

Peripheral blood lymphocyte subpopulations in schoolchildren born very preterm

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Abstract

Aim—To investigate whether lymphocytes or serum inflammatory markers are associated with obstructive lung disease and bronchial lability in schoolchildren born very preterm.

Method—Lymphocyte subsets were studied in the peripheral venous blood of 29 such children (median age 8.8 years). Serum eosinophil cationic protein (ECP) and myeloperoxidase (MPO) concentrations and the association between them, lymphocyte subsets, and lung function were studied. Fourteen healthy children born at term, median age 9.1 years, served as controls. T lymphocytes (CD3), T lymphocyte subpopulations (CD4 and CD8), B lymphocytes (CD19), natural killer cells (CD16+56) and activation markers of T and B lymphocytes (CD23 and CD25) were determined using flow cytometry. Lung function was measured in all children both in the clinic and at home (Vitalograph Data Storage Spirometer).

Results—Compared with the controls, schoolchildren born very preterm had significantly lower CD4⁺ T cell percentages and CD4:CD8 ratios ($p < 0.05$ for both), whereas natural killer cell percentages and serum ECP values were significantly higher ($p < 0.05$). The very preterm schoolchildren had significantly lower spirometric values than the control group ($p < 0.05$)—except forced vital capacity. When all the subjects were considered together, a weak, but significant, negative association was observed between the bronchial responsiveness in peak expiratory flow, after a β_2 agonist during home monitoring, and the CD4⁺ T cell percentage ($r = -0.45$; $p = 0.008$) and the CD4:CD8 ratio ($r = -0.50$; $p = 0.003$), indicating a relation between bronchial lability and imbalance of T cell subpopulations.

Conclusions—These results suggest that there is an inflammatory basis for lung function abnormalities in schoolchildren born very preterm.

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Keywords: very preterm; schoolchildren; T and B lymphocytes; bronchial lability

Very preterm infants with or without diagnosed chronic lung disease may have abnormal lung function¹ and increased respiratory morbidity during infancy.² The pulmonary function of these children is believed to improve rapidly during the first few years of life.³ However,

studies performed at school age have shown that bronchial obstruction, increased bronchial responsiveness, and bronchial lability are common in these children.^{4,5} Cellular and biochemical studies have shown neutrophilic inflammatory changes in the lungs of ventilated preterm infants as early as the first few days of life.⁶ Eosinophils might also have a role in the early inflammatory process in chronic lung disease and contribute to the lung injury.⁷

It is not known whether chronic inflammation has a role in the increased bronchial responsiveness and bronchial lability of schoolchildren born very preterm, as it does in asthma. The beneficial effect of glucocorticoids has been shown in ventilator dependent neonates⁶ and in some wheezy preterm infants.² At school age, inhaled glucocorticoids did not influence basic lung function or bronchial responsiveness in children born very preterm.⁸ However, some schoolchildren born very preterm with bronchial lability may benefit from this treatment (Hakulinen *et al*; abstract presented at the international conference of the American Thoracic Society in 1995).

Eosinophil mediated damage to the respiratory epithelium is a major pathogenetic mechanism in asthma.⁹ Activated eosinophils secrete several granule derived proteins—for example, eosinophil cationic protein (ECP),¹⁰ which is increased in asthma.^{9,11} Neutrophils are prominent in the airways in chronic obstructive pulmonary disease (COPD) in adults.¹¹ Activated neutrophils secrete several proteins including myeloperoxidase (MPO),¹⁰ which is increased in bronchoalveolar lavage fluid in COPD.¹¹ Numerous studies have shown that lymphocytes have an important role in the pathogenesis of both asthma^{12,13} and COPD.^{14,15}

This study aimed to investigate whether the obstructive lung disease and bronchial lability of schoolchildren born very preterm is associated with changes in peripheral blood, suggesting inflammatory lung disease.

Methods

In our previous cross sectional study¹⁶ pulmonary function was investigated in 40 schoolchildren born very preterm (birthweight < 1500 g and/or gestational age < 30 weeks) with lung immaturity. All had participated in randomised bicentre (University of California, San Diego, CA, USA, and University of Helsinki, Finland) trials of human surfactant in the Children's Hospital, University of Helsinki, Finland, between 1983 and 1987. Thirty four children (24 boys and 10 girls) participated in this

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follow up study at a median (range) age of 8.8 (7.4–11.7) years. The median (range) birth-weight of these children was 1090 (595–1730) g and the median gestational age 27.4 (24.9–29.9) weeks. The median (range) duration of mechanical ventilation and duration of supplemental oxygen treatment during the neonatal period were 5.5 (1–129) and 10 (1–512) days, respectively. Six children were dependent on supplementary oxygen at the age of 36 postconceptional weeks, which was used as the criterion of chronic lung disease.¹⁷ Twenty six had been treated with exogenous surfactant.

