

Airway Inflammation in Asymptomatic Children With Episodic Wheeze

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Summary. Airway pathologies have been comprehensively researched in adult asthma, but in children, the extent of airway inflammation associated with episodic wheeze, often diagnosed as asthma, has not been fully characterized. It is not clear whether persistent airway inflammation is present in the absence of wheezing symptoms, and there is controversy regarding the role of age and atopy. This study assessed cellular and cytokine markers of airway inflammation in asymptomatic children with a history of episodic wheeze. Children with a history of episodic wheeze and cough (study group) and nonasthmatic patients requiring elective surgery (control group) were recruited. All subjects in the study group had a history of significant episodic wheezing (>2 episodes per year), and used only as-needed beta-agonist treatment. Bronchoalveolar lavage (BAL) was obtained using bronchoscopic lavage (study group) and nonbronchoscopic lavage (control group). Differential cell counts of BAL and flow cytometry were performed to identify T-lymphocyte phenotypes, and intracellular cytokine profiles were measured after phorbol-12-myristate 13-acetate (PMA) stimulation of BAL fluid T-cells. Twenty-one children with a history of 2–12 episodes of wheeze per year and 21 nonasthmatic subjects without respiratory symptoms were recruited. Study and control subjects were matched for age (median age, 5 years) and demographic characteristics. Study subjects had higher IgE levels, but their measurements were still within normal range. No significant differences in BAL differential cell counts were noted, and in both groups, the majority of T-cells were CD3+ CD8+, with a median CD4:CD8 ratio of 0.6. There was no significant difference in T-cell expression of the activation markers HLA-DR and CD25 (IL-2 receptor), or in PMA-induced production of the intracellular cytokines IFN- γ , IL-2, IL-4, IL-5, and IL-10. The results of this study suggest that significant T-cell-driven airway inflammation is absent in mild or nonatopic, asymptomatic children of this age group who have episodic wheeze. Our findings support asthma management guidelines that do not recommend long-term treatment of this group of patients with anti-inflammatory medications. **Pediatr Pulmonol.**

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INTRODUCTION

In Australia, 1 in 4 children will have at least one episode of wheeze per year, triggered in many cases by viral infections. Of children with wheezing, 75% have infrequent episodes, 20% have frequent symptoms (at least every 4 weeks), and 5% have daily persistent symptoms.¹ Airway inflammation is presumed to be the underlying pathological process associated with symptoms, but it is not clear whether inflammatory changes persist during asymptomatic periods.

Airway inflammation in adults with asthma has been well-characterized, and T-cells are believed to play a key role in the orchestration of the inflammatory process.^{2,3} Inflammatory changes can be detected in asthmatics with relatively sparse symptoms, and studies in adults with mild asthma, using bronchoalveolar lavage (BAL) and mucosal biopsy, showed increased activation of T-lymphocytes and excess production of Th2 cytokines.^{4–6} Raised mast-cell and eosinophil mediator production is also present.

Few comparable studies have been performed in children with recurrent wheeze, who are often diagnosed as mildly asthmatic. The extent and level of persistent airway inflammatory changes in this group of patients are controversial, and may depend on age and atopy.^{7,8} For example, a study of infants with persistent wheeze

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demonstrated significant influx of most inflammatory cell types in BAL,⁹ while a comparable study in older children failed to find differences except in neutrophils.⁷ Persistent airway inflammation in episodic wheeze is important because it may influence whether corticosteroids will be prescribed, on the basis of evidence suggesting that early treatment of inflammation with daily inhaled corticosteroids may prevent airway remodeling and the development of irreversible structural change to the airways.¹⁰

In this study, we conducted detailed assessments of T-cell-driven airway inflammation in a group of patients with episodic wheezing and cough who were asymptomatic at the time of the study. BAL fluid was analyzed to evaluate inflammatory cell numbers and detect T-cell phenotypes, activation status, and intracellular cytokine production.

