

Airway Remodeling and Inflammation in Symptomatic Infants with Reversible Airflow Obstruction

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Rationale: We hypothesized that the epithelial reticular basement membrane (RBM) thickening and eosinophilic inflammation characteristic of asthma would be present in symptomatic infants with reversible airflow obstruction. **Methods:** RBM thickness and numbers of inflammatory cells were determined in ultrathin sections of endobronchial biopsies obtained from 53 infants during clinical bronchoscopy for severe wheeze and/or cough. Group A: 16 infants with a median age of 12 months (range 3.4–26 months), with decreased specific airway conductance (sGaw) and bronchodilator reversibility; Group B: 22 infants with a median age of 12.4 months (5.1–25.9 months), with decreased sGaw but without bronchodilator reversibility; and Group C: 15 infants with a median age of 11.5 months (3.4–24.3 months) with normal sGaw. Additional comparisons were made with the following groups. Group D: 17 children, median age 10.3 years (6–16 years), with difficult asthma; Group E: 10 pediatric control subjects without asthma, median age 10 years (6–16 years); and Group F: nine adult normal, healthy control subjects, median age 27 years (21–42 years). **Main Results:** There were no significant differences in RBM thickness or inflammatory cell number between the infant groups. RBM thickness was similar in the infants and Groups E and F. However, the RBM in all infant groups (Group A: median 4.3 μm [range 2.8–9.2 μm]; Group B: median 4.15 μm [range 2.7–5.8 μm]; Group C: median 3.8 μm [range 2.7–5.5 μm]) was significantly less thick than that in the older children with asthma (Group D: median 8.3 μm [range 5.3–12.7 μm]; $p < 0.001$). **Conclusion:** RBM thickening and the eosinophilic inflammation characteristic of asthma in older children and adults are not present in symptomatic infants with reversible airflow obstruction, even in the presence of atopy.

Keywords: asthma; inflammation; pathology; pediatric; remodeling

Asthma may present at any age but its incidence is highest in childhood (1). Many children outgrow their preschool wheeze, but in some, symptoms persist and they go on to develop asthma. We cannot yet discriminate with certainty the future individual with asthma from the individual with transient wheeze (2–5). When it is safe and ethically permissible, direct examination of bronchial tissue may allow early identification of asthma and the introduction of preventative therapy. It may also discriminate

those who do not require inhaled corticosteroids, with their attendant risks (6).

Structural changes of the airway wall, referred to as remodeling, likely play an important role in the pathophysiology of asthma (7, 8). Thickening of the epithelial reticular basement membrane (RBM) is a characteristic feature of the remodeling process in asthma in adults, which can be determined relatively easily by examination of bronchial biopsies (9, 10). Biopsy studies of children with asthma demonstrate that the RBM is already thickened (11–14) and maximally so between the ages of 6 and 16 years (11). However, as asthma develops, it is not known exactly when RBM thickening begins (15). Moreover, biopsy studies in school-aged children with asthma have shown that there are inconsistent relationships between RBM thickening, inflammation, symptoms, and variable airflow obstruction (12, 13, 16).

There have been no biopsy studies in infants. However, in bronchoalveolar lavage, an overall increase in inflammation has been reported in infants and preschool children with wheeze (17, 18), but interestingly, eosinophils were rarely observed (18, 19).

An appreciation of the pathology of early infant wheeze may be invaluable in predicting future asthma (20). In the present study, we have been afforded a unique opportunity to investigate the airway pathology of groups of infants with recurrent symptoms, in whom bronchoscopy had already been indicated for their clinical evaluation. We have tested the hypothesis that RBM thickening and eosinophilic inflammation are already present when asthma often begins, in infants younger than 2 years with severe symptoms and reversible airflow obstruction. We believed it reasonable to consider as potentially asthmatic the group of infants with recurrent wheeze and/or cough together with decreased specific airway conductance (sGaw) and evidence of bronchodilator reversibility (BDR). We have compared our results in the symptomatic infant group with BDR with two other symptomatic infant groups, one without reversibility and another with normal lung function. To take account of the effects of normal development, disease, and age, we have also included for our quantitative comparison of RBM thickness, biopsies obtained previously from older children with and without asthma, and a further adult normal, healthy control population (11, 21).

METHODS

Full details of the methods are described in the online supplement.

