

Short communication

Nonatopic wheezy children have reduced interferon-gamma

Viruses cause asthmatic exacerbations in schoolchildren. We tested the hypothesis that children who wheezed with viral respiratory tract infections secrete higher levels of the type 1 cytokine interferon-gamma (IFN- γ) in the peripheral circulation than children who had never wheezed. Blood was taken from 13 children (eight atopic) with episodic wheeze and 11 controls. CD4 and CD8 cells were separated from peripheral blood mononuclear cells and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 24 h. IFN- γ , IL-4, and IL-5 were measured in the supernatant by ELISA. IFN- γ production by CD4 and CD8 cells was lower in children with a history of wheeze (CD4, $P=0.046$; CD8, $P=0.037$). These children were then analysed according to atopic status. CD4 and CD8 IFN- γ production in nonatopic wheezy children was reduced (CD4, $P=0.009$; CD8, $P=0.003$). IFN- γ production by atopic wheezy children was lower than by controls, but the differences were not significant (CD4, $P=0.2831$; CD8, $P=0.1372$). CD8 IL-5 was lower in children who wheezed ($P=0.012$). Release of IL-4 and IL-5 by CD4 cells did not differ between the three groups. We propose that defective IFN- γ secretion by CD4 and CD8 cells may contribute to viral-induced wheeze in nonatopic children.

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Wheezing illness in children is commonly accompanied by symptoms of viral respiratory infection. The mechanism is poorly understood, but in pre-school children it may be related to developmental variation in airway size and function. Reduced airway calibre, identified by lung-function testing soon after birth, is associated with wheezing in the first 3 years of life (1–3). However, in most of these children, the wheezing illnesses are transient and have resolved by the age of 6 years (4). Symptoms of viral upper respiratory infection also frequently accompany wheezing illness in school-age children; 80–85% of asthma exacerbations in children aged 9–11 years are associated with viral colonization of the upper respiratory tract (5). At this age, factors other than developmental variation in airway calibre may contribute to virus-induced wheezing. Atopy is important and becomes more important with increasing age. However, a community survey in southern England found that 53% of 7-year-olds and 30% of 11-year-olds with a history of wheezing illness in the previous year had negative skin tests to common aeroallergens (6). There is some evidence that wheezy schoolchildren have both increased susceptibility to viral upper respiratory tract infections (7) and a higher incidence of symptoms when they contract these infections (8). It is not known whether these observations indicate an underlying immune abnormality.

In this study, our aim was to test the hypothesis that production of interferon-gamma (IFN- γ) by stimulated CD4 and CD8 cells is reduced in school-age children with a history of wheezing with viral infections compared with children who have never wheezed. We also measured stimulated levels of the type 2 cytokines IL-4 and IL-5 secreted by CD4 and CD8 cells in these children. Our further aim was to determine whether there were differences in cytokine production between atopic and nonatopic children who wheeze with viral infections.

Material and methods

Study subjects

Permission from the Research Ethics Committee of King's College Hospital was obtained prior to the start of the study. Informed consent was obtained from the parent and, where appropriate, the child upon entry to the study. Thirteen children (nine boys) aged 5–15 years with a past history of wheeze on three or more occasions were recruited from the paediatric chest clinic. All were receiving 200–400 μ g a day of inhaled corticosteroid. Eleven children (eight boys) aged 5–14 with no history of wheezing illness, attending for day case surgery, acted as controls. All children were free from recent respiratory infections. A volume of 6 ml of blood was taken from each subject.