MATERIALS AND METHODS

Patient Groups

All children had been referred to Monash Medical Centre, a tertiary care center, for investigation of recurrent respiratory symptoms including wheeze, cough, and breathlessness. As part of their clinical assessment, they underwent bronchoscopy, and children and their parents were invited to participate in the study. Although all bronchoscopies were clinically indicated, BAL was performed for research. Written consent was obtained from a parent or guardian. A validated respiratory illness questionnaire¹¹ was administered to determine the frequency of recent wheeze, and those children with >2 episodes of wheeze in the past 12 months were included in the study group. All children were asymptomatic for >4 weeks at the time of the study, and used only as-needed beta-agonist medications. Inhaled corticosteroids were taken irregularly by 8 patients (maximum dose, 400 µg beclomethasone dipropionate), but had not been used for >4 weeks prior to the study. Nonasthmatic subjects attending for elective surgery were recruited to serve as controls, and written consent was obtained from a parent or guardian. The children were matched for age, and did not have a history of asthma or chronic respiratory symptoms. Exclusion criteria included children having cardi thoracic surgery, those with congenital malformations, cystic fibrosis, congenital heart disease, and proven recurrent aspiration, and children who were born at less than 37 weeks of gestation. Subjects were also excluded if they had an upper or lower respiratory tract infection or treatment with oral or inhaled corticosteroids in the preceding 4 weeks. The Human Research Ethics Committee of the Southern Health Care Network, Monash Medical Centre (Melbourne, Australia) approved the study.

Bronchoscopy and BAL

Recently, nonbronchoscopic BAL was performed in children presenting for elective surgical procedures. Several studies showed nonbronchoscopic BAL to be a safe and reliable technique, with no postoperative complications, adequate BAL fluid return for analysis of inflammatory cells, and results comparable to conventional bronchoscopic BAL.^{7,12,13} Normal reference values for cell types and numbers in pediatric airways were derived and found to be similar to adult values.^{7,12–14} Although the BAL procedure samples predominantly luminal tissues, the cells so obtained are deemed to provide an accurate indication of tissue events in the airway wall itself.¹⁵

Bronchoscopic Lavage

No premedication was given, and beta-agonists were not used within 2 hr of the procedure. Anesthesia was induced with halothane and maintained with a halothane/oxygen mixture and Propofol (1%) boluses of 1 mg/kg. Liquid lignocaine hydrochloride (2–4%) was applied directly to the laryngeal and tracheal mucosa. Flexible bronchoscopy was performed transnasally or via a laryngeal mask airway, with the subject breathing spontaneously. The bronchoscope was wedged in a segmental or subsegmental bronchus of the right middle lobe or right lower lobe, and three aliquots of 1 ml/kg of saline up to 20 ml were instilled and immediately aspirated into a specimen trap, using 150 mm Hg negative pressure. During anesthesia, heart rate and oxygen saturation were continuously monitored, and an experienced pediatric anesthetist was in attendance.

Nonbronchoscopic Lavage

The procedure was performed in volunteers undergoing general anesthesia for elective surgery. Premedication with 30 mg/kg of paracetamol was given, and a relaxant general anesthetic technique was used. Immediately after intubation, and with the child's head in the midline, a 60-cm end-hole suction catheter (Vygon S.A., Ecouen, France) was inserted into the endotracheal tube (ET). The catheter size was adjusted to the ET diameter (8 French catheter for size 3.5–5.0 ET, and 10 French catheter for size >5.5 ET), and was advanced until wedged in a lung segment. Saline at room temperature was instilled (1 ml/kg body weight up to 20 ml), and BAL fluid was immediately aspirated into a specimen trap (Sherwood Medical, St. Louis, MO), using 150 mm Hg negative pressure and a three-way tap (Discofix, Braun, Melsungen AG, Germany). With the catheter remaining wedged in situ, two further aliquots of 1 ml/kg saline were instilled and aspirated, giving a total instilled volume of 3 ml/kg.¹⁴

Antibodies and Reagents

RPMI-1640 and fetal calf serum (FCS) were obtained from Gibco (Melbourne, Australia). The fluorescence-activated cell sorter (FACS) wash consisted of phosphate-buffered saline (PBS), 1% bovine serum albumin (Sigma, Melbourne, Australia), and 0.01% sodium azide. Paraformaldehyde, saponin, phorbol 12-myristate 13-acetate (PMA), ionomycin, and brefeldin A were obtained from Sigma. Anti-CD3-cyanine 5 (Cy5), CD8-Alexa Fluor 488, and HLA-DR-Alexa Fluor 488 were sourced from our own laboratory. Other antibodies used were CD4-allophycocyanin (Exalpha, Maynard, MA), CD4-FITC (DAKO, Copenhagen, Denmark), anti-IFN γ -FITC, IL-4-PE, IL-2-FITC, IL-10-PE, IL-5-PE, and CD8-PE/Cy5 (Pharmingen, Franklin Lakes, NJ).