Subjects

Infants aged 3 months to 2 years. Full-term infants (> 37 weeks gestation) who were symptomatic for at least 4 weeks were included. All had been referred to a tertiary center for investigation of recurrent respiratory symptoms, including dyspnea, cough, and wheeze. As part of their clinical assessment, they underwent lung function tests and bronchoscopy. Functional residual capacity (FRC), sGaw, and BDR were measured by total body plethysmography (22). It has been routine clinical practice at the Hospital for Children and Adolescents and Skin and

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Allergy Hospital, University of Helsinki, to evaluate infants with either chronic or recurrent respiratory difficulties using rigid bronchoscopy (23, 24). Bronchoscopy was performed to exclude structural airway abnormalities, such as subglottic stenosis, laryngo-, tracheo-, or bronchomalacia, and other diagnoses, such as foreign body inhalation or mucus plugging. If a structural airway abnormality was identified, patients were excluded from the present study. Bronchoscopy with endobronchial biopsy was performed under general anesthesia, with a 3.5-mm rigid bronchoscope, using biopsy forceps (No. 10378L; Karl Storz, GmbH and Co., Tuttingen, Germany). Up to two endobronchial biopsies were taken from the main carina.

Although all bronchoscopies were clinically indicated, endobronchial biopsy was performed for research. The study was approved by the local ethics committee, and written, informed consent was obtained from parents.

Patients were excluded if they had used corticosteroids within 8 weeks of their lung function visit. Other methodologic details and exclusion criteria are described in the online supplement. Infants were divided into three groups according to lung function: Group A: decreased sGaw with BDR; Group B: decreased sGaw without BDR; and Group C: normal sGaw.

Children with difficult asthma, children without asthma, and adult control subjects. There were 17 children with difficult asthma (16 atopic), 10 children without asthma (2 atopic), and 9 healthy, nonatopic, nonsmoking adult control subjects. Clinical details, bronchoscopy, and endobronchial biopsy have been described previously for these subjects (11, 21). For these groups only, biopsy tissue collected in previous studies was reexamined. All counts and data presented in the present study were, however, the result of a new evaluation and quantification by a single observer (S.S.).

Atopy

Atopy was defined by a positive skin-prick test to food or aeroallergens.

Biopsy Processing, Quantification, and Statistical Analyses

Biopsies were processed, and plastic sections (1 μ m) stained with toluidine blue (11). RBM thickness was measured by light microscopy in coded sections using computer-aided image analysis (25). Intraobserver repeatability and within- and between-biopsy variability were determined (26). The proportion of each biopsy occupied by key structures was assessed to see whether biopsies obtained by two different techniques (rigid and flexible bronchoscopy) were comparable. Ultrathin sections containing areas of epithelium, RBM, and subepithelium were cut for analysis of inflammatory cells (27). The numbers of subepithelial inflammatory cells (eosinophils, neutrophils, mast cells, plasma cells, and lymphomononuclear cells) and fibroblasts, identified by their ultrastructure (27), were assessed in ultrathin sections of infant biopsies using transmission electron microscopy. Nonparametric tests were applied to test for intergroup differences.

RESULTS

Subjects

Sixty-one infants met the inclusion criteria for the present study: 18 in Group A, 25 in Group B, and 18 in Group C. Only one had received steroids within the defined period (*see later*). Biopsies were suitable for RBM measurement from all but eight patients, leaving for analysis 53 full-term infants (mean gestation 39.5 weeks): 16 in Group A (8 were atopic), 22 in Group B (7 were atopic), and 15 in Group C (4 were atopic). The demography and lung function data of the included infants is shown in Table 1. By