Specific IgE measurement

Serum was removed from 1 ml of blood and frozen. At a later date, levels of specific IgE to cat, grass pollen, and house-dust mite were measured with a semiquantitative kit enzyme immunoassay test (Dr Fooke Laboratories, Neuss, Germany). A volume of 5 μ l serum was added to a well containing a paper allergen disc. The plate was incubated at room temperature for 30 min. The discs were washed, and 5 μ l anti-human-IgE alkaline phosphatase-conjugate was added and incubated for 60 min at room temperature. After washing, 200 μ l chromogenic substrate (PNPP) was added and incubated for 30 min at room temperature. A volume of 50 μ l stop solution (3 N NaOH) was added to each well. Absorption was measured by OD at 405 nm, and the results were compared to standard sera. The lower limit of detection was 0.35 RAST units/ml. Levels of specific IgE were graded from class 0 (undetectable) to class 4 (very high) (9). Atopy was defined as a class 2 (moderate) response or above to one or more allergens.

Cytokine measurement

Gibco BRL supplied all reagents unless otherwise stated. Peripheral blood mononuclear cells (PBMC) were separated by density-gradient centrifugation of heparinized blood over Lymphoprep. The PBMC were washed and resuspended in 5 ml Hanks' medium. Viable cells were counted with trypan blue (Sigma). The PBMC were centrifuged at 200 g for 10 min at 4°C and resuspended in 100 μ l phosphate-buffered saline containing 1% foetal calf serum. Dynabeads (Dynal) were used to purify first the CD8 and then the CD4 cells according to the manufacturer's instructions. Purified cells were counted with trypan blue.

The cells were cultured in complete medium; RPMI-1640 containing 1% MEM ($\times 100$), 1% sodium pyruvate (100 mM), 0.5% gentamicin (10 mg/ml), 10% human AB serum (Serotec), and 0.1% 2 mercaptoethanol (50 mM) with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 400 ng/ml ionomycin (Sigma) at a concentration of 1×10^6 cells per ml. The cells were incubated at 37°C in 5% CO₂ for 24 h, and the supernatants were removed and frozen at -30°C until analysed.

An ELISA using antibody pairs (Pharmingen) was used to quantitate IFN- γ , IL-4, and IL-5 in the cell-culture supernatant. Cytokine levels (pg/ml) were calculated from standard curves calibrated against a Quantikine kit standard assay. The lowest detectable concentration was 5 pg/ml.

Statistical analysis

Because the data for IFN- γ , IL-4, and IL-5 production by CD4 and CD8 cells were non-normally distributed,

nonparametric statistics were used. Subjects who wheezed (atopic and nonatopic) were compared to nonwheezing controls by the Mann-Whitney U test. Cytokine concentrations are expressed as 95% confidence intervals (CI) for the geometric mean concentration, calculated by standard methods based on taking log₁₀ of individual values.

Results

Specific IgE measurement

Eight children with episodic viral wheeze were atopic. Three showed raised IgE to house-dust mite, and five showed raised IgE to two or more allergens. Five children with episodic viral wheeze did not have elevated IgE to any of the allergens tested and were classified as nonatopic. Two children who had never wheezed had raised specific IgE to more than one allergen and were classified as atopic. One child who had never wheezed had specific IgE to cat and house-dust mite, while the other had raised IgE to all three allergens tested.

IFN- γ production

Comparison of children with episodic wheeze and controls. IFN- γ production by CD4 cells was lower in children with a history of wheeze (geometric mean 16023 pg/ml, 95% CI 15 723–16 328, $P=0.046$) than in controls who had never wheezed (geometric mean 33 684 pg/ml, 95% CI 33 136–34 242). IFN- γ production by CD8 cells was also lower in children with a history of wheeze (geometric mean 17 254 pg/ml, 95% CI 17 036–17 474, $P=0.037$) than in controls who had never wheezed (geometric mean 37 464 pg/ml, 95% CI 36 940–37 995). Further analysis of children with a history of wheeze was performed according to atopic status.

Comparison of atopic children with episodic wheeze and controls. Secretion of IFN- γ by CD4 (geometric mean 24 649 pg/ml, 95% CI 24 084–25 228, $P=0.2831$) and CD8 cells (geometric mean 22 190 pg/ml, 95% CI 21 814–22 665, $P=0.1372$) of atopic children with episodic wheeze was slightly, but not significantly, lower than in children who had never wheezed (Fig. 1).

