

Markers of inflammation and bronchial reactivity in children with asthma, exposed to animal dander in school dust

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Several studies have confirmed the presence of animal dander allergens in school dust but the effect of this indirect animal exposure on health has not been evaluated. In this study we investigated bronchial reactivity and markers of eosinophil activity and inflammation during two separate weeks of school in 10 children with mild asthma and a positive skin prick test to cat and dog. At the beginning and the end of the first week the children underwent bronchial challenges with methacholine, and at the beginning and the end of the second week they underwent nasal lavages (NAL) and induced sputum samplings. Blood and urine samples for analysis of inflammatory markers were obtained before and after both school weeks. Peak expiratory flow (PEF) and symptoms of asthma and allergy were recorded daily, and spirometry was performed on each visit. The exposure to animal dander allergens was estimated from dust samples obtained in the subjects' schools and homes. Bronchial sensitivity to methacholine increased in the week when this was measured. The proportion of eosinophils in peripheral blood, and urinary eosinophil protein X (EPX), decreased in both weeks. There was a trend towards an increase of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) in sputum in the week when these proteins were measured. The concentrations of cat (Fel d1) and dog (Can f1) allergens were higher in dust collected in schools than in homes. Our results show that in children with mild asthma and animal dander allergy, there is a significantly increased bronchial sensitivity to methacholine after one school week. There is also a significant decrease in the number of circulating eosinophils and a trend towards an increase of sputum EPO, which could correlate with the early phase of eosinophil recruitment to the lungs. These effects may be related to the continuous exposure to animal allergens in school dust.

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Asthma is present in 7–8% of schoolchildren in Sweden (1), and 50% of these children have allergy to furred pets (2). Exposure to animal dander triggers asthma in the sensitized child, and it is possible that indirect animal exposure plays a role in maintaining the allergic inflammation in the airways (3). The concentration of animal dander allergen in school dust frequently exceeds the amount measured in homes with no furred pets (4). This could be one of the reasons why the

prevalence of allergy to furred pets is increasing (5). It may also explain why children with asthma and animal dander allergy often require continuous therapy with inhaled steroids to control symptoms, even when they avoid all direct contact with furred animals. Experimental studies suggest that repeated exposure to low doses of allergens increases bronchial hyper-reactivity in adults with allergy and mild asthma (6, 7). Increased recruitment of eosinophils occurs at

sites of inflammation in both allergic and non-allergic asthma (8). Released eosinophil proteins, such as eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), or eosinophil protein X (EPX), can be used as markers of inflammation, as can the expression of intracellular ECP. The monoclonal antibody (mAb) EG2 is thought to detect activated eosinophils by expressing intracellular ECP (9). Data have also been presented which suggest that mAbs CD9 and CD11b, both *in vitro* and *in vivo*, can be used as activity markers for eosinophils (10). The cysteinyl leukotrienes (LTC₄, D₄ and E₄) are lipid mediators that are involved in inflammatory and airway obstructive events in asthma. Urinary excretion of LTE₄ is a marker of *in vivo* production of cysteinyl leukotrienes and possibly reflects an ongoing inflammatory activity in the airways (11).

The aim of this study was to investigate the effect of exposure to cat and dog allergens, during one school week, on allergic inflammation in the airways of children with mild asthma and animal dander allergy, not treated with inhaled corticosteroids. Our intention was to measure the allergic inflammation in various compartments by analyzing serum, urine, nasal lavage (NAL), and sputum for leukotrienes, eosinophil proteins, proportion of eosinophils and eosinophil activity, on a cellular basis, and to relate eosinophil activity and leukotriene formation to bronchial methacholine sensitivity.

Materials and methods

Subjects

Ten children: six boys and four girls, aged between 9 and 14 years (median age 12 years); attending the Paediatric Asthma and Allergy Clinic at Huddinge Hospital, participated in the study (Table 1). The inclusion criteria were: a history of mild allergic asthma; normal lung function; a positive skin prick test (SPT) to cat and dog; and no current treatment with inhaled corticosteroids. Exclusion criteria were house dust mite or mould allergy and ongoing symptoms of asthma. The children did not have furred pets at home. Asthma had previously been diagnosed by a physician, according to the American Thoracic Society (ATS) guidelines. None of the patients included in the study had been treated with inhaled corticosteroids or cromolyn sodium for the last 2 months, and had not experienced asthmatic or allergic symptoms during the last month. Spirometric measurements confirmed normal lung function with

Table 1. The 10 children with asthma, but normal lung function, who participated in this study

Patient no.	Age	Gender	FEV ₁ (%)
1	12	M	101
2	12	F	82
3	9	M	90
4	12	M	81
5	14	F	93
6	12	F	83
7	9	F	109
8	12	M	80
9	10	M	91
10	9	M	94
Mean	11.1		90.4
Median	12		90.5

forced expiratory volume in 1 s (FEV₁) over 80% of the predicted values.