Differential Cell Counts

A volume of 1–2 ml of retrieved specimen was separated for differential cell counting. This was performed on 200 μ l of unfiltered fluid, cytocentrifuged at 500g for 5 min. Two slides were fixed in 95% ethanol and stained with Papanicolaou reagent, and two were air-dried and stained with May-Grünwald-Giemsa reagent. Total counts were done, and differential counts were performed by counting 400 cells at high power and expressing them as relative percentages. Ciliated and squamous cells were not counted.¹⁴

Surface Molecule and Intracellular Cytokine Detection

Cells from BAL fluid were washed twice in RPMI, and resuspended in RPMI/10% FCS. Total cell counts were performed using a Neubauer hemocytometer counting chamber (Weber, Lutterworth, UK). Depending on the number of cells, the specimen was separated into two equal volumes: one for cell surface staining, and the other for T-cell cytokine measurements (see below). If enough cells were not obtained, only one of the techniques was performed, and hence study numbers are lower for studies other than BAL cell counts.

For surface labeling, the cells were spun and resuspended in 100- μ l aliquots of FACS wash in 1.5-ml Eppendorf tubes. Appropriate antibodies were added at the recommended volumes, and cells were incubated on ice for 30 min, and then washed and centrifuged. Cells were resuspended in 400 μ l of FACS wash, and left at 4°C before analysis by flow cytometry.

Measurement of intracellular cytokines by flow cytometry is a validated measure of T-cell activation.^{16–18} BAL cells were incubated in a 24-well tissue culture plate, in 1 ml of RPMI/10% FCS plus PMA (25 ng/ml), ionomycin (1 μ g/ml), and 10 μ g/ml brefeldin A, using a protocol modified from Picker et al.¹⁸ Preliminary studies

found that intracellular cytokines were not detectable in unstimulated T-cells, and therefore cytokines were measured only in stimulated cells. Cells were incubated for 3 hr at 37°C and 5% CO₂, rinsed with FACS wash, centrifuged, and resuspended in 2% paraformaldehyde in PBS and left on ice for 20 min. After centrifugation, cells were resuspended in 0.2% saponin in PBS, left on ice for 5 min, and again centrifuged and resuspended in 100 μ l of 0.2% saponin plus the recommended volume of antibodies. As stimulation with PMA and ionomycin causes reduction in the surface expression of CD4, anti-CD4 was added after permeabilization to maximize staining. After incubation on ice for 30 min and washing in 1 ml 0.2% saponin, cells were resuspended in 0.5 ml of FACS wash for analysis.

Flow Cytometry

A MoFlo (Cytomation) flow cytometer equipped with Cyclops SUMMIT software was used for studies. Cells were excited with a 488-nm argon ion laser and a 633-nm HeNe laser. Up to six parameters were collected at one time, including forward and 90° light scatter, FITC or Alexa 488 green fluorescence, PE orange fluorescence, PE-Cy5 red fluorescence, and the 633-nm excited allophycocyanin (APC) or Cy5 red fluorescence. Lymphocytes were gated by both size and fluorescence, following labeling with anti-CD3 for cell surface staining and anti-CD4 for cytokine staining, with at least 2,000 lymphocytes counted for surface staining, and 500 CD4+ counts for cytokine staining. Results are expressed as a percentage of T-cells expressing the surface markers, and CD4+ cells producing intracellular cytokines. Fluorescence-positive cutoffs for each sample were determined using the appropriate isotype control antibodies.

IgE Measurements

Total IgE was measured using an enzyme immunoassay, with a normal range of 0–150 katal units/l (KU/l) at age 4–7 years (Bipharco, Brussels, Belgium).

Statistical Analyses

Previous studies indicated a standard deviation (SD) of 3.5 in CD25 and HLA-DR expression on T-cells.⁶ A 50% increase in T-cell activation (measured by CD25 and HLA-DR expression), an alpha of 0.05, and 80% power required a sample size of 11 patients per group to demonstrate a significant difference in children with episodic wheeze compared to controls. For assessments of IFN- γ , repeated measurements indicated an SD of 7,¹⁹ and hence 12 study subjects (6 in the study group, and 6 controls) would be needed to detect a 50% increase in cells positive for IFN- γ , with 80% power at the 5% level. It was not possible to calculate accurate study patient numbers

for IL-4, and to account for this uncertainty as well as for patients in whom enough BAL may not have been obtained, a total of 21 patients per group was recruited. Repeat FACS measurement of intracellular cytokines on the same BAL samples had a coefficient of variation <3%.