A group of 14 healthy schoolchildren of the same age (median (range) age 9.1 (5.8–13) years), born at term, were recruited as a control group. These children were attending school at the time of study and had neither respiratory symptoms nor symptoms of atopy. The study was approved by our institutional ethics committee. Informed consent was obtained from the parents.

All 34 children born very preterm and 14 control children made two visits to the outpatient department, University of Helsinki Central Hospital. They all underwent the following lung function and blood sample testing protocol. At the first hospital visit, a physical examination was performed, body height and weight were measured, and questionnaires focusing on respiratory symptoms during the preceding year were completed. Flow volume spirometry and a bronchodilation test were performed, and each child was trained to use a home spirometer. Lung function was monitored twice daily at home for four weeks. The effect of a β_2 agonist was measured using spirometry before and after terbutaline inhalation every morning and evening for the first two weeks of home monitoring. At the second visit, flow volume spirometry was performed, the data of the home spirometer were downloaded, and a blood sample was taken to determine serum ECP, MPO, and lymphocyte subsets.

Atopy was evaluated by skin tests or by any evidence of specific serum IgE (positive Phadiatop Combi test, Pharmacia Diagnostics, Uppsala, Sweden). Skin tests were performed using the skinprick technique, for eight common allergen extracts. A negative vehicle solution and a positive histamine hydrochloride (10 mg/ml) solution were used as controls. Atopy was defined as a wheal reaction of at least 3 mm in diameter in the absence of a response to the negative control solution.

Flow volume spirometry was carried out in the office with a pneumotachograph (Spirotrac IIIR, Vitalograph Ltd, Buckingham, UK). In accordance with the acceptability criteria of the American Thoracic Society, at least three technically correct forced expiratory curves were recorded during each measurement. The curve was considered to be reproducible if the highest forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) and the second highest FVC and FEV_1 of the acceptable curves differed by 5% or less (expressed as percentages of the highest observed FVC and FEV_1 , regardless of the curve on which they

occurred).¹⁸ The curve with the highest sum of FEV_1 and FVC values was selected for statistical analysis. The results of the lung function tests were expressed as percentages of the predicted values reported by Polgar and Promadhat.¹⁹ The following spirometric parameters were recorded: FVC, FEV_1 , peak expiratory flow, and forced expiratory flow at 50% of FVC (FEF_{50}). Spirometric parameters were measured in the clinic before and 15 minutes after inhalation of 0.5 mg of terbutaline from a dry powder inhaler (terbutaline sulfate 0.25 mg, Bricanyl Turbuhaler, Astra Draco AB, Lund, Sweden). Peak inspiratory flow through a Turbuhaler device was recorded during each terbutaline inhalation. Neither FEV_1 nor peak expiratory flow changes were recorded when peak inspiratory flow was insufficient—that is, <30 l/min.²⁰ The changes in FEV_1 and peak expiratory flow values after terbutaline were expressed as percentages of the values recorded before terbutaline inhalation.

Lung function was recorded at home every morning and evening for four weeks, using a Vitalograph Data Storage Spirometer (Vitalograph Ltd, Buckingham, UK) especially designed for long term recording and storage of lung function parameters. The device consists of a pneumotachograph and a computer capable of recording FVC, FEV_1 , peak expiratory flow and peak inspiratory flow values. Before use, the device was calibrated with a standard volume (variation within $\pm 1\%$). After home recordings the calibration was checked. The difference between these two calibration values (before and after home monitoring) was $\leq 5\%$. The test was repeated until the results of the two best curves met the criteria, a maximum of five times.¹⁸ When this was not achieved after five attempts, failure was recorded. These recordings were not used in the analyses. The Data Storage Spirometer stored the curve with the largest sum of FEV_1 and FVC in the built-in electronic diary. During the first two weeks, lung function was monitored before and 15 minutes after inhalation of terbutaline 0.25 mg twice daily every morning and evening and home monitoring was continued for four weeks. The data were then analysed. Compliance of inhalation treatment was monitored by measuring peak inspiratory flow.

