

Parental tobacco smoking is associated with augmented IL-13 secretion in children with allergic asthma

Wojciech Feleszko, MD, PhD,^{a,*} Anna Zawadzka-Krajewska, MD, PhD,^a
Katarzyna Matysiak, MD,^b Dorota Lewandowska, MD,^b Joanna Peradzińska, MD,^a
Q. Thai Dinh, MD, PhD,^c Eckard Hamelmann, MD, PhD,^d David A. Groneberg, MD, PhD,^c
and Marek Kulus, MD, PhD^a Warsaw, Poland, and Berlin, Germany

Background: Exposure to environmental tobacco smoke (ETS) has been shown to increase symptoms of allergic bronchial asthma, but direct effects on the expression of inflammatory markers have not been demonstrated thus far.

Objective: The aim of this study was to assess the correlation of ETS exposure with the expression of proinflammatory mediators in airway secretions, including IFN- γ and IL-12, as well as IL-5 and IL-13, in allergic asthmatic schoolchildren and healthy control subjects.

Methods: By using the nasopharyngeal aspiration technique, airway secretions were collected from 24 atopic children with asthma (age, 6-16 years) and 26 healthy control subjects, and the concentration of cytokines was measured with immunoenzymatic methods.

Results: IL-13 levels were highly increased in patients with asthma ($P < .005$), and parental tobacco smoke resulted in a significant increase in airway IL-13 secretion in these children compared with that seen in nonexposed children and healthy control subjects (median, 860 pg/mL vs 242 pg/mL and 125 pg/mL, respectively). Furthermore, a positive correlation between IL-13 levels and serum IgE concentrations ($r_s = 0.55$) was found in children with allergic asthma.

Conclusions: These results indicate that ETS augments the expression and secretion of IL-13 in allergic asthma and that nasopharyngeal aspiration is a suitable method to assess cytokine measurements in airways in children. Measurements of IL-13 in secretions might be taken into account as a noninvasive marker of airway inflammation and to assess

the detrimental effects of ETS. (*J Allergy Clin Immunol* 2006;117:97-102.)

Key words: Children, IL-13, smoking, pediatric asthma, IgE, environmental tobacco smoke, nasopharyngeal aspirates, IL-5, IL-12, IFN- γ

Reliable evidence has been provided that exposure to environmental tobacco smoke (ETS) is linked with impaired lung function and aggravation of asthma in childhood.¹ Asthmatic children with mothers who smoke were found to have more severe asthma when compared with those whose mothers did not smoke. Although parental smoking has not consistently been found to correlate with the risk of allergic sensitization in children, it has been suggested that maternal smoking during pregnancy and children's ETS exposure might cause asthma through increased bronchial hyperreactivity, alterations in circadian variations in pulmonary function, heightened sensitivity to allergens, or an irritant effect.^{2,3} These effects might be due to an increase of the inflammatory burden of the lower respiratory tract through the recruitment of increased numbers of inflammatory cells, alteration in cell subtypes, and proinflammatory mediator release.⁴ However, direct correlation between ETS exposure and single mediators has not been explored thus far. Tobacco smoke in animal models seems to increase neurogenic inflammation and intensify oxidative stress, which could potentially lead to an amplification of the airway inflammation already present in asthmatic subjects.⁴ Asthma is an inflammatory disease of the airways that is thought to originate as a result of polarized immune response to ubiquitous inhaled allergens. Proinflammatory cytokines derived from T_H2 lymphocytes, such as IL-4, IL-5, IL-6, and IL-13, are considered to orchestrate the asthmatic phenotype.^{5,6} The overexpression of these cytokines results in the recruitment and activation of a wide variety of effector cells, which leads to airway inflammation and hyperresponsiveness. Recently, IL-13 has been proposed to play a pivotal role in the T_H2-polarized immune response to inhaled allergens in adults.⁶⁻⁸ It has already been shown to be a critical mediator of the effector arm of the allergic response, which acts in concert with other T_H2 cytokines, including IL-4 and IL-5.⁸⁻¹⁰ For these reasons, numerous studies investigated the exaggerated production of the T_H2-type cytokines in the lungs of

From ^athe Departments of Pediatric Pneumology and Allergy and ^bthe Unit of Anaesthesiology, The Medical University Children's Hospital, The Medical University of Warsaw; ^cPneumology and Immunology, Charité School of Medicine, Free University and Humboldt-University, Berlin; and ^dthe Department of Pediatric Pneumology and Immunology, Charité Universitäts Medizin, Berlin.