The study was approved by the local Ethics Committee at Huddinge Hospital. The patients and parents gave their informed consent.

Study design

The children attended the clinic on the Monday and Friday of two different weeks to take part in various procedures. The first week was in January or February and the second in March or the first week of April. By following the weekly pollen counts we were able to eliminate the risk that birch pollen would influence the results. The children came to the clinic before school on Monday morning and after school on Friday, in the same week. In the first week, the children underwent methacholine bronchial challenge on Monday morning at 09.00 h and on Friday at noon. In the second week, NAL and sputum induction were carried out at the same times as methacholine challenges had been performed in the first week. Blood and urine samples for analysis of inflammatory markers were obtained before and after both school weeks. The children's contact with any furred animal and their use of bronchodilators were documented throughout the study, by means of a structured questionnaire. Lung function was followed by flow-volume curves at each visit. The children recorded their daily morning and evening peak expiratory flows (PEF), by using a Mini-Wright peak flow meter (Clement Clarke International Ltd, Harlow, Essex, UK). Dust samples were collected from the classrooms, and from every child's mattress, according to a standardized protocol. SPTs were performed in duplicate, with a panel of cat, dog, horse, rabbit, guinea-pig, and rat allergens (Soluprick 10,000 SQ; ALK, Hørsholm, Denmark). A mean wheal diameter

≥ 3 mm was considered to be a positive result. Immunoglobulin E (IgE) antibodies to hazel, alder, and birch pollen were analyzed with the Pharmacia CAP system, RAST FEIA (Pharmacia & Upjohn AB, Diagnostics, Uppsala Sweden).

Methods

Methacholine challenge. The patients inhaled increasing concentrations of methacholine (12), delivered by a dosimeter (Spira Elektro 2 nebulizer system, Respiratory Care Center, Hameenlinna, Finland). The concentrations of methacholine were: 0.075, 0.15, 0.31, 0.62, 1.25, 2.50, 5.0, and 10.0 mg/ml. Five breaths of each concentration resulted in cumulative doses of methacholine, from a minimum of 3 μ g to a maximum of 700 μ g. The aerosol was inhaled through a mouthpiece (13). The patients were asked not to take any β_2 agonists for 8 h prior to the methacholine challenge. The methacholine challenge was stopped when a 20% fall in FEV₁ occurred or a maximum dose of 700 μ g was reached. The bronchial sensitivity to methacholine (PD₁₀ or PD₂₀ value) was determined from the cumulative log dose-response curve by linear interpolation. When the challenge was completed, all patients were treated with inhaled β_2 -agonist.

Nasal lavage. The NAL was performed with the patient sitting and leaning forward (14). This position prevented fluid from reaching the throat. One nasal cavity was lavaged with 6 ml of lukewarm 0.9% saline solution by using a syringe (10 ml) to which a nasal cone was adapted. To avoid leakage of fluid, the cone was gently applied to the external part of the nostril. The saline solution was injected into the nasal cavity, left there for 1 min and then aspirated back into the syringe. Thereafter, the nasal wash was centrifuged at $530 \times g$, at $+4^\circ\text{C}$, for 10 min.

Sputum induction. Sputum was induced by inhalation of nebulized hypertonic saline, at concentrations of first 3% and then 5%, for 10 min each, until a sputum sample had been obtained (15). The sample was examined within 2 h of sampling. Salivary contamination was removed macroscopically from the specimen. The selected portion was weighed and treated with fresh dithiothreitol (DTT) in a balanced salt solution (Sputalysin 10%; Calbiochem, San Diego, CA, USA), diluted to 1% by the addition of distilled water. The selected portion

was mixed with an equal volume of the DTT solution. The mixture was then placed in a plastic tube and shaken in a vortex for 15 s. It was then placed in a 37°C shaking water-bath for 15 min. Every 5 min, it was vortexed again for 5 s and then gently aspirated in and out of a pipette to ensure mixing, strained through a nylon gauze and centrifuged for 10 min at $530 \times g$. Spirometry was performed before and after the 3% saline inhalation, and after the 5% saline inhalation.