Statistical methods with independent groups were used. The data were not normally distributed, and are presented as medians and interquartile ranges. Statistical comparisons were performed using the Mann-Whitney U-test or unpaired *t*-test as appropriate, and $P < 0.05$ was considered significant. MINITAB release version 10.2 for Windows was used for data analysis.

RESULTS

Patient Demographics

Demographics of the two study groups are shown in Table 1. In total, 21 children between ages 3–8.6 years (mean, 5 years) with episodic wheezing and 21 age-matched controls undergoing general anesthesia were recruited. Mean total IgE was higher in the study group than in controls (25 vs. 7 KU/l, $P < 0.01$), but fell within normal range for this age group.

BAL and Differential Cell Counts

No complications were observed during the bronchoscopic and nonbronchoscopic BAL procedures or in the postoperative period. The characteristics of the BAL fluid recovered and differential cell counts are shown in Table 2. There was no difference in volume of BAL and number of cells in BAL obtained from subjects in the study groups.

Cell populations sampled by BAL revealed no evidence of inflammation in subjects with episodic wheeze (Table 2). As expected, there was a predominance of macrophages in BAL (mean, 86% in both groups), and although neutrophil counts were more variable, no significant differences were found. There was also no significant difference observed between study groups in numbers of lymphocytes and eosinophils. Steroid-naïve subjects with episodic wheeze were separately analyzed, and also had similar cellular profiles to the control group (data not shown).

T-Cell Subgroups and Activation Markers

Cell surface markers were measured in 12 children with episodic wheezing and 15 control subjects. Similar percentages of cells were identified as CD3+ CD4+ and CD3+ CD8+. The median CD4:CD8 ratio was 0.57 (range, 0.33–0.74) in patients with wheeze, and 0.61 (range, 0.41–0.87; $P = 0.8$) in the control group.

T-cell activation markers HLA-DR and CD25 (IL-2 receptor) were also examined. Again, no significant differences between the two groups were found in the percentages of T-cells expressing these surface receptors (Table 2). An analysis of steroid-naïve subjects with episodic wheeze gave similar results.

Intracellular Cytokine Measurements

Intracellular cytokines were measured in 9 patients with wheeze, and in 6 control subjects. After cells were stimulated with PMA and ionomycin in the presence of brefeldin A, the percentage of CD4+ cells producing detectable intracellular cytokines was determined. The cytokine most frequently detected was IFN- γ , but this was not increased in either group. Similar observations were made for IL-2, IL-4, IL-5, and IL-10 following stimulation (Fig. 1). Again, an analysis of steroid-naïve subjects with episodic wheeze gave similar results.

A majority of CD3+ cells in BAL were CD8+, and their cytokine expression following PMA stimulation was also assessed in a smaller group of patients (episodic wheezing, $n = 6$; controls, $n = 5$). Again, the only cytokine present in significant numbers of CD3+ CD8+ cells was IFN- γ (episodic wheezing: median, 36%; range, 12–65%; controls: median, 47%; range, 2–58%; $P > 0.5$). The cytokines IL-2, IL-4, and IL-5 also did not differ significantly between groups.

Association of Inflammatory Indices With Serum IgE

To assess whether levels of serum total IgE may be linked to the presence of inflammation, correlations with various indices of inflammation were examined. No significant link was observed (data not shown), but the

TABLE 1—Patient Demographics and Treatment¹

	Episodic wheezing (n = 21)	Control subjects (n = 21)
Age, median (range; min-max.)	5 (3–8.6)	5 (2.3–7.5)
Sex (M:F)	17:4	15:6
IgE, IU/l, median (IQR)	25 (6–87)	7 (3–163)*
Episodes wheeze reported per year	2–4 episodes (13 patients) 5–12 episodes (8 patients)	0/21
Inhaled β_2 -agonists prn ²	16	0

¹IQR, interquartile range.

²Five patients reported never using β_2 -agonists in spite of having wheezing symptoms.

* $P < 0.01$.