*Dr Feleszko is on a leave of absence at the Department of Pediatric Pneumology and Immunology, Charité Universitäts Medizin, Berlin, Germany.

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Reprint requests: Wojciech Feleszko MD, PhD, Department of Pediatric Pneumology and Allergy, The Medical University Children's Hospital, ul Działdowska 1/3, PL-01-184 Warsaw, Poland. E-mail: wfeleszko@klinikum.pl.

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TABLE I. Characteristics of study populations

	Asthmatic subjects		Control subjects	
	Passive smokers*	Nonsmokers	Passive smokers	Nonsmokers
Subjects, n	10	14	14	12
Sex, male/female	7/3	10/4	8/6	7/5
Age, y	10.1 (6-15)	11.1 (7-16)	11.5 (6-16)	11 (5-14)
Duration of asthma, y	5.7 (2-9)	5.8 (6 months to 11 years)	0	0
Reversibility (12% of baseline)	10/10	14/14	ND	ND
Serum IgE, IU/mL	771 (24-2000)	729 (53-2000)	35.5 (10.5-125.9)	50 (10.8-124.9)
Steroids inhaled, n; $\mu\text{g}/\text{d}^\dagger$	4/10; 250 $\pm$ 64.5 (250)	6/14; 217 $\pm$ 44 (200)	0/14; –	0/12; –
Cromones inhaled	5/10	7/14	0/14	0/12
Antileukotriene oral	6/10	6/14	0/14	0/12

Data are presented as absolute numbers or as means, with ranges in parentheses.

ND, Not done.

*Passive smokers are defined as children exposed to parental tobacco smoke at home (≥ 10 cigarettes per day).

† Budesonide or equivalent (mean steroid dose $\pm$ SEM [median]).

Abbreviations used

DTT: Dithiothreitol

ETS: Environmental tobacco smoke

NPA: Nasopharyngeal aspirate

experimental animals and adult human asthmatic subjects (for review, see Wills-Karp⁸). However, the role of proinflammatory cytokines in allergic airway inflammation in childhood asthma is less well established,¹¹⁻¹³ and the mechanisms whereby passive smoking in childhood might modify local immune responses and thereby contribute to pediatric asthma remain obscure.¹⁴⁻¹⁶

The aim of the current study was to determine the local cytokine profile in pediatric atopic asthmatic patients with special respect to the effects of ETS exposure.

METHODS

Patients

The study population consisted of 24 atopic asthmatic school-children (age, 6-16 years) attending the Allergy Clinic of the Department of Pediatric Pneumology and Allergy of the Medical University Children's Hospital in Warszawa. Inclusion criteria were mild-to-moderate stable asthma, a positive skin prick test response to at least one common aeroallergen, and an FEV₁ value of greater than 75% of predicted value. Asthma severity was classified by using a modified version of the 1997 National Asthma Education and Prevention Program algorithm and the Global Initiative for Asthma.^{17,18} The diagnosis of asthma was made on the basis of clinical history, symptoms, physical findings, and the presence of reversible airflow obstruction demonstrated by an improvement in FEV₁ of greater than 12% after bronchodilator use or positive methacholine bronchial challenge result, as suggested by the Global Initiative for Asthma.¹⁸ All patients in the study presented with reversible airflow reduction, and therefore methacholine testing was not needed (Table I). The mean duration of symptoms in the group of asthmatic patients was 5.8 years (range, 6 months to 11 years). Parents of asthmatic patients were asked for their smoking status, and children were defined as "passive smokers" or "ETS exposed" when they were exposed to

tobacco smoke at home and the parents smoked 10 or more cigarettes per day, which corresponds to 4 to 36 $\mu\text{g}/\text{L}$ urinary cotinine.¹⁹ Seven (29%) asthmatic patients had allergic rhinitis, but it was inactive at the time of assessment. Subjects were excluded if they had either active upper respiratory tract infections within the last 3 weeks, asthma exacerbation, active allergic rhinitis, acute sinus disease requiring antibiotic treatment within 1 month before the visit, or any other major clinically significant disease.

The control group consisted of children admitted for elective surgery, such as noncomplicated hernia or phimosis. Children with a history or physical findings consistent with asthma, allergic rhinitis, or both were excluded. In the operating theater, as soon as the child was anesthetized, nasopharyngeal aspirates (NPAs) were obtained as a part of routine procedure before intubation, with the same method used for asthmatic patients. None of the patients or control subjects had any symptoms of active infections.

