

Difficult asthma in children: An analysis of airway inflammation

Jacques de Blic, MD,^{a*} Isabelle Tillie-Leblond, MD, PhD,^{b*}

André Bernard Tonnel, MD, PhD,^b Francis Jaubert, MD,^c Pierre Scheinmann, MD,^a
and Philippe Gosset, PhD^b Paris and Lille, France

Background: Difficult asthma in children displays distinct clinical patterns, and its physiopathology remains poorly understood.

Objective: To determine the characteristics of the bronchial inflammatory profile in children with difficult asthma.

Methods: We performed endobronchial biopsy and bronchoalveolar lavage in 28 children with persistent bronchial obstruction despite high doses of inhaled corticosteroids and regular treatment with long-acting β_2 -agonists: 13 had persistent symptoms and 15 had few or no symptoms.

Results: The number of eosinophils ($P = .03$) and neutrophils ($P = .04$) in the epithelium was significantly higher in symptomatic children than in children with few symptoms. Reticular basement membrane thickening was similar in both groups. IFN γ levels ($P = .03$) and IFN γ /IL-4 ratio ($P = .01$) were significantly higher in children with few symptoms.

Conclusions: In symptomatic children, T_H2-type inflammation was associated with the presence of activated eosinophils in the epithelium, whereas asthma in children with few symptoms was associated with an increase in T_H1 cytokine levels. The high levels of IFN γ suggest that this T_H1 cytokine may modulate the local inflammatory response. (*J Allergy Clin Immunol* 2004;113:94-100.)

Key words: Difficult asthma, child, bronchoalveolar lavage, bronchial biopsy, interferon gamma, eosinophils, neutrophils, reticular basement membrane

Refractory asthma is a heterogeneous condition that encompasses severe asthma, difficult-to-control, poorly controlled, irreversible asthma.^{1,2} Children whose asthma remains poorly controlled despite treatment with at least 800 μ g/d inhaled budesonide or equivalent should be classified as having "difficult asthma." Although it affects probably <5% of all children with asthma, difficult asthma has a large impact on morbidity.³

Wenzel et al⁴ identified two groups of adult patients with refractory asthma. Inflammation-associated

Abbreviations used

BAL: Bronchoalveolar lavage

CS: Corticosteroids

ECP: Eosinophil cationic protein

RBM: Reticular basement membrane

eosinophils were present in only one of these groups. The heterogeneity of difficult asthma in children was identified retrospectively in 21 children with severe asthma who did not respond to a trial course of oral glucocorticosteroids.⁵ Spirometry patterns were described as chaotic and nonchaotic, according to variability in daily pulmonary function. The children in the chaotic group might have ongoing airway inflammation or an intrinsic resistance to glucocorticoids, whereas the children in the nonchaotic group might have undergone airway remodeling leading to airway fibrosis, but no specimens collected by bronchoscopy were studied.⁵ Payne et al⁶ demonstrated a correlation between exhaled nitric oxide and eosinophil score in bronchial biopsy samples from patients with difficult asthma, with higher values recorded for children with persistent symptoms. The aim of this study was to determine whether, in a subgroup of children with persistent obstructive pattern despite optimal treatment, children with persistent symptoms and children with few symptoms presented different bronchial inflammatory profiles. We used bronchial biopsy and bronchoalveolar lavage (BAL) specimens to identify the inflammatory cells present, reticular basement membrane (RBM) thickness, and to investigate the balance of T_H1-versus T_H2-type cytokines.