TABLE 2—BAL, Differential Cell Counts, and T-Cell Activation Markers¹

	Episodic wheezing (n = 21)	Control subjects (n = 21)
Saline return (ml) median (IQR)	18 (15–21)	16 (12–20)
Cells × 10 ⁴ /ml BAL	11.2 (8.2–12.9)	9.8 (6.5–15.4)
Neutrophils (%), median (IQR)	3 (1–16)	3 (2–9)
Eosinophils (%)	0 (0–1)	0 (0–0.25)
Lymphocytes (%)	8 (2–12)	9 (4–11)
CD3+ (%) ²	73.9 (64.4–86) (n=12)	85.7 (71.9–90.3) (n=15)
CD3+ CD25+ (%)	17.6 (11–25) (n=10)	16.8 (11.6–21.1) (n=13)
CD3+ HLA-DR+ (%)	14.3 (8.8–21.7) (n=10)	16.9 (11.6–21.1) (n=13)

¹IQR, interquartile range.

²Cell surface receptors could only be assessed in those subjects with adequate BAL fluid remaining after initial cell counts.

analyses were restricted by the small number of wheezing children with higher levels of serum IgE.

DISCUSSION

This study employed BAL cell counts, T-cell activation markers, and intracellular cytokine production to assess airway inflammation in children with wheeze. No evidence of persistent airway inflammation in asymptomatic young children with infrequent episodic wheeze was found, and our findings in this particular cohort suggest that significant bronchial inflammation is not present during periods when symptoms are absent. It implies that daily anti-inflammatory (preventer) therapies may not be required in this particular group of patients, who are often diagnosed as having mild, infrequent, episodic asthma.

Previous studies in children evaluated airway inflammation principally on the basis of cell populations in BAL, but the use of newer techniques to detect T-cell intracellular cytokine production has not been reported.¹⁸ We used nonbronchoscopic techniques to obtain BAL in a control group of volunteers without airway symptoms, and found no differences between methods of lavage with

respect to BAL volumes and total cells counts. BAL was analyzed employing laboratory methods, providing accurate measurements of inflammatory indices in peripheral blood during both childhood and adult asthma.^{6,20} The percentage of CD3+ T-cells in BAL in both study groups was comparable to T-cell numbers in a population of children having bronchoscopy for the investigation of stridor,¹⁴ normal BAL cellular data by nonbronchoscopic¹² and bronchoscopic²¹ procedures, and adult reference values.⁵ Moreover, similar inverse CD4:CD8 ratios in BAL fluid (median, approximately 0.6 in both groups) from nonasthmatic children of a similar age were reported.¹⁴ The number of eosinophils in BAL obtained from the group with wheeze and the control group was similar (and low), and suggests an absence of eosinophil bronchitis, a condition that has been considered one of the more consistent markers of asthmatic airway inflammation.^{22,23} Numbers of other cell types also did not differ between study groups. Mast-cell numbers in BAL may be increased in asthma,²⁴ and were not specifically identified. However, a small percentage of cells was not accounted for in the BAL differential cell counts, and may possibly have been mast cells or epithelial cells; their omission is unlikely to mask changes in other cell types. Overall, using inflammatory-cell populations as a measure of inflammatory burden, our results and that of a recent bronchial biopsy study²⁵ suggest the absence of significant airway inflammation in children with mild asthma.

Activation of T-cells is reflected by the expression of surface membrane receptors such as HLA-DR and CD25 (IL-2 receptor).⁶ Significant increases were demonstrated in the peripheral blood and BAL of adult asthmatics, as well as in the peripheral blood of children with asthma.²⁰ In the latter study, 17 children were studied, aged 7–16 years, with moderate to severe asthma. A significant elevation in peripheral blood CD4+ cells expressing CD25 and HLA-DR, and CD8+ cells expressing CD25, was found in asthmatics compared to controls. Furthermore, activation was correlated with numbers of eosinophils and disease severity. Based on these results, we postulated that increased airway T-cell activation may

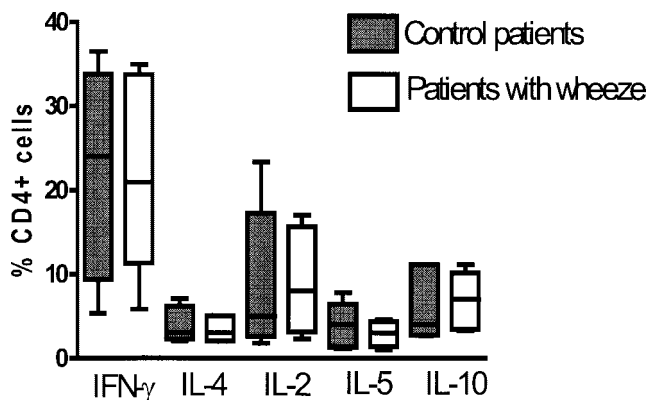


Fig. 1. Box-and-whisker plots show intracellular CD4+ cytokine measurements in control subjects and in patients with episodic wheeze. No significant differences were noted.