Comparison of nonatopic children with episodic wheeze and controls. CD4 IFN- γ (geometric mean 8043 pg/ml, 95% CI 7863–8227, $P=0.009$) and CD8 IFN- γ (geometric mean 11 563 pg/ml, 95% CI 11 380–11 695, $P=0.003$) production in nonatopic children who wheezed was reduced compared to children who had never wheezed (Fig. 1).

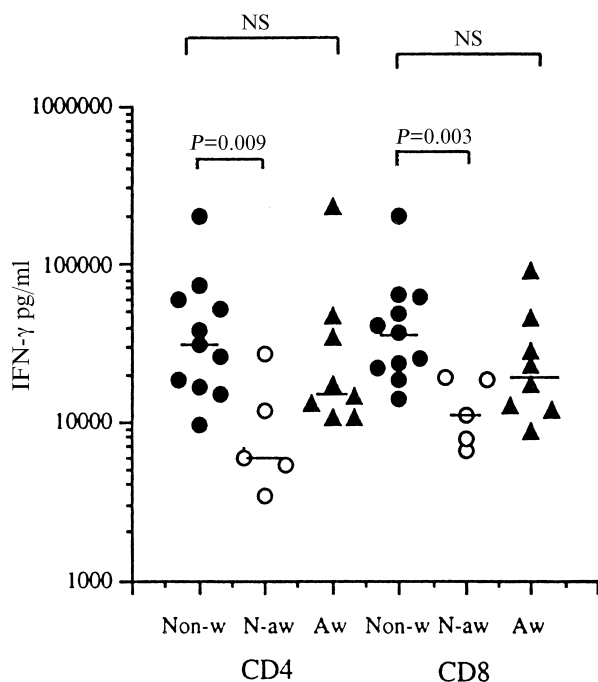


Figure 1. IFN- γ production by children with viral-induced wheeze (atopic and nonatopic) and nonwheezy controls. Nonatopic children who wheezed produced less IFN- γ from separated CD4 and CD8 cells, stimulated with PMA and ionomycin for 24 h, than nonwheezy controls. Reduction was more marked in CD8 cells. Statistics were performed with Mann-Whitney U test. Horizontal bars indicate median values. Non-w: nonwheezy control, N-aw: nonatopic wheezy children, Aw: atopic wheezy children, NS: not significant.

IL-4 production

Release of IL-4 by CD4 and CD8 cells did not differ between children with episodic wheeze and children who had never wheezed, nor when the children with episodic wheeze were analysed according to atopic status (Fig. 2).

IL-5 production

Secretion of IL-5 by CD8 cells of children with episodic wheeze (geometric mean 4.0 pg/ml, 95% CI 3.8–4.1, $P=0.012$) was lower than IL-5 secretion by CD8 cells of children who had never wheezed (geometric mean 266 pg/ml, 95% CI 219–233). Release of IL-5 by CD8 cells was lower in nonatopic children with episodic wheeze (0 pg/ml, $P=0.004$) than in children who had never wheezed. Release of IL-5 by CD4 cells did not differ between groups (Fig. 2).

Discussion

We demonstrated that

- 1) IFN- γ secretion by isolated CD4 and CD8 T cells was reduced in nonatopic children with recurrent wheeze

- 2) IL-5 secretion by CD8 cells in nonatopic children with recurrent wheeze was reduced.

These differences remained significant when the outliers were removed and the results reanalysed.

This is a small study which may have been insufficiently powered to detect some differences in cytokine production. Inability to demonstrate a reduction in IFN- γ production in the atopic wheezy group or an elevation in IL-4 or IL-5 secretion by children who wheezed may represent a type 2 error.