The study was formally approved by The Medical University of Warsaw Ethical Committee. Informed consent was obtained from the parents or guardians of all patients.

Collection of respiratory secretions

NPAs were collected from patients with asthma during a remission phase. Samples of NPAs were obtained by placing a catheter (diameter, 3.3 mm) of the pediatric mucus extractor (Uno, Maersk Medical A/S, Denmark) into the nasopharynx and applying suction according to the method of Xiang et al.²⁰ The secretions were rinsed into traps and stored frozen (-72°C). The volume of the samples collected from each group of patients varied between 50 and 500 μL .

Cytokine determinations

NPAs were processed according to the method of Pizzichini et al.²¹ All portions of the secretion that macroscopically appeared free of salivary contamination were placed in a polystyrene tube, treated with a 4 $\times$ volume of 0.1% dithiothreitol (DTT; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and gently vortexed at room temperature. All cytokines were measured with specific OptEIA ELISA kits obtained from PharMingen (PharMingen, San Diego, Calif), according to the manufacturer's instructions. The recovery of relevant cytokines spiked to NPAs and reagents used was evaluated, and the recovery was determined. Average recovery was 89% for all cytokines (range, 75% to 114%). Minimum detection levels were calculated with KJC Junior software (Bio-Tek Instruments Inc, Winooski, Vt) and were as follows: IL-5 and IL-12, 7.8 pg/mL; IL-13 and IFN- γ , 4.7 pg/mL. The IgE concentrations were determined

TABLE II. IL-5, IL-12, IL-13, and IFN- γ and IL-5/IFN- γ , IL-13/IFN- γ , and IL-13/IL-12 ratios in NPAs from allergic asthmatic children and control subjects

	Control subjects				Asthmatic subjects			
	N	% > detection limit	Mean $\pm$ SEM	Median	N	% > detection limit	Mean $\pm$ SEM	Median
Cytokine (pg/mL)								
IL-5	26	67	45.1 $\pm$ 9.0	31.1	20	79	40.1 $\pm$ 8.5	22.1
IL-12	26	96	35.3 $\pm$ 7.0	31.5	20	100	39.5 $\pm$ 3.2	40.6
IL-13	26	72	203.8 $\pm$ 48.1	125.0	24	100	590.3 $\pm$ 83.9 $\dagger$	540.0
IFN- γ	26	100	72.6 $\pm$ 6.1	68.0	20	100	43.3 $\pm$ 2.8 $\dagger$	43.1
Cytokine ratio								
IL-5/IFN- γ	26	67	0.6 $\pm$ 0.2	0.3	20	79	1.2 $\pm$ 0.3	0.5
IL-13/IFN- γ	26	72	3.5 $\pm$ 0.9	2.0	20	100	15.6 $\pm$ 2.9 $\dagger$	14.3
IL-13/IL-12	26	72	6.8 $\pm$ 1.5	3.8	20	100	17.4 $\pm$ 3.3*	13.9

* $P < .01$, $\dagger P < .005$ compared with control subjects (Mann-Whitney test).

by using the automatic analyzer UniCAP 100 (Pharmacia & Upjohn, Uppsala, Sweden).

Statistical analysis

Data are expressed as means $\pm$ SEM (with 95% CIs). Comparisons between groups were performed with the Mann-Whitney test, and the analysis of correlation was done with the Spearman rank correlation coefficient (Instat version 3.00; GraphPad Software Inc, San Diego, Calif, www.graphpad.com). The χ^2 test was used to analyze the proportions of patients and control subjects with cytokine responses greater than the detection limit. In all tests the 2-tailed method was applied, and a P value of less than .05 was considered to be statistically significant.

RESULTS

IL-13 is highly expressed in NPAs in children with allergic asthma

IL-13 concentrations in NPAs obtained from children with allergic asthma were increased compared with those seen in the control group ($P = .005$, Mann-Whitney test; Table II and Fig 1). In contrast, a decreased level of IFN- γ was detected in asthmatic versus control patients ($P = .0008$). All cytokines were detected more frequently in asthmatic patients than in control subjects (Table II), but the difference was statistically not significant (χ^2 test). We determined also the IL-5/IFN- γ , IL-13/IFN- γ , and IL-13/IL-12 ratios. There was no difference in the IL-5/IFN- γ ratio, but a nearly 3-fold higher IL-13/IL-12 ratio was noted in allergic asthmatic patients ($P < .01$, Mann-Whitney test). A stronger difference was observed for the IL-13/IFN- γ ratio, where a 4.5-fold difference was revealed ($P < .005$, Table II).