Assays. Sera, NAL, and sputum were assayed for ECP, EPO, and myeloperoxidase (MPO). Urine samples were assayed for EPX and LTE₄. In blood, the EG2 epitope on intracellular ECP, the surface expression of CD9 and CD11b, and the proportion of eosinophils, were analyzed by flow cytometry.

ECP was measured with the Pharmacia CAP system, ECP fluorescent enzyme immuno-assay (FEIA) having an intra- and inter-assay coefficient of variation of 5–10%. The detection limit was 2 μ g/l.

MPO was measured with the Pharmacia competitive radioimmunoassay (RIA), which is a double antibody-based RIA (16). The inter-assay coefficient of variation was below 13%, and the detection limit was 8 μ g/l.

EPO was assessed with a prototype immuno-fluorometric assay, utilizing the Pharmacia CAP SystemTM. The EPO standard curve of Carlsson et al. (17) was used and it covered the range from 0.5 to 200 μ g/l. Cross-reactivity with ECP and MPO in the EPO assay was less than 0.3 and 0.01%, respectively. The intra- and inter-assay coefficients of variation were below 10% and the detection limit of the assay was 0.5 μ g/l.

Urinary EPX was determined with Pharmacia's commercial, specific, competitive RIA (18). Cross-reactivity of the assay with ECP and EPO was less than 0.03%. The inter- and intra-assay coefficients of variation were less than 10%. The creatinine levels in urine were used to adjust the EPX levels. Results were expressed as ng EPX/mmol creatinine in the urine.

Urinary LTE₄ was determined with an enzyme immunoassay (EIA) utilizing a rabbit polyclonal antiserum (Cascade Biochemicals, Reading, UK) and acetylcholine esterase-linked LTE₄ tracer (Cayman Chemicals, Ann Arbor, MI, USA), as described previously (19). Cross-reactivity of the assay with LTC₄ and LTD₄ was 27% and 39%, respectively. The intra- and inter-assay coefficients of variation were less than 18%, and the detection limit was 8 pg/ml. Results were expressed as ng LTE₄/mmol creatinine in the urine.

Cell membrane permeabilization (FOG method). We wished to analyze intracellular ECP, in the separated eosinophils, by using flow cytometry. This was performed after the peripheral blood leucocytes were prepared using the FOG cell-membrane permeabilization method (9). The cell-surface antigens were detected by using fluorescein isothiocyanate (FITC)-conjugated mAbs against CD9 (DAKO, Glostrup, Denmark) and phycoerythrin (PE)-conjugated mAbs against the adhesion molecule CD11b (DAKO). To obtain optimal binding of mAb to the cell-surface antigens, and to avoid intracellular binding, these antibodies were added to the cells first, i.e. before the FOG treatment. Measurements of intracellular ECP were performed by using FITC-EG2 (Pharmacia & Upjohn Diagnostics AB), and this mAb was added after the FOG treatment.

Analysis with flow cytometry. The final preparations of immunostained FOG-treated mixed leucocytes were analyzed and counted in an EPICS XL flow cytometer (Coulter, Inc., Hialeah, FL, USA). A minimum of 1000 cells were accumulated in the eosinophil gate for analysis.

Standard-Brite (Coulter), a fluorosphere with controlled fluorescence intensity, was used to standardize the mean fluorescence intensity (MFI) before each experiment. The fluorescent signals were expressed in logarithmic-scaled histograms and a quantitation of the expression of CD9, CD11b, and the EG2 epitope was obtained by measuring the MFI units of the positive cell population. The proportion of eosinophils was also determined.

Sampling and processing of dust. Dust samples were collected from the children's classrooms and bedrooms. In the classroom, the child's chair, desk, and textiles (such as curtains and upholstered furniture) were vacuumed for 10 min/m². The children's bedroom, mattresses, and other textiles were also vacuumed for 10 min/m². Each sample was weighed and 100 mg of dust was extracted by rotation overnight at 4°C with 2 ml of phosphate-buffered saline, pH 7.4, containing 0.1% Tween-20. The content of cat (Fel d1) allergens was measured with a mAb-based enzyme-linked immunosorbent assay (ELISA), according to the protocols from Indoor Biotechnology (Charlottesville, VA, USA), except that peroxidase-labelled conjugates were substituted with alkaline phosphatase (ALP)-labelled streptavidine (DAKO) (20). The content of dog

(Can f1) allergens was analyzed with a RIA, using partially purified anti-Can f1 mAbs (provided by Professor R. Aalberse and Professor M. D. Chapman), and coupled to cyanogen-bromide (CNBr)-activated discs (Pharmacia), as described previously (21). One international unit (1 IU) of the International dog standard corresponds to ≈ 1 ng/ml purified Can f1. The detection limits of the Fel d1 and Can f1 assays were 55 and 80 ng/g dust, respectively, and the coefficient of variation $< 8\%$ and 15% , respectively.