Blood for ECP and MPO was taken in gel tubes (SST, Becton Dickinson, England) and was allowed to clot at room temperature for 60 (± 10) minutes. Serum was separated by centrifugation at $1350 \times g$ for 10 minutes and stored at $-20^\circ C$ until analysed for ECP²¹ and MPO,²² using a radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Blood eosinophils were counted using a haematological analyser (Advia 120, Bayer).

For flow cytometric analysis, peripheral venous blood samples were drawn in heparin. Thereafter, mononuclear leucocytes were isolated using Ficoll-Paque centrifugation at $400 \times g$ for 30 minutes. Subsequently, a mononuclear blood cell suspension at a cell concentration of $1 \times 10^6/ml$ in RPMI 1640, with

Table 1 Clinical characteristics of schoolchildren born very preterm and controls

Variable	Very preterm children N=34	Controls N=14	p Value
Gender (F/M)	10/24	9/5	0.09
Age (years)	8.8 (7.4–11.7)	9.1 (5.8–13)	0.73
Height (SD units)	-0.5 (-2.5–2)	-0.1 (-3–0.7)	0.96
Atopy	6/34 (18%)	3/14 (22%)	0.55
Parental smoking	11/34 (32%)	0/14 (0%)	0.03
FVC	96 (39–121)	100 (84–124)	0.31
FEV ₁	84 (39–105)	94 (79–118)	0.01
PEF	74 (38–91)	86 (73–111)	0.0008
FEF ₅₀	70 (35–110)	106 (75–145)	0.0001

Values are expressed as medians (range) or as numbers (%) in the given category. Lung function tests are expressed as percentages of the predicted values.

Table 2 Peripheral blood lymphocyte subsets in schoolchildren born very preterm and controls

Cell surface antigen	Very preterm children N=29	Controls N=14	p Value
CD3	67.9 (54.8–78.9)	71.3 (60.9–78.2)	0.15
CD4	38.4 (25.7–52.5)	42.2 (35.8–53.1)	0.03
CD8	28.8 (18.7–37.6)	25.3 (17.5–36.3)	0.18
CD4:CD8 ratio	1.4 (0.8–2.2)	1.8 (1.0–2.6)	0.03
CD19	11.8 (5.2–25.8)	9.8 (6.9–17.0)	0.18
CD23	10.6 (4.3–20.4)	7.7 (2.6–22.4)	0.66
CD19/CD23	9.3 (3.6–20.3)	5.8 (2.4–12.4)	0.06
CD16+CD56	11.5 (3.8–21.5)	5.5 (4.0–18.0)	0.03
CD4/CD25	8.5 (5–14)	9.1 (7.5–11.2) ³⁰	
CD8/CD25	3 (0–15)	2.4 (1.9–2.7) ³⁰	

Values of lymphocyte subsets are expressed as percentages of lymphocytes. Values are expressed as medians and ranges.

antibiotics, glutamine, and 5% fetal calf serum, was incubated at 4°C for 10 minutes with monoclonal antibodies against CD3, CD4, CD8, CD19, CD23, CD25, and CD16+56 (which are natural killer cells) (Beckton Dickinson Immunocytometry Systems, Mountain View, California) according to the manufacturer's instructions.²³ Flow cytometric analysis was then performed, using the same instrument settings in each analysis. The lymphocytes were finally gated to study the numbers of cell subsets within the gate. The numbers of cells positive for the surface markers are expressed as percentages of all the cells within the gate.

Bronchial obstruction was defined as at least two of the following spirometric parameters recorded in the clinic: FEV₁ < 80%, peak expiratory flow < 75%, or FEF₅₀ < 62% of the predicted values.¹⁹ A positive response to terbutaline was defined as an FEV₁ or peak expiratory increment of ≥ 15% after terbutaline in the clinic or at home,²⁴ and the numbers of these responses at home were recorded and used in the analysis. The numbers of diurnal peak expiratory flow variations of ≥ 20% during home monitoring were calculated as the differences between the morning and evening peak expiratory flow values, and were expressed as percentages of the higher peak expiratory flow value, and used in the analysis. Earlier studies have shown that a diurnal peak expiratory flow variation of ≥ 20% is a useful screening test for asthma and a good indicator of bronchial lability.²⁵

Results of lung function tests were analysed as percentages of the predicted values (Polgar and Promadhat).¹⁹ The data of the children born very preterm and the controls were compared using the Mann-Whitney or Fisher's exact tests. A Kruskal-Wallis non-parametric

test was used to determine the differences between the three study groups (preterm schoolchildren with and without bronchial obstruction and the controls). A p value of < 0.05 was considered to be significant. Spearman's rank correlation (r_s) was used to evaluate the association between lung function and the results of the blood tests overall and within the groups. Statistical analyses were performed using the Macintosh Stat View computer program.