ETS augments IL-13 secretion in children with allergic asthma

To elucidate the possible role of IL-13 as a marker of allergic inflammation, we investigated whether IL-13 concentration might correlate with age, IgE level, parental smoking, prescribed medication, and lung function tests. Although influence of the patient's age, present spirometric results, or drug intake on IL-13 concentration was not

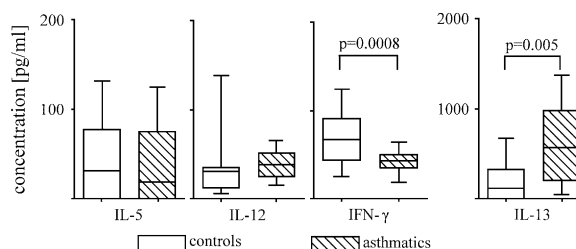


FIG 1. Levels of IL-5, IL-12, IL-13, and IFN- γ in the fluid phase of NPAs from asthmatic children and healthy control subjects. Boxes indicate the 25th to 75th percentile of the data, the horizontal line across the box shows the median for the data, and whiskers (lines extending from the boxes) indicate the 5th and 95th percentiles (Mann-Whitney test).

observed (data not shown), a significant difference came to light when the effect of ETS was considered. Aspirates from asthmatic subjects exposed to tobacco smoke revealed higher concentrations of IL-13 in comparison with aspirates from non-ETS-exposed asthmatic and control subjects (median, 860 pg/mL vs 241 pg/mL and 125 pg/mL, respectively; $P = .03$ and $P < .0001$, respectively, Mann-Whitney test; Fig 2). There were no significant differences observed among the clinical characteristics in the children with allergic asthma from the smoke-exposed families compared with the offspring from the nonexposed families (Table I). An increased concentration of IL-13 in ETS-exposed control patients compared with that seen in unexposed patients was also observed (median, 176 vs 50 pg/mL); however, this difference was statistically nonsignificant (Table III). Also, asthmatic children not exposed to ETS differed from healthy subjects ($P = .02$, Fig 2).

Correlation between cytokine concentrations and serum IgE levels

A correlation analysis was performed to examine whether cytokine concentration in aspirates might correlate with the level of allergic sensitization expressed as serum IgE concentration. There was a significant correlation between the concentration of IL-13 in nasopharyngeal secretions and the serum IgE concentration ($r = 0.55$;

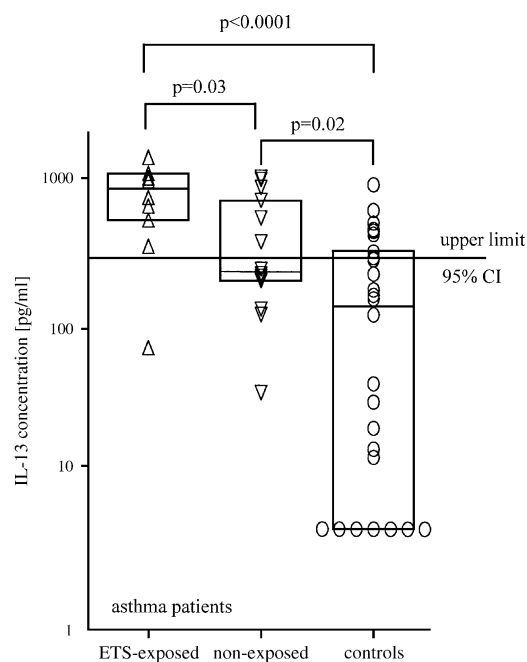


FIG 2. Concentration of IL-13 in ETS-exposed (passive smokers) and nonexposed (nonsmokers) asthmatic patients and healthy control subjects. *Markers* indicate each of the samples in the group (undetectable levels of IL-13 were plotted at the detection limit, 4.7 pg/mL). *Boxes* indicate 25th to 75th percentiles of the data, and the *horizontal line* across the box represents median value. The *line* across the chart represents the upper limit of the 95% CI of the mean in the control group (Mann-Whitney test).

95% CI, 0.18-0.79; $P = .005$; Fig 3, A). The IL-13/IFN- γ and IL-13/IL-12 ratios were also significantly higher in asthmatic subjects with high serum IgE concentrations. They correlate closely with IgE level, and their correlation coefficients were an r value of 0.61 (95% CI, 0.21-0.83; $P = .004$) and an r value of 0.57 (95% CI, 0.17-0.82; $P = .008$), respectively (Fig 3, B and C).