Statistical analysis

Non-parametric tests were used for statistical analysis of results. Wilcoxon's signed rank test was used to test for differences. Relationships between variables were evaluated by the Spearman rank correlation coefficient; a p-value < 0.05 was considered significant.

Results

Metacholine challenge

The maximum dose of 700 μ g was too low to reach PD₂₀, at both challenges, in seven of the children. PD₁₀ was therefore calculated; one child was still a non-responder, but the remaining eight showed a significant increase in bronchial sensitivity to methacholine after one school week ($p=0.01$) (Fig. 1). One patient was withdrawn from the methacholine challenge because of an airway infection, on the Monday when the first bronchial provocation had been performed. He

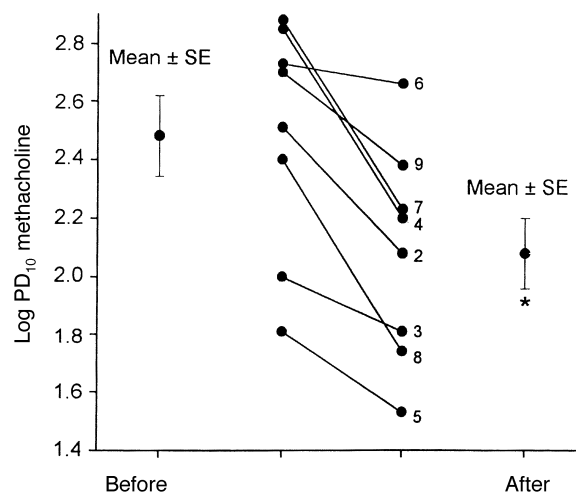


Fig. 1. The bronchial sensitivity to metacholine, expressed as PD₁₀, decreased in eight children after one school week. One child (no. 1) was a non-responder, and one child (no. 10) refused to take part in more than one methacholine challenge.

later returned, on another week, and completed the protocol on Monday and Friday, but declined further methacholine challenges.

Inflammatory markers

The urinary EPX decreased significantly (Table 2) in the first week ($p=0.005$) and the second week ($p=0.007$). The proportion of eosinophils in peripheral blood showed similar decreases in both weeks, i.e. not significant in the first ($p=0.07$) but significant in the second week ($p=0.005$). The reproducibility for the two weeks was good, with no significant difference between the two Monday samples. No significant changes were seen in serum ECP or serum EPO or in the eosinophil activity in blood, as measured by the binding of mAbs CD9, CD11b, and EG2. The concentrations of markers in sputum were generally low with a trend towards an increase of sputum EPO ($p=0.06$) and sputum MPO ($p=0.07$). There was a trend, although not significant, of an increase in urinary LTE₄ in the second week, but not in the first week. The inflammatory markers in NAL showed no changes.

Lung function and symptoms

All the children had normal lung function with FEV₁ > 80% (Table 1) when entering the study.

The FEV₁ and FEF50% values did not change significantly during the course of the study. The children reported no asthmatic or other allergic symptoms, such as rhinoconjunctivitis. According to the diaries and the personal questionnaires, all the children avoided contact with furred pets. No increased use of β 2-agonists and no significant variability regarding the PEF curves, were recorded during the study.

Dust samples

The concentrations of Fel d1 and Can f1 were higher in dust collected in classrooms than in homes (not significant (ns) for Fel d1, $p=0.07$; but significant for Can f1, $p<0.05$) (Fig. 2). The geometric means for the Fel d1 content in classrooms and homes were: 976 ng/g dust and 306 ng/g dust, respectively; and for Can f1, 1375 ng/g dust and 255 ng/g dust, respectively.

Three patients had higher levels of Fel d1 at home than at school, but they still had markedly higher levels of Can f1 at school than at home, thus their total cat + dog allergen exposure was higher at school.