METHODS

Patients

We studied 28 children with difficult asthma. These children attended the clinic every 2 to 3 months for at least 1 year, and symptoms, bronchodilator use, exacerbations, and pulmonary function test (PFT) results were regularly recorded. Asthma was diagnosed according to American Thoracic Society criteria.⁷ All children had a history of recurrent episodes of airway obstruction and reversibility of bronchial obstruction or nonspecific bronchial hyperresponsiveness. Difficult asthma was defined as persistent obstructive pattern (FEV₁ <80% of predicted values), regardless of symptoms, despite treatment with at least 800 μ g/d inhaled beclomethasone or equivalent, associated with regular long-acting β_2 -agonist treatment.^{8,9}

Cystic fibrosis, primary ciliary dyskinesia, bronchopulmonary dysplasia, postinfectious pulmonary sequelae, immune deficiencies, and cord vocal dysfunction were excluded. Clinical history, family

From ^aService de Pneumologie et d'Allergologie Pédiatriques, Hôpital Necker-Enfants Malades, Paris; ^bUnité INSERM U 416, Institut Pasteur de Lille, and ^cService d'Anatomopathologie, Hôpital Necker-Enfants Malades, Paris.

*J. de Blic and I. Tillie-Leblond contributed equally to this work.

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Reprint requests: Prof Jacques de Blic, Service de Pneumologie et d'Allergologie pédiatriques, Hôpital Necker Enfants Malades, 149 rue de Sèvres, 75015 Paris, France.

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history of allergy, allergy data, and PFT results were recorded. Atopic status was determined by standard skin prick tests and by measuring total serum IgE levels and levels of IgE specific for house dust mite, cat, grass pollen, and cockroach. Test results were considered positive if there was a skin reaction ≥ 3 mm in size to one or more allergens or specific IgE levels were at least 3.5 kU/L.

We assigned the 28 children to 2 groups: group S ($n = 13$), children with persistent symptoms (defined before the study as the use of more than 3 bronchodilator rescue treatments per week) and/or exacerbations defined as asthma attacks requiring systemic corticosteroids (CS) and/or hospitalization⁹ (more than 3 exacerbations in the past year); and group PS ($n = 15$), children with few symptoms (defined as the use of less than 1 bronchodilator rescue treatment per month) and no more than 1 exacerbation in the past year.

All children had a negative trial course of CS (2 mg/kg per day of prednisolone for 14 days), defined as an increase in FEV₁ of no more than 15%. Bronchoscopy was performed 4 to 6 weeks after this trial, during a period free from exacerbation and acute respiratory infection.

Ethical approval

The study was approved by the ethics committee (CCPPRB) of Necker Enfants-Malades University Hospital, Paris. Written informed consent was obtained from the parents.

Spirometry

Spirometry and flow volume curves were performed according to the ATS guidelines (Gould 9000; Sensormedics; Dayton, Ohio).¹⁰

Bronchoscopy with endobronchial biopsy and bronchoalveolar lavage

All flexible bronchoscopy procedures were performed under deep sedation, as recently described.¹¹ Children were continuously monitored by pulse oxymetry (SAO₂) and electrocardiography. Flexible bronchoscopy was performed with a 4.9-mm external diameter bronchoscope (Olympus Winter IBE GMBH, Hamburg, Germany). BAL was performed in the right middle lobe, using 3×1 mL/kg aliquots of normal saline.¹² Return fluid was collected into sterile polypropylene vials (Falcon 50 mL, Becton Dickinson, Rutherford, NJ). Four to 6 endobronchial biopsy samples were taken from segmental or subsegmental bronchi of right or left lower lobes, using cupped forceps.

Lavage and tissue processing

The first aliquot was used for bacterial cultures (the diagnosis of lower respiratory tract infection required $>10^4$ CFU/mL)¹³ and immunofluorescence testing for RSV, parainfluenza, influenza virus, adenovirus, and cytomegalovirus. Subsequent aliquots were pooled and used for cytologic analysis. Mucus was removed from the fluid by filtration through a monolayer of sterile gauze. BAL samples were immediately placed on ice and centrifuged to separate fluid from cells. We counted the total number of cells with a hemocytometer (Malassez, Preciss, Strasbourg, France). Aliquots of centrifuged BAL samples were subjected to May-Grunwald-Giemsa staining for the determination of differential cell counts. Two investigators blinded to clinical history read the slides, counting at least 500 cells in each case. The mean differential cell counts for the two investigators are reported. Macrophages, neutrophils, eosinophils, lymphocytes, mast cells, and epithelial cells were counted, and results are expressed as a percentage of total cells. The BAL fluid was aliquoted and frozen at -70°C .