TABLE 1. DEMOGRAPHY AND LUNG FUNCTION DATA OF INFANTS

	Group A	Group B	Group C	All
No. subjects	16	22	15	53
Age, mo				
Median	11.7	12.4	11.5	12.1
Range	3.8–23.3	5.1–25.9	3.4–24.3	3.4–25.9
Sex, no. male	8 (50%)	18 (82%)	11 (73%)	37 (70%)
Atopy (SPT+)	8 (50%)	7 (32%)	4 (27%)	19 (36%)
Parental asthma	8 (50%)	10 (45%)	3 (20%)	21 (40%)
Parental smoking	5 (31%)	6 (27%)	5 (33%)	16 (30%)
Symptoms				
Wheeze ever	13 (81%)	15 (68%)	8 (53%)	36 (68%)
Wheeze frequently	9 (56%)	10 (45%)	4 (27%)	23 (43%)
Cough ever	8 (50%)	14 (64%)	12 (80%)	34 (64%)
Wheeze and/or cough between colds	14 (88%)	16 (73%)	13 (87%)	43 (81%)
Age at onset of symptoms, mo				
Median	4.5	5.0	2.0	5.0
Range	0–16	0–14	0–13.5	0–16
Duration of symptoms, % of age				
Median	60	50	80	60
Range	22–100	27–100	19–100	19–100
FRC, ml				
Mean	296	313	292	296
Range	198–416	199–562	197–358	197–562
FRC, z-scores				
Mean	0.6	0.7	−0.1	0.5
Range	−0.8–3.2	−0.6–3.4	−0.9–0.8	−0.9–3.4
sGaw, kPaL ^{−1} s ^{−1}				
Mean	1.4	1.4	2.7	1.8
Range	0.6–2.3	0.7–2.3	2.0–3.2	0.6–3.2
sGaw, z-scores				
Mean	−3.6	−3.5	−0.2	−2.6
Range	−5.5 to −1.8	−5.2 to −1.8	−1.6–1.1	−5.5–1.1
Change in sGaw, %				
Mean	46	6	−4	15
Range	30–66	−34–26	−37–25	−37–66

Definition of abbreviation: FRC = functional residual capacity; sGaw = specific airway conductance; SPT = skin-prick test.

definition, the infants in Groups A and B had significantly lower baseline sGaw than the infants in Group C. Infants in Group A had significantly greater BDR than the infants in Groups B and C. The infants in Groups A and B had a higher FRC than the infants in Group C (Table 1). Fewer biopsies were suitable for analysis of inflammatory cells: 13 in Group A (5 atopic), 17 in Group B (3 atopic), and 11 in Group C (1 atopic). Those unsuitable for analysis were equally represented in the three groups.

Bronchoscopy

No complications were associated with bronchoscopy and endobronchial biopsy. A total of 92 biopsies were obtained from 61 infants. Of these, at least one biopsy was suitable for assessment of RBM in 53 (82.8%) subjects and inflammation in 41 (67.2%) subjects. There were six patients from whom two biopsies were evaluable. The area of the biopsies varied from 0.12 to 1.2 mm². Surface epithelium, RBM, and subepithelial tissue were present in all evaluable biopsies (Figure 1); bronchial smooth muscle was present in 50% and submucosal glands were present in 35% of biopsy specimens.

Variability

The intraobserver repeatabilities as percent coefficient of variation (%CV) for RBM thickness in three different biopsies were 2.3, 3.2, and 7.2%, giving a mean of 4.2%. Within a single biopsy, the between-section CV for four different sections was 1.9%. The average difference for the between-biopsy variability in five of six patients ranged from 0 to 1 μ m; that for the sixth patient was 2.4 μ m. The intraobserver %CV for total inflammatory cells in three different biopsies ranged from 7.8 to 15%. The between-section CV for four sections from the same biopsy ranged from 2 to 15%.

RBM Thickness

There were no significant differences in RBM thickness between the three infant groups: (Group A median 4.3 [range 2.8–9.2] vs. Group B median 4.15 [range 2.7–5.8] vs. Group C median 3.8 [range 2.7–5.5] μ m). RBM thickening in each of the infant groups was significantly less than in the older children with established asthma: Group D median 8.3 μ m (range 5.3–12.7;

$p < 0.001$ for each) and similar to RBM thickness in the children without asthma (Group E) and the adult healthy control subjects (Group F; Figure 2).

There were no significant differences in RBM thickness between patients with atopy in the three infant groups: Group A median 4.3 (range 2.8–5.7) vs. Group B median 3.3 (range 2.7–5.1) vs. Group C median 4.7 (range 3.8–5.3) μ m. When all infants were considered together (i.e., Groups A, B, and C), there was no significant difference in RBM thickness between infants with and without atopy (Figure 3). Nor was RBM thickness significantly different when the infants with atopy with reduced sGaw and BDR were compared with infants without atopy with normal lung function (Group A patients with atopy, median 4.3 [range 2.8–5.7], vs. Group C patients without atopy, median 3.6 [range 2.7–5.5] μ m). The duration of symptoms was not associated with RBM thickness (Pearson correlation: Group A, $r = 0.095$, $p = 0.73$; Group B, $r = 0.309$, $p = 0.16$; and Group C, $r = 0.085$, $p = 0.76$).