Pollen forecast specific IgE and SPT

Six of the children had specific IgE to tree pollen (birch, hazel and alder). According to the pollen counts, there were no measurable levels of birch

Table 2. Granulocyte markers

Granulocyte markers	At the start of the first week (median)	At the end of the first week (median)	p-value	At the start of the second week (median)	At the end of the second week (median)	p-value
Cellular						
Eosinophils (%)	10.5	7.8	0.07	9.5	8.5	0.005
EG 2	23.8 MFI	27.3 MFI	NS	28.4 MFI	31.2 MFI	NS
CD9	104.0 MFI	109.5 MFI	NS	105.2 MFI	109.9 MFI	NS
CD 11b	9.1 MFI	8.8 MFI	NS	8.8 MFI	7.9 MFI	NS
Serum						
S-ECP	15.3 μ g/l	11.3 μ g/l	NS	12.9 μ g/l	18.0 μ g/l	NS
S-EPO	30.0 μ g/l	23.0 μ g/l	NS	28.5 μ g/l	32.5 μ g/l	NS
S-MPO	309.5 μ g/l	341.0 μ g/l	NS	239.5 μ g/l	356.0 μ g/l	0.06
Urine						
U-EPX	121.0 μ g/mmol	69.5 μ g/mmol	0.005	102.0 μ g/mmol	84.0 μ g/mmol	0.007
U-LTE ₄	73.4 ng/mmol	66.5 ng/mmol	NS	60.4	68.9 ng/mmol	NS
Sputum						
Sputum ECP				28.0 μ g/l	37.9 μ g/l	NS
Sputum EPO				0.3 μ g/l	0.9 μ g/l	0.06
Sputum MPO				60.5 μ g/l	104.0 μ g/l	0.07
NAL						
NAL ECP				11.7 μ g/l	10.1 μ g/l	NS
NAL EPO				11.8 μ g/l	5.35 μ g/l	NS
NAL MPO				103.5 μ g/l	22 μ g/l	NS

The granulocyte markers represent the median values from the first and the second school week.

ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; EPX, eosinophil protein X; LTE₄, leukotriene E₄; MFI, mean fluorescence intensity; MPO, myeloperoxidase; NAL, nasal lavage; NS, not significant.

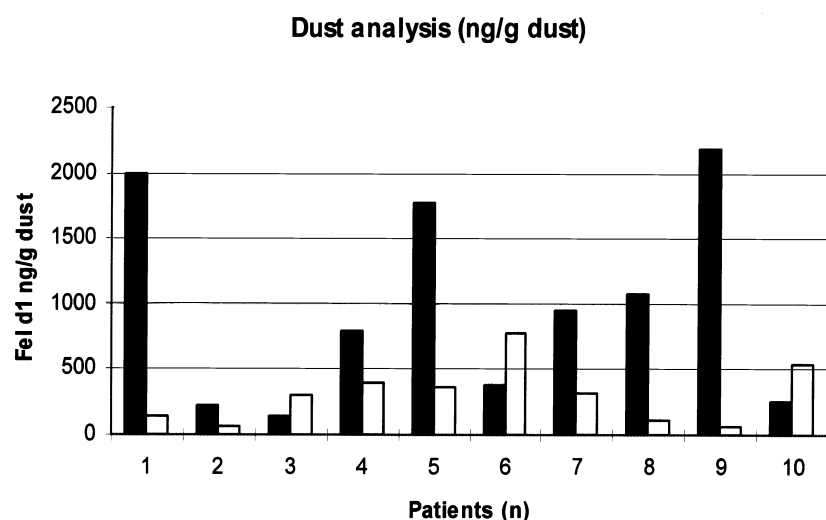


Fig. 2. The concentrations of cat (Fel d1) and dog (Can f1) allergens were higher in school dust than in dust collected in the childrens' homes. The results were not significant for Fel d1 ($p=0.07$) but were significant for Can f1 ($p<0.05$). ■, classroom; □, home.

pollen during the course of the study. In March, but not in April, there were, however, low levels of alder and hazel pollen in the air. Four of the children sensitized to tree pollen came for their second week of investigation in March but there was no indication that pollen exposure affected their airways. All children had, as required, a positive SPT to cat and dog but also to horse and rabbit. Six children had a positive SPT to guinea-pig, and five had a positive SPT to rat.