Biopsy samples were fixed in 10% formaldehyde and embedded in paraffin blocks. They were stained with hematoxylin and eosin

staining and May-Grunwald-Giemsa by standard methods. The number of T lymphocytes was determined by using anti-CD3 antibody (Dako, Glostrup, Denmark). Two independent observers assessed the numbers of inflammatory cells in the epithelium and in the subepithelium area (the area beneath the epithelial basement membrane).¹⁴ Biopsy specimens were examined at $\times 500$ magnification. For each biopsy specimen, the count was performed on at least the area of one grid (surface 0.59 mm² for subepithelial area; side length 0.4 mm for intraepithelial count). The entire section was evaluated. Mucus gland and muscle areas were excluded for the counting. Results are expressed as the number of cells per millimeter length of RBM and the number of cells per mm² of submucosal area.¹⁴ RBM thickness was determined as the average of 20 measurements in sections in which the epithelium was oriented in such a way that measurement artifacts did not occur.¹⁵

Evaluation of mediator concentrations in BAL supernatants

We determined the concentrations of IL-4, IFN- γ (Diacione, Besançon, France), IL-5 (Coulter-Immunotech, Villepinte, France) and neutrophil elastase (Bender Medsystems, Vienna, Austria) by enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's protocols. Eosinophil cationic protein (ECP) (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) levels were evaluated by means of a competitive radioimmunoassay (RIA). Results are expressed in picograms per milliliter for IL-4, IL-5, and IFN γ and in nanograms per milliliter for neutrophil elastase; the sensitivity of these assays was 0.3, 1, and 3 pg/mL and 0.1 ng/mL, respectively. For ECP, the results are expressed in micrograms per liter, and the limit of detection was 1 $\mu\text{g/L}$.

Statistical analysis

Group data are expressed as medians, with 25th and 75th centiles. Differences between groups were assessed by using the Mann-Whitney *U* test for continuous variables and Fisher exact test for qualitative variables. We used the Spearman rank correlation test to assess correlations between ECP and eosinophil levels and between neutrophil elastase and neutrophil levels in BAL. Probability values $<.05$ were considered statistically significant.

RESULTS

Patient characteristics and tolerance of bronchoscopy

The characteristics of the patients are described Table I. None of the children were active smokers. Bronchoscopy, BAL, and endobronchial biopsy were well tolerated. One child required treatment with nebulized β_2 -agonist after the procedure.

Microbiological results

No bacteria present at concentrations $>10^4$ CFU/mL were identified, and immunofluorescence detected no viruses.

Cell counts/differentials in BAL

The interobserver and intraobserver variation for the differential cell counts in BAL (and bronchial biopsy) was 8% and 5%, respectively. There was a trend for higher percentage of neutrophils in the S group (Table II). The percentage of lymphocytes was significantly higher in the PS group ($P = .03$).