Results

The schoolchildren born very preterm and the controls did not differ in age, height, or weight. Eighteen of the 34 (53%) children born preterm had had dyspnoeic symptoms at least once and/or had had a continuous cough for more than three weeks during the previous year. Three children had asthma diagnosed by a physician; two of these were receiving continuous disodium chromoglycate and one budesonide inhalation. The groups did not differ in individual atopies. Atopy was found in 18% of schoolchildren born very preterm and in 22% of the control group. The percentages exposed to parental smoking were 32% in the preterm children and 0% in the control group (p = 0.03) (table 1).

All the children performed a complete set of spirometric tests. Home monitoring was not acceptable in seven children (three preterm and four control children) because of technical problems with the device (four children) or non-compliance (three). For the 34 preterm children screened, the median (range) spirometric values in the clinic were: FVC 96 (39–121)%, FEV₁ 84 (39–105)%, peak expiratory flow 74 (38–91)%, and FEF₅₀ 70 (35–110)% expressed as percentages of the predicted values. In the control group the corresponding median (range) spirometric values were: FVC 100 (84–124)%, FEV₁ 94 (79–118)%, peak expiratory flow 86 (73–111)%, and FEF₅₀ 106 (75–145)% of the predicted values. The schoolchildren born very preterm had significantly lower FEV₁, peak expiratory flow, and FEF₅₀ values than the control group (p < 0.05) (table 1). Fifteen of the very preterm children (44%) and none of the controls had spirometric values, indicating bronchial obstruction.

In the bronchodilator test in the clinic, two of the children born very preterm had a significant response to terbutaline (Δ FEV₁ ≥ 15%). The median Δ FEV₁ was 2.6% (range -4.1 to 29%). During the two weeks of recording at home, an increment in peak expiratory flow of ≥ 15% after terbutaline was observed at least three times in 44% of very preterm children and in none of the control children. The median (range) of the number of positive bronchodilator tests at home was 3 (0–8) in the very preterm children and 0 (0–2) in the control children (p = 0.0006). Eight of the children born very preterm (24%) and none of the controls had a diurnal peak expiratory flow variation of ≥ 20% at least four times during four weeks of home monitoring. The median number (range) of abnormal peak expiratory

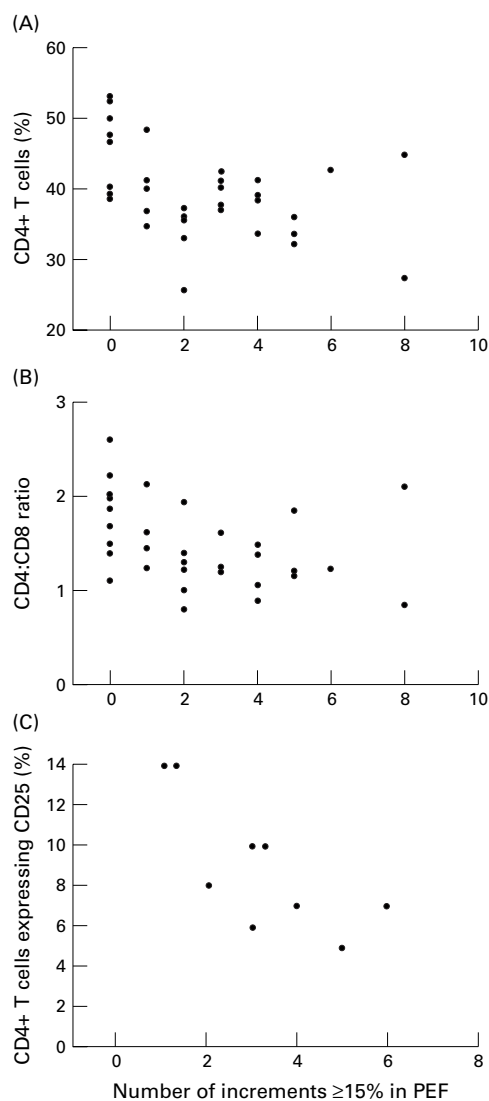


Figure 1 Relation between the number of increments of $\geq 15\%$ after terbutaline during home monitoring and the percentage of CD4⁺ T cells (A), CD4:CD8 ratio (B) and the percentage of CD4⁺ T cells expressing CD25 (C).

flow variations at home was 1 (0–9) in the very preterm children and 0 (0–1) in the control children ($p = 0.006$).