characterize children with episodic wheeze, as it appears to do in children with chronic asthma.²⁰ However, our findings are similar to those reported by Harmanci et al., who found no differences of CD25 and HLA-DR expression in the peripheral blood of 32 stable, mild asthmatics compared with controls.²⁶

Although the numbers of BAL T-cells may be similar in asthmatic and nonasthmatic persons, those with asthma may be predisposed to produce predominantly Th2 cytokines on stimulation. To assess the production of intracellular cytokines after stimulation with PMA, T-cells were examined by flow cytometry,^{14,17} employing a method previously used by our group to demonstrate differences in cytokine profiles in patients with bronchiectasis.²⁷ Median numbers of approximately 25% of CD4+ T-cells and of CD8+ T-cells produced IFN- γ , but no differences were found between children with and without episodic wheeze. IL-4, IL-5, and IL-10 were produced on stimulation by small numbers of T-cells (medians, <5%) in both groups, again without detectable differences. No comparable investigations of BAL cells were reported, although limited studies were done in peripheral blood. Noma et al. measured the production of peripheral-blood IFN- γ and IL-4 in adolescent asthmatics during exacerbations and at recovery, and found that subjects with stable asthma produced little IL-4.²⁸ IFN- γ production was high compared with nonasthmatic "normal" subjects, and it was concluded that increased IFN- γ in stable asthma suppressed IL-4 production. Our results are consistent with this possibility, if both the nonasthmatic controls and subjects with episodic wheeze represent a group of "normal" nonasthmatic individuals. It should be remembered that cytokine production might originate from sources other than T-cells, such as macrophages, eosinophils, and airway epithelium. Studies in BAL demonstrated that macrophages spontaneously release tumor necrosis factor- α (TNF- α) in infants with wheeze,²⁹ and eosinophil cationic protein (ECP) is increased in some infant wheezers.³⁰

Several factors may influence the interpretation of our results. First, there is evidence that wheezing during childhood may be a heterogeneous condition, and that different forms of wheezing may be associated with diverse risk factors and prognosis. Stein et al. described three phenotypes: transient early wheezing, nonatopic wheezing, and IgE-associated wheeze/asthma, the latter associated with airway hyperresponsiveness (AHR), peak flow variability, and markers of atopy.³¹ It was not possible to assess AHR or peak flow variability in this study, but total serum IgE was higher in the study group, suggesting that at least some patients with wheeze were drawn from the third group. However, IgE elevations in the study group were modest (median, 25 KU/l) and fell within normal range, implying that most patients were of a nonatopic or mildly atopic phenotype.³¹ Second, we

considered that prior use of inhaled corticosteroids may have suppressed airway inflammation, and hence we performed analyses of steroid-naïve subjects. No differences were observed. Third, inflammatory indices may have been overlooked, because study and control subject numbers were too low to detect differences in T-cell cytokine production. However, power calculations for IFN- γ confirmed adequate numbers, and other measurements of inflammation were very similar in both groups. For example, T-cell numbers were approximately the same, and activation markers such as CD25 were almost identical. It is also possible that the techniques used to quantify T-cell activation and cytokine production were not of the requisite sensitivity, but similar studies using the same methods found differences in both adults and children with asthma^{6,20} and in other diseases.²⁷ Finally, cellular findings in BAL may not accurately reflect airway tissue events revealed by direct tissue sampling using mucosal biopsy. A number of studies reported that the overall correlation between BAL and biopsy methods was reasonably good, and it seems unlikely that significant airway-wall inflammation will not be reflected in studies of BAL cells.^{5,15,23}

In conclusion, we found no evidence of persistent T-cell-driven airway inflammation in BAL obtained from young, predominantly mild or nonatopic children with episodic wheeze. It was suggested that daily use of inhaled glucocorticosteroids should be considered in wheezy children to reduce chronic airway inflammation and the possibility of airway remodeling, and to improve pulmonary function.¹⁰ The results of our study provide some support for the current guidelines of the British Thoracic Society^{32,33} and Australian National Asthma Campaign,^{34,35} which state that long-term daily anti-inflammatory medication is not indicated in children with infrequent episodic wheeze.

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