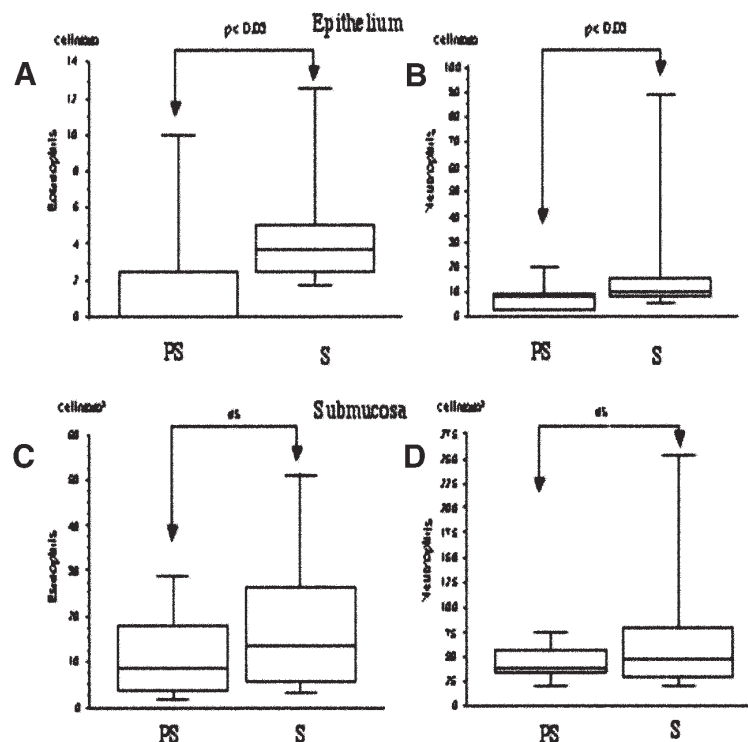


FIG 1. Eosinophil and neutrophil cell numbers in epithelial (A, B) and submucosal (C, D) areas of endo-bronchial biopsy tissue in paucisymptomatic (PS) and symptomatic (S) children. Data are expressed as box plots. Horizontal lines indicate median; squares indicate 25th and 75th percentiles, and error bars indicate 10th and 90th percentiles.

TABLE I. Clinical characteristics of children

	Group PS (n = 15)	Group S (n = 13)	P value
n	15	13	
Sex ratio (M/F)	12/3	6/7	.06
Age (y)	13.3 (10.1-15)	10.4 (9.6-12.5)	.055
Age at onset (y)	1 (0.5-2)	1.5 (1-2)	NS
Duration of asthma (y)	12.7 (9-14)	9.4 (8-10.5)	NS
Acute exacerbations (number per y)	0 (0-2)	5 (5-7)	
Duration of inhaled corticosteroid treatment (y)	6 (3-9)	5 (4-7)	NS
Inhaled corticosteroids ($\mu\text{g/d}$)	1000 (800-1000)	1000 (800-1000)	NS
Long-acting β_2 agonist (n)	15	13	
Leucotriene antagonist (n)	2	2	
FEV ₁ (% predicted)	75 (69-77)	73 (65-76)	NS
FEF ₂₅₋₇₅ (% predicted)	48 (36-53)	48 (32-57)	NS
Total serum IgE (kU/L)	60 (19-93)	249 (101-722)	.07
History of atopic dermatitis (n)	10	6	NS
Familial allergy history (n)	6	8	NS
Positive SPT or positive specific IgE (n)	11	10	NS

PS, Paucisymptomatic children; S, symptomatic children.

Data are expressed as medians and 25th and 75th centiles or absolute results (n).

Bronchial biopsy

Within the epithelium, eosinophil and neutrophil counts were significantly higher in children with persistent symptoms. The eosinophil counts for groups S and PS were 4 (2.5 to 5) and 0 (0 to 2.5), respectively ($P = .03$), and the neutrophil counts were 10 (7 to 15) and 7 (2 to 9), respectively ($P = .04$) (Figs 1 and 2). T-lymphocyte counts were comparable in group S (25 [16 to 52]) and PS (22 [8 to 45];

$P = .35$). In the submucosal area, eosinophil and neutrophil counts were not significantly different between groups.

Taking both groups together, intraepithelial and submucosal counts of neutrophils ($r_s = 0.53$; $P = .006$) and eosinophils ($r_s = 0.4$; $P = .02$) were significantly correlated.

RBM thickness in children was similar in the two groups: 7.2 (4.9 to 8.2) μm in group S and 6.7 (5.5 to 9.7) μm in group PS.