Inflammation

The median (range) areas of tissue assessed for each patient were as follows: Group A = 0.29 (0.06–0.44) mm², Group B = 0.32 (0.16–0.7) mm², and Group C = 0.13 (0.06–0.36) mm². There were no significant differences between groups regarding the total numbers of inflammatory cells. Eosinophils were infrequent in all sections, accounting for an average of 0.1% of the total inflammatory cells, with no significant group differences. Similarly, no between-group differences were seen regarding the numbers of neutrophils, mast cells, or plasma cells (Table 2). The median ratio of inflammatory cells to fibroblasts was 1.5 for Group A, 1.87 for Group B, and 1.15 for Group C.

One subject in Group A, a 10-month-old boy, did have a thickened RBM at this early time point (see Figure 2). His RBM thickness was 9.2 μ m and well into the asthma range. He also had the highest eosinophil (0.35/0.1 mm²), neutrophil (0.45/0.1 mm²), and mast cell (2.21/0.1 mm²) count of Group A. Eosinophils accounted for 1.16% of the total inflammatory cells in his biopsy, approximately 10-fold greater than the other infants. His wheeze had begun at 4 months and persisted for 6 months. Seven days after plethysmography, he had an exacerbation. Corticosteroids were administered for 1.5 days, after which he underwent bronchoscopy and biopsy procedures. His parents did not have

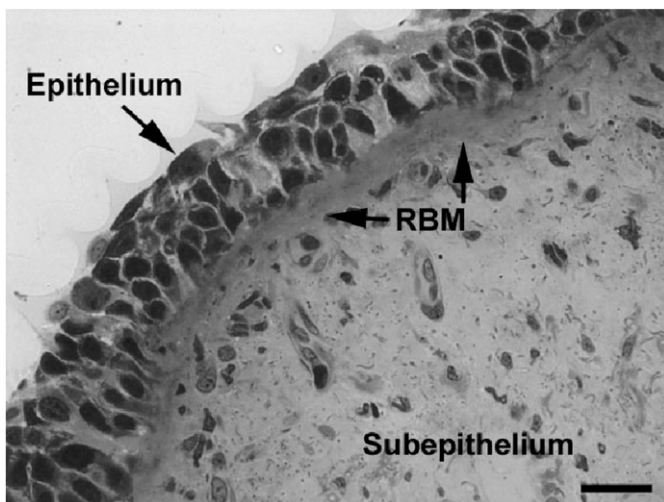


Figure 1. Endobronchial biopsy from the infant with the thickest reticular basement membrane (RBM; arrows) adjacent to squamoid epithelium (stained with toluidine blue; magnification $\times 400$). Scale bar represents 20 μ m.

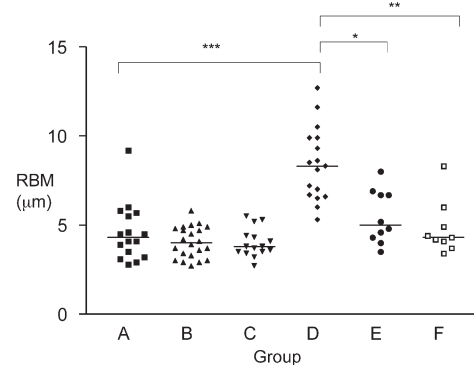


Figure 2. RBM thickness in symptomatic infants with bronchodilator reversibility (Group A), infants without bronchodilator reversibility (Group B), and infants with normal lung function (Group C), compared with children (age 6–16 years) with difficult asthma (Group D), children without asthma (age 6–16 years; Group E), and adult control subjects (Group F). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Groups A, B, C vs. D).

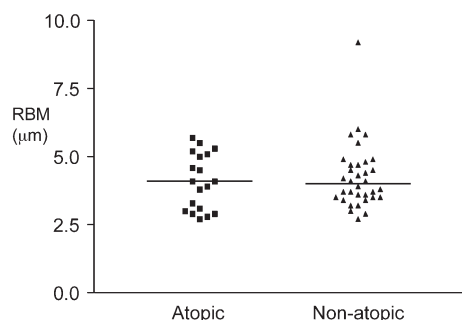


Figure 3. RBM thickness in all infants with atopy compared with infants without atopy. No significant difference between groups.

asthma, and although he was initially not found to be atopic, he subsequently developed positive skin-prick tests to dog and cat.

