the homogenous genetic background in Italy, further studies using this methodology in other countries is warranted.

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