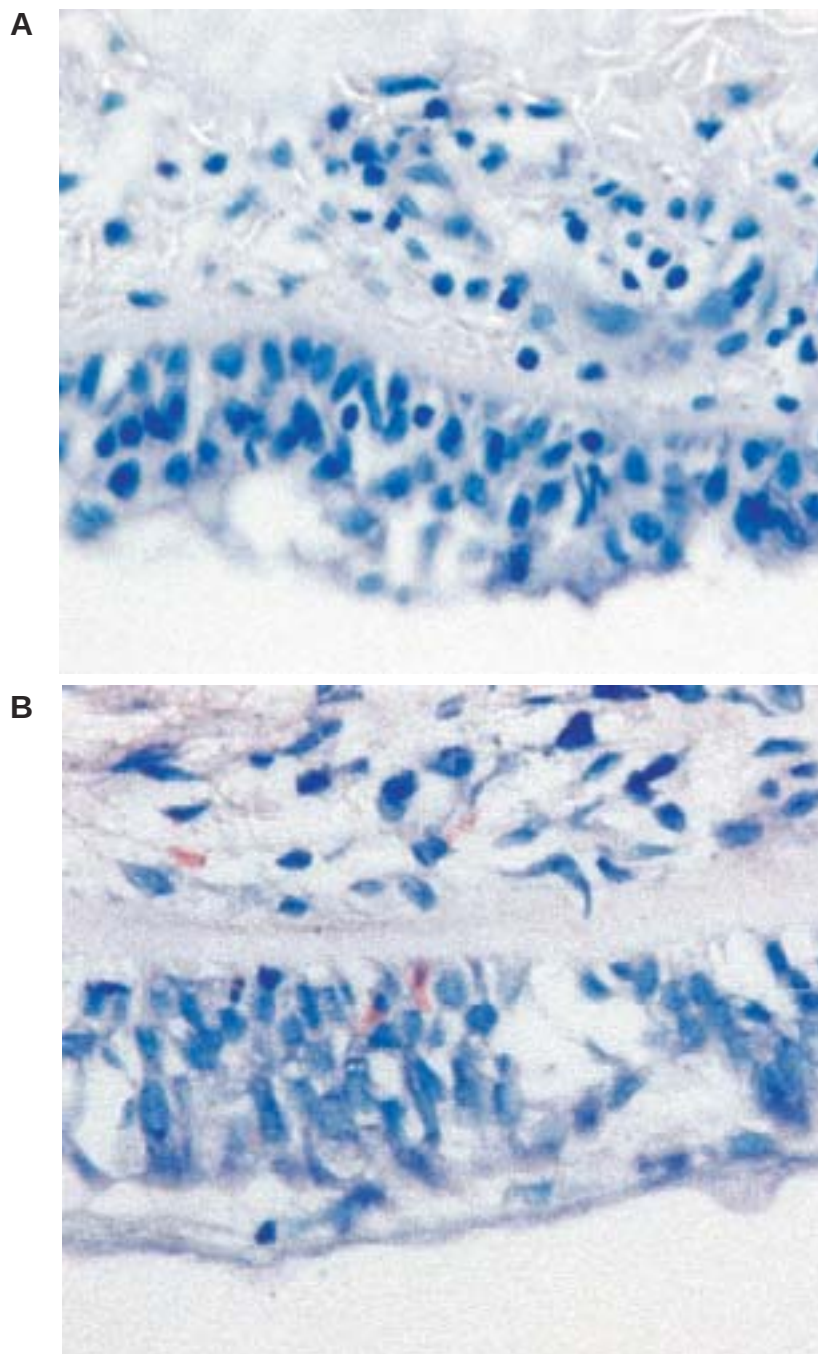


FIG 2. Bronchial biopsy stained with May-Grunwald Giemsa from a paucisymptomatic child showing intraepithelial and submucosal mononuclear cells (**A**) and from a symptomatic child showing eosinophils in the intraepithelial and submucosal area (**B**). Magnification $\times 500$.