DISCUSSION

Until now, there has been no pathologic study of symptomatic infants: the youngest patients biopsied previously had been 3 years old (12). We demonstrate here that, in infants undergoing a clinically indicated bronchoscopy (in this particular hospital, rigid bronchoscopy is the usual practice), the additional procedure of endobronchial biopsy for the purpose of research is safe, providing the procedure is performed at a tertiary center by experienced personnel. Our experience is in agreement with previous reports of the safety of performing clinical flexible bronchoscopy with biopsy in preschool children (28, 29). The results of our examination of the bronchial mucosa of symptomatic infants, some with reversible airflow obstruction, and considered by us to be potentially asthmatic, do not support the hypothesis that RBM thickening and eosinophilic inflammation are present in this group at a time when asthma often begins.

The infants participating in our study had respiratory symptoms, on average, for 6 months. Wheeze was their primary symptom, and we excluded other diseases, including reflux and aspiration, foreign body inhalation, anatomic or cardiac abnormalities, cystic fibrosis, and bronchopulmonary dysplasia. The likelihood of a clinical diagnosis of asthma in our Group A infants was strengthened by including tests of pulmonary function and reversibility. We acknowledge that the measurement and interpretation of pulmonary function testing in infancy are difficult. However, we used infant body plethysmography, enabling simultaneous measurement of airway conductance and FRC (30). After inhaled salbutamol, our Group A infants demonstrated an

increased sGaw of at least 30% from baseline, with the nominal increase exceeding 2 SDs of the baseline between-test variability after inhaled salbutamol. Group B infants did not demonstrate such a response. Therefore, we considered those infants with recurrent wheeze and/or cough and decreased sGaw with a bronchodilator response most likely to represent asthma. Yet, despite the functional differences between Groups A and B, neither the RBM thickening nor numbers of inflammatory cells discriminated between them.

RBM Thickening

Thickening of the RBM is considered characteristic of asthma in adults and older children. Yet, by comparison with the older children with asthma, such thickening was not apparent in our group of symptomatic infants with reversible airflow obstruction. Thus, we have demonstrated for the first time that the symptoms, airflow obstruction, and reversibility characteristic of asthma in older children and adults can develop independently of RBM thickening in infants. RBM thickening in our infants was also not associated with atopic status, a finding recently reported in older atopic children without asthma (12).

RBM Thickening and Inflammation

There is debate about the relationship between inflammation and remodeling in infants and preschool children (15), and there are no previous biopsy assessments of RBM thickness or airway inflammation in infants (31). We did not find differences in the numbers of inflammatory cells between the symptomatic infant groups. Previous bronchoalveolar lavage studies of infants with wheeze have shown an increase in total inflammatory cells (17, 18), but this was in comparison with a nonwheezing control group. It could be argued that the presence of symptoms in all of our infants might explain the lack of a difference in the numbers of inflammatory cells between groups. However, despite symptoms, the numbers of inflammatory cells were low relative to the number of structural cells (i.e., fibroblasts).

Our biopsy results are in agreement with previous studies of lavage, demonstrating a sparsity of eosinophils in infants and preschool children with wheeze (18, 19). Considering the emerging view and recent proposal for a causal link between eosinophilia and remodeling (32), we propose that the absence of eosinophilic inflammation in our infants probably best explains the absence of RBM thickening. The paucity of eosinophilic inflammation in the biopsies of our infants might be taken to indicate that the use of inhaled corticosteroids in this group, even in the presence of severe symptoms, is unlikely to be clinically beneficial.