This study was performed on peripheral blood lymphocytes because bronchoalveolar lavage studies are difficult to carry out in children for ethical reasons. Isolated CD4 and CD8 cells were studied in order to understand the relationship between cytokines produced by CD4 cells (normally associated with allergy) and CD8 cells (normally associated with infection). Other investigators have looked at cytokine production in serum (10) by mononuclear cell populations (10–17), by mixed T-cell populations (18), and by CD4 cells and monocytes together, as well as at cytokine production at the single-cell level (17). There have been no previous reports of cytokine secretion by CD8 T cells isolated from children with episodic viral-associated wheeze.

The possibility that the findings in this study were influenced by the use of inhaled corticosteroids in the children with recurrent wheeze needs to be discussed. Recent evidence suggests that, in asthma, glucocorticoids exert their therapeutic effect, partly by reducing synthesis of IL-4 and IL-5, and by upregulating IFN- γ , and that changes can be detected in mRNA (19). It is unlikely that meaningful levels of steroids remain in the isolated and washed PBMC preparations. However, they may have affected the ability of the T cells to generate cytokines in response to stimulation or have affected cell differentiation (by inhibiting the development of Th2 cells). Nurse et al.'s study (16) detected no difference in IL-4 or IFN- γ production between patients who were and who were not taking inhaled steroids. If inhaled steroids had any effect on cytokine production, it would have been to reduce synthesis of IL-4 and IL-5 and upregulate IFN- γ production. This may have contributed to an absence of elevation of IL-4 production by the episodic wheezy children and to the suppression of IL-5 seen in the CD8 cells of the nonatopic wheezy children. The observed reduction in IFN- γ production occurred despite a possible elevation in IFN- γ produced by inhaled corticosteroids.

Reduced IFN- γ in atopic patients is well recognized. Experiments on cord blood (11–13, 20) suggest that a defect in IFN- γ production is a primary component of the atopic state, resulting in an excess of the type 2 cytokines IL-4 and IL-5, elevated IgE levels, and mast-cell degranulation on exposure to antigen. In atopic asthma, this is thought to initiate the asthmatic response, as modelled by the response to allergen

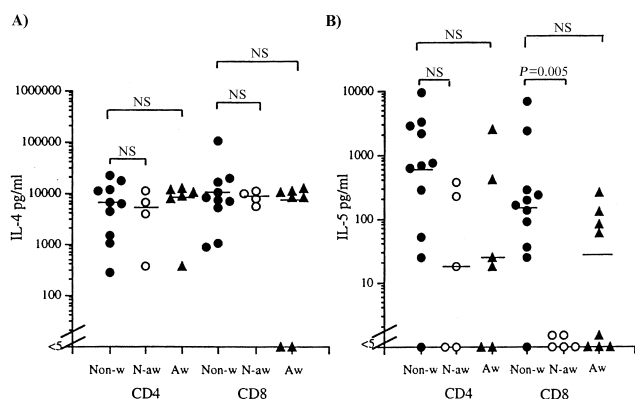


Figure 2. IL-4 and IL-5 production by children with viral-induced wheeze (atopic and nonatopic) and nonwheezy controls. A) No difference in IL-4 production by CD4 and CD8 cells, stimulated with PMA and ionomycin for 24 h, was demonstrated between nonwheezy controls, nonatopic and atopic wheezy children. B) Nonatopic children who wheezed produced less IL-5 from CD8 cells, stimulated with PMA and ionomycin for 24 h, than nonwheezy children. There was no difference detected in IL-5 production by stimulated CD4 cells. Statistics were performed with Mann-Whitney U test. Horizontal bars indicate median values. Non-w: nonwheezy controls, N-aw: nonatopic wheezy children, Aw: atopic wheezy children, NS: not significant.