Mediator levels

ECP levels but not neutrophil elastase levels were significantly higher in group S ([Table III](#)). ECP levels were correlated with the percentage of eosinophils in BAL samples ($r_s = 0.45$; $P = .04$). Neutrophil elastase and neutrophil counts were positively correlated in BAL samples ($r_s = 0.5$, $P = .01$).

IFN γ levels and the IFN γ /IL-4 ratio were significantly higher in the PS ([Table III](#)).

DISCUSSION

In a subgroup of children with difficult asthma, we identified 2 different bronchial inflammatory profiles. Difficult asthma is probably a spectrum of phenotypes,^{1,2}

TABLE II. Total cell counts and differential cell counts in BAL fluid from paucisymptomatic and symptomatic children

	Group PS	Group S
Total cell count (10 ³ /mL)	130 (100-150)	125 (115-175)
Macrophages (%)	72 (50-85)	42 (28-54)
Lymphocytes (%)	4.6 (3.7-8)*	3.5 (2.2-4.4)
Neutrophils (%)	3.2 (1.3-6)	4 (3-14.4)
Eosinophils (%)	1 (0.7-2.4)	1 (0.4-6.5)
Mastocytes (%)	0 (0-0)	0 (0-0)
Epithelial cells (%)	15 (5-32)	27 (5-48)

PS, Paucisymptomatic children; S, symptomatic children.

Results are expressed as median and 25th and 75th centiles.

**P* < .05.

suggesting the involvement of various cell types and mediators. The use of bronchoscopy and biopsy to clarify diagnosis and guide treatment is only justified in children with refractory asthma but is considered unethical in normal children and in children with controlled asthma.¹⁶⁻¹⁸ All children had persistent obstructive pattern despite optimal treatment, and oral CS treatment did not reverse airway obstruction. One potential weakness of this study is the lack of objective criteria for assessing compliance during the trial of oral steroids. This may have resulted in a selection effect during recruitment. However, in contrast to the Payne et al study,⁶ our study was carried out 4 to 6 weeks after the end of corticosteroid treatment, and so our results are unlikely to be affected by that treatment. Children were assigned to 2 groups on the basis of the presence or absence of clinical symptoms. Another weakness may be altered compliance in long-term treatment. However, these children regularly attended the clinic for at least 1 year. Symptoms, bronchodilator use, exacerbations, and PFT results were recorded, and partnership was developed with the children and their parents. Finally, these children may represent 2 true distinct phenotypes of asthma or 2 parts of a continuum of the disease.

In adults with "steroid-resistant asthma," clinical studies have demonstrated increases in numbers of eosinophils and levels of T_H2-type cytokines, such as IL-4 and IL-5.^{19,20} The factors responsible for chemotactic activity for eosinophils in BAL include IL-5 and CC chemokines.²¹ As IL-5 concentrations were similar in the 2 groups of children, eosinophil recruitment probably involved CC chemokines.

In our study, the 2 groups of children had persistent inflammation with large numbers of eosinophils and neutrophils and a T_H2-type cytokine profile in BAL. Intraepithelial eosinophils were significantly more frequent in children with persistent symptoms, as shown in adults.²²⁻²⁵ Wenzel et al defined 2 types of refractory asthma on the basis of different pathological processes. The group with bronchial eosinophils was associated with a larger number of exacerbations requiring intubation and thicker RBM than the group without eosinophils.⁴ As in the Payne et al study,²⁶ RBM thickening in the children with persistent

TABLE III. Mediator levels in BAL fluid from paucisymptomatic and symptomatic children

	Group PS	Group S	<i>P</i> value
ECP (μg/L)	2 (1-11.7)	30 (17.5-36.40)	.006
Neutrophil elastase (ng/mL)	103 (38-134)	130 (28-652)	NS
IL-4 (pg/mL)	1.4 (0.925-1.750)	1.3 (1-1.7)	NS
IL-5 (pg/mL)	2 (2-4.25)	2 (2-4.85)	NS
IFNγ (pg/mL)	11.9 (6-17)	2.6 (2-55)	.03
IFNγ/IL-4	8.2 (4.5-11.7)	2.8 (1.4-6.5)	.01

PS, Paucisymptomatic children; S, symptomatic children.