There are potential criticisms of our study. Only infants with

TABLE 2. COUNTS OF INFLAMMATORY CELLS AND FIBROBLASTS IN ULTRATHIN SECTIONS OF THE THREE INFANT GROUPS

	Group A	Group B	Group C	p Value
No. patients with evaluable sections	13	17	11	
Total inflammatory cells	14.2 (6.5–31.5)	22.1 (6–76.2)	17.1 (9.5–126.9)	NS
Lymphomononuclear cells	4.5 (1.7–18)	10.7 (1–60.7)	9.7 (2.4–104.5)	NS
Eosinophils	0 (0–0.35)	0 (0–0.3)	0 (0–0.2)	NS
Neutrophils	0.06 (0–0.45)	0.09 (0–0.9)	0.06 (0–0.71)	NS
Mast cells	0.46 (0–2.21)	0.53 (0.23–0.95)	0.28 (0–0.98)	NS
Plasma cells	2.4 (0.2–13.8)	1.7 (0.18–8.7)	1.5 (0–2.6)	NS
Difficult-to-classify inflammatory cells	5.2 (2.6–8.9)	6.2 (1.9–20.7)	6.0 (3.2–20.9)	NS
Fibroblasts	12.1 (7.7–23.9)	11.8 (6.8–18.6)	11.4 (5.2–14.5)	NS

Definition of abbreviation: NS = not significant.
Values are shown as median (range)/0.1 mm².

severe, prolonged, or atypical symptoms underwent bronchoscopy. We acknowledge that our patients differed from most infants with relatively mild wheeze and/or cough, who would not normally undergo bronchoscopy. We also accept that a potential criticism is the absence, because of ethical considerations, of a healthy, age-matched infant control group. However, in young children, bronchoscopy cannot be performed solely for research purposes (33). Accordingly, the "control" infants in our study only underwent bronchoscopy as part of their clinical evaluation for troublesome symptoms. Although they had respiratory symptoms, typically cough, their measures of lung function were within the normal range and thus presented us with a functional control for comparison. The absence of an asymptomatic control infant group without asthma for a baseline comparison allows us only to conclude that there was no difference in RBM thickness between the infants, grouped according to their lung function, and that the RBM was not as thick as that in older schoolchildren and adults with established asthma (11, 25). However, the RBM thickness of infant control subjects was the same as that of the normal, healthy adult control subjects, indicating that the RBM of the infants of Group C had reached its normal maximum value regarding normal development.

Some of our infants may also have developed their recurrent wheeze as a result of an episode of acute bronchiolitis, and we would not expect this phenotype to have the same pathology as asthma. But only two of our infants had previously been hospitalized with acute bronchiolitis (respiratory syncytial virus was the causal organism in one). Also, not all of the infants had frequent wheeze; however, there was no difference in RBM thickening or inflammatory cell counts when only the patients with persistent wheeze from Group A were compared with the control subjects.

Yet, we would consider it reasonable to predict that the 10-month-old boy with persistent wheeze and airways reversibility, who had a thickened RBM (of 9.2 μm , well into the asthma range) and the highest number and proportion of eosinophils and mast cells, will develop asthma in later childhood. Although the short period of corticosteroid therapy received by this patient before bronchoscopy may have contributed to his neutrophilia (34), it would not explain the remodeling and rise in the proportion of other inflammatory cells; rather, the numbers of eosinophils and mast cells would have been expected to be lower. We plan to follow up each of our clinically and histologically well-characterized infants to see who, if any, develop persistent wheeze and eventual asthma at school age.

In conclusion, examination of the bronchial mucosa of infants younger than 2 years demonstrates that recurrent wheeze and/or cough, even in the presence of reversible airflow obstruction, can develop independently of eosinophilic inflammation and RBM thickening, the latter an established characteristic feature of remodeling in both older children and adults with asthma.

Conflict of Interest Statement: S.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.S.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.P.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.V.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.N.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.J.M. does not have a financial relationship with a commercial entity that has

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**Airway remodelling and inflammation in symptomatic infants with
reversible airflow obstruction**

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Online Data Supplement

Methods

Subjects

Infants aged 0-2 years

Full-term infants (>37/40 gestation at birth) symptomatic for at least 4 weeks were included. All patients had been referred to The Skin and Allergy Hospital and the Hospital for Children and Adolescents, Helsinki, tertiary paediatric centres, for further investigation of chronic respiratory symptoms including dyspnoea, cough and wheeze. After assessment at the centre, it was decided, on clinical grounds, that the patients needed further investigations, including lung function tests and bronchoscopy, in order to guide future management. It has been routine clinical practice at the Hospital for Children and Adolescents and Skin and Allergy Hospital, University of Helsinki to evaluate infants with either chronic or recurrent respiratory difficulties by rigid bronchoscopy.^{E1,E2} Bronchoscopy was performed to exclude structural airway abnormalities such as subglottic stenosis, laryngo- tracheo- or bronchomalacia, and other diagnoses such as foreign body inhalation or mucus plugging. If a structural airway abnormality was identified, patients were excluded from the present study. None of the patients had received corticosteroids within 8 weeks of their lung function. Subsequently, one patient received oral steroids 2 days prior to bronchoscopy. Exclusion criteria were: non-caucasian ethnicity; prematurity (<36/40) or small for gestational age (birth weight < -2 SD); height at the time of investigation < -2.5 SD; respiratory distress syndrome; bronchopulmonary dysplasia; respiratory infection in the 14 days preceding lung function; severe atopic dermatitis (>20% of area at the first visit); obvious