Flow cytometric measurements were obtained for all the control children and for 29 of the 34 children born preterm. Of these 29 very preterm, 14 had bronchial obstruction without an earlier diagnosis of asthma. The percentages of peripheral blood CD4⁺ and CD8⁺ T lymphocytes expressing the activation marker CD25 were determined in 20 children born very preterm. The percentage of CD4⁺ T lymphocytes and the CD4:CD8 ratio were both significantly lower in the children born very preterm, and the percentage of CD16+CD56

cells (natural killer cells) was significantly higher than in the controls ($p < 0.05$). The percentage of B cells expressing CD23 tended to be higher in the children born very preterm than in the healthy controls ($p = 0.06$). Otherwise, the percentages of lymphocyte subsets in the two groups were comparable (table 2).

In all the children significant negative associations were observed between the number of $\geq 15\%$ increments in peak expiratory flow values after β_2 agonist inhalation during home monitoring and the percentage of CD4⁺ T lymphocytes ($r = -0.45$, $p = 0.008$) and the CD4:CD8 ratio ($r = -0.50$, $p = 0.003$) (fig 1). In the very preterm schoolchildren with bronchial obstruction, the number of $\geq 15\%$ increments in peak expiratory flow values after β_2 agonist inhalation had a significant negative association with the percentage of CD4⁺ T cells expressing CD 25 ($r = -0.75$; $p = 0.03$) (fig 1). In all children the number of $\geq 15\%$ increments in peak expiratory flow values after β_2 agonist inhalation and the percentage of CD19⁺ lymphocytes expressing CD 23 tended to have an association ($r = 0.30$; $p = 0.08$). Neonatal variables, basic spirometric values, diurnal peak expiratory flow variation, parental smoking, and atopy were not significantly associated with the lymphocyte subpopulations.

The number of blood eosinophils and the ECP and MPO values were measured in all the children. Serum ECP values were significantly higher in the very preterm schoolchildren than in the controls ($p < 0.05$) (table 3). The number of blood eosinophils and the MPO values were similar in the two groups (table 3). The number of blood eosinophils was significantly associated with ECP values ($r = 0.56$; $p = 0.001$). Parental smoking and ECP values were not significantly positively associated. There were no significant associations between serum markers of inflammation and the other measured variables.

Discussion

Compared with the controls, the schoolchildren born very preterm with lung immaturity had significantly lower CD4⁺ T cell percentage and CD4:CD8 ratio, whereas the natural killer cell percentage and the ECP values were significantly higher. A weak but significant negative association was observed between the bronchial responsiveness in peak expiratory flow after β_2 agonist inhalation during home monitoring and the CD4⁺ T cell percentage and the CD4:CD8 ratio, suggesting a relation between bronchial lability and imbalance in T cell subpopulations. Our results suggest that the changes found in peripheral blood lymphocytes have a role in the pathogenesis of airway disease in schoolchildren born very preterm.

Numerous studies have suggested that lymphocytes are involved in the pathogenesis of asthma^{12 13} and also in COPD.^{14 15} In adults with mild to severe asthma, both activated T cells (activated CD4⁺ T lymphocytes, reflected by an increase in CD25 expression) and activated eosinophils have been observed in

Table 3 Numbers of blood eosinophils and serum ECP and MPO concentrations in schoolchildren born very preterm and controls

	Preterm children N=34	Controls N=14	p Value
Blood eosinophils ($10^9/l$)	0.25 (0.05–0.96)	0.15 (0.03–0.65)	0.3
Serum ECP ($\mu g/l$)	6.1 (1.8–50.6)	2.9 (1.8–10.2)	0.03
Serum MPO ($\mu g/l$)	238 (43–1065)	168 (127–320)	0.3

Values are expressed as medians and ranges.

bronchial biopsy specimens,^{9,13} in bronchoalveolar lavage (BAL) fluid, and in the peripheral blood.^{9,12,26} The degrees of T lymphocyte activation and eosinophilia were closely correlated with the severity of the asthma and the degree of bronchial responsiveness.^{9,12,13} The percentages of activated T cells decreased with inhaled glucocorticoid treatment.²⁶ These findings suggest that T cell activation and eosinophil recruitment represent a pathway leading to the development of airway narrowing, hyperresponsiveness, and symptoms of atopic asthma.^{9,12,13,26} The properties of peripheral blood cells in asthmatics closely resemble those of cells in the bronchial mucosa and BAL fluid, suggesting that peripheral blood could be used for immunological studies.²⁷ Childhood asthma, like asthma in adults, is associated with activation of T cells and increased expression of eosinophil active cytokines. Furthermore, glucocorticoid treatment results in clinical improvement and in a decrease of T cell activation.²⁷