Discussion

It would have been interesting to study the children during the first week of term, but this was not possible for practical reasons. On the other hand, we might then have had other confounding factors interfering with the results, such as complete change of environment after a school holiday.

The children were asymptomatic and took no daily medication. In spite of this, the bronchial sensitivity to methacholine had increased in the airways by the end of the school week. Some changes in inflammatory markers were also found by the end of the school week, for instance a decrease of eosinophils in peripheral blood, a decrease of EPX in urine, and a tendency for an increase of EPO and MPO in sputum. The concentrations of cat and dog allergens were higher in school dust than in dust obtained from the childrens' own bedrooms, which means that the children were more exposed to animal allergen during school hours than in their homes. Other studies have confirmed the presence of animal dander allergens in school dust but the effect on health of this indirect animal exposure has not been evaluated (4, 5).

The increased methacholine sensitivity in the airways that we observed during a school week may be a sensitive indicator of allergen exposure at school and is, incidentally, very similar to how a week at work worsens occupational asthma (22). It corresponds also with other reports, where repeated exposure to low doses of allergens were found to increase bronchial hyper-reactivity in adults with allergy and mild asthma (6, 7). Our challenges were performed on Monday at 09.00 h and on Friday at noon. The 3 h difference between sampling times on the different days might theoretically have affected our results, but previous studies on diurnal variation have shown no changes in methacholine sensitivity during the time period we studied (23).

All of the children whose PD_{10} showed a decrease also presented a decrease in EPX, and all, except two, also showed a decrease in eosinophils in peripheral blood. It therefore seems likely that the increased methacholine sensitivity in our study reflects a gradual increase in sensitivity in the airways of the children.

The decrease in the proportion of eosinophils in peripheral blood, after both weeks, may be the result of an early recruitment from the blood to the airways. Both the decrease in the number of eosinophils in peripheral blood (24) and the increase in bronchial reactivity are known to coincide with the onset of the late allergic response (25). Similar findings have been presented in a recent study where eosinophils in blood decreased 5 h after allergen provocation while a slight increase was seen after 24 h (26). In the same study, a marked increase was seen in the number of eosinophils in sputum and in the number of progenitor cells (Eo/B-CFU) in bone marrow. The decrease of circulating eosinophils in this early stage of inflammation can be explained by the fact that airways inflammation

is a dynamic process and that the pooling of eosinophils in peripheral blood is only a transient stage before recruitment to the target organ. The children in our study had mild asthma, not requiring steroid treatment and they might be in this 'early recruitment phase' for a long period of time without showing clinical symptoms. Accordingly, eosinophils in blood and urinary EPX decreased after both school weeks.

A diurnal variation of the number of eosinophils in blood has been reported, with the highest values in the early morning, at $\approx$ 04.00 h (27). One could therefore argue that the decrease in the proportion of eosinophils was a consequence of diurnal variation but, on the other hand, the time difference was only 3 h and the morning samples were taken at 09.00 h. Recent studies also suggest a diurnal variation in urinary EPX (28); this finding needs further investigation.

Assays in blood samples are an indirect way of evaluating the inflammatory process in the airways. A better way is probably to evaluate markers of inflammation in sputum (14). This method has also been modified for children (29). We found low concentrations of inflammatory markers in sputum; this was not unexpected because the children had mild asthma and no spontaneous sputum production. Turner and colleagues also obtained the same results when studying subjects with mild exacerbation of asthma, almost one-half of whom had no sign of sputum eosinophilia (30). Our finding of a trend towards increased concentrations of EPO and MPO in sputum could be another sign of an ongoing, mild, airways inflammation.

The inflammatory processes in the nose could also reflect an ongoing process in the lower airway (31), and NAL is easy to perform with little discomfort for the patient (32). We found low concentrations of inflammatory markers in NAL, and no specific changes after the school week. Although eosinophils in peripheral blood decreased significantly after both school weeks, it was disappointing that mild inflammation in the lower airways was not reflected in NAL.

In this study, the number of participants was restricted for practical reasons, although higher numbers might have given more significant results. The population was, however, very well defined and homogenous, and repeated measurements were performed in blood and urine.

In conclusion, our results show that, in children with mild asthma and animal dander allergy, there is a significantly increased bronchial sensitivity to methacholine after one school week. There is also a significant decrease in the number of circulating eosinophils, and a trend towards an

increase in sputum EPO, which may correlate with eosinophil recruitment to the lung. These effects might be related to the continuous exposure to animal allergens in school dust.

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