bronchomalacia or other structural defect; specific airways conductance (sGaw) <35% of predicted combined with dyspnoea at rest.^{E3} Infants were divided into 3 groups according to their lung function: A: decreased sGaw with bronchodilator reversibility (BDR); B: decreased sGaw without BDR; C: normal sGaw. A chest x-ray was obtained to look for structural abnormalities or atelectasis. Infants underwent bronchoscopy within a median of 3 weeks from plethysmography.

Children with difficult asthma, non-asthmatic children, and adult controls

There were 17 children with difficult asthma and 10 non-asthmatic children, as previously described,^{E4} and 9 normal, healthy, non-smoking adult control subjects as described previously.^{E5} The children with difficult asthma had persistent symptoms requiring rescue bronchodilator therapy > 3 days per week, despite > 1600 micrograms per day of inhaled budesonide (or equivalent), and long acting β_2 -agonists, and/or regular oral steroids. There were also 10 children without asthma undergoing bronchoscopy for other respiratory indications.^{E4} Biopsies for these groups had been collected in previous studies, but all data presented in the present study were the result of re-evaluation and quantification by a single observer (SS).

Informed Consent

All bronchoscopies were clinically indicated. However, endobronchial biopsy (EB) was performed for the purposes of research. The study was approved by the Ethics Committee at the Helsinki University Central Hospital, and written,

informed consent for the bronchoscopy itself, and the additional research biopsies, was obtained from parents prior to EB.

Atopy

Atopy was defined by a positive skin prick test (Solu-Prick SQ, ALK-Abello, Horsholm, Denmark) for one of the test allergens (birch, timothy, cat, dog, *Dermatophagoides pteronyssinus*, latex, egg, cow- milk).

Lung Function

Functional residual capacity (FRC) and specific airways conductance (sGaw) were measured with an infant body plethysmograph (BabyBody, Jaeger GmbH, Wurtzburg, Germany).^{E6} Paired sets of measurements were performed to assess mean baseline FRC and sGaw, and their individual between-test variability. FRC higher than that of the 95th centile (z-score > +1.65) and sGaw lower than that of the 5th centile (z-score < -1.65) of the reference range were considered abnormal.^{E3} BDR was assessed by repeating the measurements 15 minutes after inhalation of salbutamol 0.6 mg (Ventolin®, Glaxo, United Kingdom) delivered with a metallic spacer (Nebunette®, Astra, Sweden). BDR was considered significant if an increase in sGaw of at least 30% was observed and if the nominal increase exceeded two standard deviations of the baseline between-test variability.^{E6}

Bronchoscopy

Bronchoscopy with EB was performed in the infants under general anaesthesia. All infants underwent bronchoscopy with a size 3.5mm rigid

bronchoscope (Karl Storz GMBH & Co, Tuttingen, Germany). Up to two endobronchial biopsies were taken from the main carina using biopsy forceps (No 10378L, Karl Storz GMBH & Co, Tuttingen, Germany). In the older children and adults, flexible bronchoscopy was performed, endobronchial biopsies being taken from the sub-carinae of the right lower lobe. Details of bronchoscopy and EB in the older children and adults have already been described.^{E4,E5}

Processing of biopsies

Biopsies were fixed in 2.5% glutaraldehyde in 0.05-M sodium cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide (in the same buffer), and dehydrated and embedded in epoxy resin (Araldite). Plastic sections (1 μ m thick) were cut and stained with alkaline toluidine blue. We used this processing and staining technique as it was the same as that used in the school children and adults. An area of the section that contained epithelium, reticular basement membrane and subepithelium, was selected. Ultra-thin sections (70nm) were cut, placed on high transmission, 200 mesh thin-bar copper grids and stained with uranyl acetate and lead citrate.