The few studies evaluating the role of lymphocytes in smoking induced airflow limitation in COPD have shown a tissue predominance of CD8⁺ over CD4⁺ T cells,¹⁴ and a parallel increase in CD8⁺ lymphocytes in peripheral blood.¹⁵ These data support the view that the pattern of inflammatory cells in COPD is different from that in asthma. In COPD there is also eosinophil migration toward the airways, but the phenomenon is more pronounced in asthma.¹¹

The lung function abnormalities in schoolchildren born very preterm have been suggested as being due to mechanical factors resulting from an abnormal pattern of lung growth in infancy. Accordingly, increased bronchial responsiveness might be exaggerated by a structural airway narrowing.⁴ Persistent airway obstruction in chronic lung disease might be related to submucosal fibrosis, or hypertrophy, or hyperplasia of bronchial smooth muscles.²⁸ However, the findings of bronchial lability in these children^{4,5} may not be explained solely by these structural changes. We propose that inflammatory processes may contribute to bronchial lability.

In our study, the low CD4:CD8 ratio observed among children born very preterm was due to a low number of CD4⁺ T cells. These preterm schoolchildren also had significantly lower baseline spirometric values than the controls. Their baseline values were not, however, associated with a low CD4:CD8 ratio. Interestingly, the children who were responsive to a β_2 agonist during home monitoring had significantly lower percentages of CD4⁺ lymphocytes and lower CD4:CD8 ratios.

In contrast to asthma,²⁹ among the schoolchildren born very preterm with bronchial obstruction, the percentage of CD4⁺ T cells expressing CD25 was inversely associated with this bronchial responsiveness. The number of CD4⁺ T cells expressing CD25 was similar to the non-asthmatic controls of the earlier study.³⁰ Our findings suggest that bronchial lability is associated with alterations in the bal-

ance of T cell subsets and possibly with abnormal CD4⁺ T cell function. CD4⁺ T lymphocytes are important immune system regulators. A low CD4:CD8 ratio is a hallmark of intense, chronic immune responses, such as allograft rejection, graft versus host disease, and haemophilia.³¹ In asthma the CD4:CD8 ratio is constant and does not differ from that of non-asthmatic groups.³²

Recent observations by Amadori and coworkers³¹ suggest that the CD4:CD8 ratio is genetically controlled. It is therefore possible that, in infants born very preterm, the early inflammatory process leading to chronic lung disease is more easily triggered in those individuals who are genetically susceptible to a low CD4:CD8 ratio. Originally, chronic lung disease was observed primarily in preterm infants with severe respiratory distress syndrome who had been exposed to high oxygen concentrations and positive airway pressures. In recent years, however, a less severe form has frequently been observed in infants with mild or no initial respiratory distress syndrome. This change in the clinical pattern suggests that additional risk factors different from those associated with the severity of acute neonatal respiratory disease are responsible for the development of lung function abnormalities in susceptible infants.³³

The percentages of natural killer cells were higher in our children born very preterm than in the controls. In children born preterm, viral infections that stimulate proliferation of natural killer cells and recurrent respiratory illnesses are common during the first few years of life.² These children continue to have an increased risk of being admitted to hospital; the risk is similar in magnitude to that seen in infancy. Among the reasons for admission, respiratory illnesses predominate.³⁴

In the present study, ECP, but not MPO concentrations, in peripheral blood were significantly higher in schoolchildren born very preterm than in controls. Increased serum ECP is considered to be a sign of activation of eosinophils during the inflammatory process in the airways.⁹ A possible explanation for the increased blood ECP concentration is the high percentage of parental smoking. It has been shown that serum ECP values in young children are related to maternal smoking.³⁵ However, in our study parental smoking and ECP values did not have a significant positive association. Studies in healthy smokers have shown a considerable influence of cigarette smoking on lymphocyte subsets, especially on CD8⁺ T cells and CD19⁺ B cells.¹⁵ In our study parental smoking and lymphocyte subsets were not significantly associated.

Our results suggest that lung function abnormalities in schoolchildren born very preterm are related to immunological changes, including an imbalance in the CD4:CD8 ratio in the peripheral blood. These findings differ from those reported in asthma.

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