challenge, in those with a genetic predisposition. Reduced IFN- γ production has been demonstrated in children with severe atopic dermatitis (14) and atopic asthma (10, 15, 16). This was confined to the CD4 T-cell subset (17). On the other hand, "mildly atopic" children, including those with asthma (14), and nonatopic asthmatics (15) have the same IFN- γ as controls. We demonstrated that although atopic asthmatic children produce less IFN- γ than nonwheezy controls, the results did not reach statistical significance. The numbers were small and this may be a type 2 error. Two children in our nonwheezy control group were atopic. The prevalence (18%) is similar to that observed, in this age group, in the general population (6). Levels of IFN- γ in the two atopic controls were, for CD4; 200 145 and 74 560 pg/ml (the highest two points in the control group); and, for CD8, 18 705 and 22 135 pg/ml (both figures lying below the median for the group). Reduced IFN- γ has been shown in nonallergic asthmatic preschool children with negatively selected T cells (which contain both CD4 and CD8 cells) (18). The reduction was assumed to be maturational, occurring in children who were in the process of developing atopic asthma, and an early indicator of atopic disease. This may also be true of some of the children in our study, although occurring at an older age. Alternatively, a reduction in IFN- γ may be involved in the generation of wheeze independently of atopy.

Increased IL-4 secretion (15) and IL-4 mRNA (18, 19) in the peripheral T cells of asthmatic children has been demonstrated. However, most studies show that

the peripheral blood monocytes of atopic children, including those with asthma, produce the same levels of IL-4 as controls (10, 14, 16–18). It is possible that an absence of elevation in IL-4 in children with recurrent wheeze in our study was due to treatment with inhaled corticosteroids. Power calculations using our data indicate that, to achieve 80% power at the 5% significance level, we would have needed at least 166 patients in each group to detect a difference in IL-4.

Previous studies have suggested that PBMC from children with atopic asthma produce the same amount of IL-5 as nonwheezy, nonasthmatic controls after stimulation (10, 16). Our study demonstrated a significant reduction in IL-5 production by stimulated CD8 cells from nonatopic wheezy children. We considered whether this may have been an effect of exogenously administered corticosteroids. *In vitro* addition of glucocorticoids inhibits synthesis of various cytokines, particularly IL-5 (21, 22). In asthma, glucocorticoids exert their therapeutic effect by reducing the synthesis of IL-5 (19). It is possible that treatment with inhaled steroids may have contributed to the low IL-5 secretion in both the atopic and nonatopic wheezy children (reaching significant levels in the CD8 nonatopic wheezy subgroup).

Asthmatic exacerbations in school-age children are mainly caused by viral upper respiratory tract infections. We demonstrated that defective IFN- γ secretion is a component of recurrent viral wheeze in nonatopic children. Reduced IFN- γ production in nonatopic children may be a primary immunologic variant or may be secondary to reduced production in response to recurrent viral infections. It could be an early event in children who subsequently develop atopy, or an immunologic abnormality independent of atopy. It occurs in the absence of elevated IL-4 and IL-5 secretion from cells in the peripheral blood but may not exclude a local increase in IL-4 and IL-5 production in the lungs, an effect which may, in itself, cause a reduction in IFN- γ secretion that can be detected in the peripheral circulation.

Reduced IFN- γ secretion was more marked in CD8 cells – the main immune response to viral infection. This poses the interesting question of whether reduced IFN- γ secretion reflects an abnormal CD8 cell population or a reduced CD8 cytotoxicity. If these cells are less able to kill and remove viruses from the respiratory tract, persistence of virus may cause damage by other mechanisms or result in more serious infection. Reduced IFN- γ production by T cells might affect their ability to activate macrophages, thus impairing antigen presentation and viral clearance during viral infections. Alternatively, a deficiency in IFN- γ may fail to suppress the development of type 2 CD4 cells and favour a type 2 response and the generation of asthma within the lungs where the cells are locally activated.

Stevenson et al. have recently found increased eosinophils and mast cells in bronchial lavage from atopic wheezy children, but no increase in cells from lavage fluid from nonatopic children with virus-associated wheeze during asymptomatic periods (23). This supports the concept that the mechanism of virus-induced wheezing differs in atopic and nonatopic

children. We postulate that an impaired immunologic response to viral respiratory tract infection, of which a reduction in IFN- γ production by CD4 and CD8 cells is a marker, offers an alternative explanation of why nonatopic children wheeze when exposed to respiratory viruses.

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