Results are expressed as median and 25th and 75th centiles

symptoms was similar to that seen in paucisymptomatic children. Our data in children provide further evidence that eosinophils play a major role in causing instability in cases of refractory asthma.

Intraepithelial eosinophils were associated with neutrophils. Neutrophils have been shown to be associated with severe asthma,²⁷ instability of asthma,²⁵ sudden-onset fatal asthma,²⁸ and status asthmaticus.²⁹ In children, the presence of neutrophils in BAL samples is correlated with the severity of asthma.^{13,30} Steroids may prolong the survival of neutrophils by decreasing the rate of apoptosis,³¹ whereas long-acting β₂-agonists might have an antineutrophilic effect.³² However, in our study, children with symptomatic asthma and higher neutrophil counts received similar doses of inhaled CS and long-acting β₂-agonists as used in paucisymptomatic children.

The role of IFNγ in children with an obstructive pattern without instability is of particular interest. IFNγ was detected in the absence of any virus. Imbalances in the T_H1/T_H2 response are less well defined in human beings than in mice,³³ and some investigators use the IFNγ/IL-4 ratio to define the phenotype of the T-cell response. IFNγ may play a role in immunomodulation of the T_H2 cell response. High levels of IFNγ production have been detected in stimulated whole blood cultures from asthmatic adults and have been correlated with asthma severity, blood eosinophil counts, and bronchial hyperresponsiveness.³⁴ An increase in circulating levels of IFNγ was also associated with asthma exacerbations.³⁵ Blood mononuclear cells from atopic children stimulated by an allergen responded by increasing both T_H1 (IFNγ) and T_H2 (IL-5 and IL-13) cytokine production.³⁶ IFNγ levels were higher in BAL samples from asthmatic children than in those from children with chronic cough.³⁷ In this study, IFNγ levels and IFNγ/IL-4 ratio were higher in children with few symptoms than in children with persistent symptoms. IFNγ is known to inhibit IL-4-dependent IgE production,^{38,39} together with IL-5-induced proliferation and eosinophil activation. IFNγ inhibits IL-13-induced goblet cell hyperplasia and the accumulation of eosinophils and neutrophils in BAL samples.⁴⁰ Low levels of IFNγ and high serum concentrations of IL-5 are associated with severe airway obstruction.^{41,42} IFNγ limits T_H2 cell activation and airway inflammation. A transcription factor, T-bet, is required to induce T_H1 cells and for T_H1 cells to

produce IFN γ . T-bet was undetectable in lymphocytes from the bronchi of allergic asthmatic subjects but was present in bronchial lymphocytes from the control group. In Tbet $^{-/-}$ mice, the lack of the T $_H$ 1 response facilitates the infiltration with eosinophils and lymphocytes and airway remodeling typical of asthma, associated with an increase in T $_H$ 2 cytokine levels.⁴³ In our study, the association of having few symptoms, mild intraepithelial inflammatory infiltration, and high levels of IFN γ in BAL samples strongly supports the hypothesis that IFN γ is involved in modulating the local inflammatory response.

In conclusion, this study confirms heterogeneity of difficult asthma in children. Despite high doses of inhaled steroids, both groups of children displayed persistent T $_H$ 2-type inflammation. In symptomatic children, this T $_H$ 2-type inflammation was associated with the presence of activated intraepithelial eosinophils and neutrophils, whereas in paucisymptomatic children, it was associated with high levels of IFN γ . Longitudinal studies are required to determine whether intraepithelial inflammation and a low T $_H$ 1/T $_H$ 2 cytokine ratio in BAL samples are associated with an acceleration in the decline of FEV $_1$.

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