Similarly, serum IgE concentration correlated with IL-5 level ($r = 0.49$; 95% CI, 0.05-0.77; $P = .026$) but not with IFN- γ or IL-12 level. Furthermore, a positive correlation between IL-5 level and the IL-13/IFN- γ ratio was found ($r = 0.50$; 95% CI, 0.06-0.78; $P = .025$), whereas a negative correlation was observed between IL-5 and IFN- γ levels ($r = -0.47$; 95% CI, -0.76 to -0.02 ; $P = .036$; data not shown).

DISCUSSION

ETS, or passive smoking, has been demonstrated to be causally associated with a large number of human diseases, although the evidence is sometimes conflicting and the tobacco industry has tried to cover up research data over the past 30 years. In adults ETS exposure has been shown to be related to respiratory symptoms, bronchial asthma, a significant impairment of lung function, and increased bronchial responsiveness. Because the consequences of ETS exposure at the workplace seemed to be

TABLE III. Mean concentration of cytokines in NPAs from tobacco smoke-exposed and nonexposed control patients

Cytokine (pg/mL)	Control subjects	
	ETS-exposed,* n = 14	Nonexposed, n = 12
IL-5	41.4 $\pm$ 11.9 (31.1)	43.0 $\pm$ 23.2 (32.8)
IL-12	38.5 $\pm$ 15.9 (31.5)	40.9 $\pm$ 18.1 (27.7)
IL-13	251.4 $\pm$ 71.2 (176)	148.3 $\pm$ 56.7 (49.9)
IFN- γ	73.4 $\pm$ 8.6 (75.5)	76.0 $\pm$ 19.1 (66.3)

Data are presented as means $\pm$ SEM (median).

*ETS-exposed children are defined as children exposed to parental tobacco smoke at home (≥ 10 cigarettes per day).

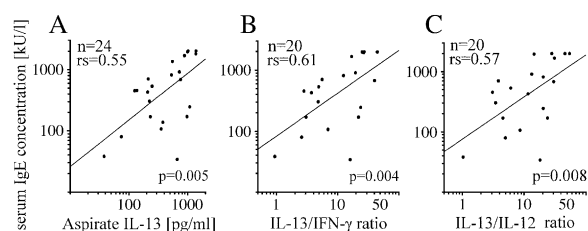


FIG 3. Correlation between cytokine levels and serum IgE concentration. IL-13 concentration (A), IL-13/IFN- γ ratio (B), and IL-13/IL-12 ratio (C) in nasopharyngeal secretion and serum IgE concentrations in children with allergic asthma are shown (log scale). Values represent individual data obtained from 20 subjects studied (as described in the Methods section). The regression line (solid line) indicates the Spearman correlation coefficient (r_s).

more serious than domestic ETS exposure, legislative measures focused on banning smoking at work. In children prenatal exposure to ETS has been shown to be associated with impaired lung function and an increased risk of bronchial asthma. Postnatal exposure mainly acts as a triggering factor for asthma attacks, and respiratory symptoms and measures focused to reduce childhood ETS exposure should have higher priority. In this respect smoking-cessation programs in pregnancy and for parents at the time of child hospitalization have been shown to have a high success rate. However, passive smoking is still widespread, and it displays an extremely important and avoidable risk factor for respiratory symptoms in children.

The present study provides evidence that the mean concentration of IL-13 in airway secretion is significantly higher in allergic asthmatic children exposed to parental tobacco smoke than that in nonexposed asthmatic subjects or control subjects. Thorough evaluation of clinical parameters among this study population did not reveal any differences between 2 groups of asthmatic children other than ETS exposure (Table I). Therefore we cannot conclude that any of the groups has more severe disease compared with any other group. Surprisingly, no effect of drug intake on cytokine levels was revealed among asthmatic patients.

The mean concentration of IL-13 in secretions corresponds to those detected in sputum of adults by Komai-Koma et al.²² Our study in children confirms the findings

from adult patients, demonstrating increased levels of both IL-13 RNA and protein in bronchial biopsy specimens, bronchoalveolar lavage cells, and induced sputum of adult asthmatic patients.^{8,22,23}

This is the first study assessing the concentration of cytokines in the airway secretion of asthmatic children exposed to ETS. We show that tobacco smoke might bias the immune system toward a T_H2 pattern, leading to enhanced IL-13 expression in human asthma, and recent animal studies support these findings.^{24,25} For maternal smoking in pregnancy, increased neonatal IL-13 levels have also been demonstrated.²⁶ Concurrently, in our study population a slight tendency toward a T_H2 -regulated cytokine response was also observed in control children exposed to tobacco smoke, although it was nonsignificant. These reports, however, remain at odds with opinion indicating that ETS is negatively associated with airway function in children, whereas it plays little or no role in atopy development (for review, see Lodrup Carlsen and Carlsen²⁷).