Quantification of RBM

Toluidine blue sections were selected for measurement of RBM thickness providing that the epithelium, RBM and the submucosa could be identified, and that the length of RBM was ≥ 1 mm.^{E7} Coded sections, from all age groups, were examined by light microscopy by one observer (SS). RBM thickness was determined using computer-aided image analysis (NIH image

1.55; National Institute of Health, Bethesda, MD) by making 40 measurements at 20 μm intervals,^{E7} and expressed as the geometric mean of the 40 measurements. Intra-observer repeatability was assessed by measuring the same section 4 times, the result being expressed as percentage coefficient of variation (% CV). Similarly, the within-biopsy variability of 4 different sections of a single biopsy was expressed as %CV. The between-biopsy variability for a single subject was determined by measuring a single section from 2 biopsies from the same subject, and was calculated using the Bland-Altman plot.^{E8} As the biopsies from the infants were taken from the main carina and obtained by rigid bronchoscopy, while those from the older children and adults were sub-carinal and obtained by flexible bronchoscopy, we assessed the proportion of each biopsy occupied by key structures, in order to see whether biopsies obtained by 2 different techniques were comparable. By morphometry, using an eye-piece graticule, at a magnification of x200, counts were performed of the proportions of the biopsy occupied by epithelium, reticular basement membrane, subepithelium, smooth muscle and submucosal glands. Apart from the subepithelium, the proportions in the infant biopsies were similar to the proportions found in the adults and school children (table E1).

Quantification of inflammatory cells

Inflammatory cells were counted in ultra-thin sections from the infant biopsies using the Hitachi H7000 (Nissei Sanyo, Tokyo, Japan) transmission electron microscope (TEM). All nucleated cells in the subepithelium that were outside vessels, gland and smooth muscle were counted. Eosinophils, neutrophils,

mast cells, plasma cells and fibroblasts were identified by their ultra-structure in ultra-thin sections of infant biopsies using transmission electron microscopy. These cells were identified using previously defined ultrastructural characteristics.^{E9} Lymphocytes were defined as mononuclear cells with a high nucleus:cytoplasm ratio, containing up to 3 mitochondria in the cytoplasm. There was a further category of mononuclear cells that had a smaller nucleus:cytoplasm ratio and more than 3 mitochondria in the cytoplasm, these were classed as lymphomononuclear cells. Data presented in table 2 of the manuscript represent the sum of lymphocytes and lymphomononuclear cells in the same category. Finally, cells that did not fit into any of the defined groups of inflammatory cells, which often had features of more than one cell type, were termed “difficult to classify inflammatory cells”. Counts of all inflammatory cells were added together to give a total inflammatory cell count for each section. In order to appreciate the proportions of inflammatory cells in relation to structural cells, in the sections, fibroblasts were also identified^{E9} and counted. Cell counts were expressed per 0.1mm^2 of the section.

Statistical Analyses

The data for analysis of RBM thickness and inflammatory cells were derived from one biopsy from each patient. Nonparametric tests were applied to test for inter-group differences. Comparison between all groups was made using the Kruskal-Wallis test, followed by a Mann-Whitney U test, if a significant difference ($p < 0.05$) was found.^{E8} The Bonferroni correction was introduced

for multiple comparisons.^{E8} Data were analysed using Statistical Package for the Social Sciences (SPSS version 11.5).

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Table E1. Comparison of structural composition of endobronchial biopsies taken by rigid and flexible bronchoscopy

Proportion of biopsy occupied*	Rigid Bronchoscopy	Flexible Bronchoscopy		p value
	Infants	Children	Adults	
Epithelium	10.3 (4.5-28.2)	11.1 (1.6-27.8)	15 (0.8-33)	NS
RBM	8.35 (1.2-15.0)	11.1 (7.1-22.4)	9.6 (1.2-25.1)	NS
Subepithelium†	73.2 (56.0-87.2)	49.4 (26.5-73.6)	49.2 (36.6-65.7)	0.003
Smooth muscle	4.8 (0-15.6)	11.8 (0-48)	13.7 (0-34.3)	NS
Submucosal mucus secreting gland	0 (0-16.9)	0 (0-40)	0 (0-23)	NS

* median (range) %

† significantly higher in infants compared to children and adults