In addition, our experiments confirm the usefulness of cytokine measurements in secretions obtained by means of nasopharyngeal aspiration and indicates the applicability of this method in asthmatic children, in whom other methods are limited. The presently used method seems to be an attractive and noninvasive alternative to obtain airway secretions and monitor the inflammatory process in bronchial mucosa in children.

Nasopharyngeal aspiration has been widely used in detecting respiratory viruses in lower airway secretion, and it has been proved to be at least as effective as bronchoalveolar lavage or induced sputum analysis in diagnostic microbiology.²⁰ Although nasopharyngeal aspiration has already been shown to effectively measure cellular characteristics, mediators, and cytokines in airways in patients with respiratory tract diseases, including severe asthma,^{20,28-31} nasal contamination might bias the analysis.

In the present study the levels of both IL-13 and IL-5, but not IL-4, were assayed because increasingly more evidence indicates that IL-13, but not IL-4, plays a crucial role in the T_H2 -driven immunopathology and bronchial hyperresponsiveness seen in asthma, and IL-5 appears to be a critical marker of eosinophilic inflammation.^{8,10,32,33} The counterbalance for T_H2 -driven immune response in asthma is achieved mainly by the classical T_H1 cytokine IFN- γ and by the monocyte/macrophage-derived T_H1 activator IL-12. In our experiments IL-12 was not down-regulated in children with asthma; however, significantly lower concentrations of IFN- γ in NPAs were present, which is concordant with previous observations in adults.²³

Unexpectedly, we did not find any association between asthma and IL-12 or IL-5 levels (Table II and Fig 1). These results are at variance with other studies, in which both sputum IL-5 levels and markers of eosinophilic inflammation were significantly greater in adult asthmatic subjects than in control subjects.^{34,35} This might be due to the preparation of the secretion in which DTT was used because

it has been postulated to affect IL-5 detection.³⁶ On the other hand, our spiking experiments demonstrated relatively high recovery, and therefore it seems unlikely that our results might be influenced significantly by the use of DTT.

The increased IL-13 expression might be attributed to a number of cellular sources. In this respect it has been reported that IL-13 is produced by T_H2 -polarized $CD4^+$ T cells, T_H1 $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells, or even non-T-cell populations that are of particular importance to the allergic response, such as mast cells, basophils, and eosinophils.⁸ IL-13 is also known to regulate serum IgE synthesis, and increased IL-13 secretion therefore results in enhanced IgE-mediated sensitization.^{8,33} Here we show a correlation between high airway IL-13 concentrations and serum IgE concentrations. These results confirm in a clinical setting the results of the pioneer study by Wills-Karp et al,³⁷ who demonstrated in mice that high airway concentrations of IL-13 result in an increase of total serum IgE levels in a time-dependent manner. IL-13 levels were also presently correlated with IL-5 concentrations. This correlation is obvious because both cytokines contribute to eosinophil tissue infiltration during the inflammatory response in asthma.^{9,37,38} Our observations are also in line with several lines of evidence, suggesting that both IL-5 and IL-13 are expressed in a highly coordinated mechanism.³⁹

In the present study allergic asthmatic children with positive skin test responses were compared with a nonallergic and nonasthmatic population. Therefore it cannot be ruled out that the findings are only specific for allergic pediatric asthma: they might be specific for an atopic diathesis in general, and future studies should address this issue.

In conclusion, the aim of the present study was to assess the correlation of ETS with the presence of proinflammatory mediators in airway secretions, including IFN- γ and IL-12, as well as IL-5 and IL-13, in asthmatic schoolchildren and healthy control subjects. Our results indicate that ETS augments the expression and secretion of IL-13 in bronchial asthma in children. Moreover, the nasopharyngeal aspiration technique might not be confined only to the diagnostic virology but might be regarded as a suitable tool to assess cytokine levels in asthmatic children and wheezy infants. NPA measurements of IL-13 in secretions can be taken into account as a noninvasive marker of airway inflammation in children.

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