

Eosinophil cationic protein in induced sputum as a marker of inflammation in asthmatic children

Sorva R, Metso T, Turpeinen M, Juntunen-Backman K, Björkstén F, Haahtela T. Eosinophil cationic protein in induced sputum as a marker of inflammation in asthmatic children.

Pediatr Allergy Immunol 1997; 8: 45–50. © Munksgaard 1997.

We evaluated the usefulness of eosinophil cationic protein (ECP) in induced sputum and in serum as markers of asthmatic inflammation in children. We measured ECP in serum and in total induced sputum samples from 14 children (7–11 years) with newly detected asthma before and after treatment, and from ten healthy non atopic controls of the same age. The patients inhaled budesonide, 800 µg/m²/day for the first month and 400 µg/m²/day for the next 5 months, both divided into two doses, and nedocromil, 4 mg three times daily for the following 6 months. In both sputum and serum, ECP levels were higher in the patients than in the controls, but the difference was more distinct in sputum. Significant clinical improvement during the treatment was accompanied by a decrease in sputum ECP, whereas serum ECP did not change. The results suggest that induced sputum is useful as a non invasive source material for evaluating asthmatic inflammation in children, total sputum ECP being more sensitive than serum ECP for diagnosing and monitoring asthma.

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Key words: asthma; budesonide; eosinophil
cationic protein; nedocromil; sputum

Received 9 July, 1996

Accepted 21 April, 1997

Sputum has recently been recognized as a non invasive source material for objective evaluation of asthmatic inflammation (1) valuable for clinicians in the diagnosis of asthma and in determining the correct choice (steroidal vs non steroidal), dose and duration of anti-inflammatory treatment. Sputum examinations have become easier because it was found that sputum production can be induced by inhalation of nebulized hypertonic saline (2). In sputum of patients with asthma, eosinophil counts have been found to be elevated (3), and to decrease after prednisolone treatment (4).

Concentrations of ECP have also been found to be increased in sputum of asthmatics (5–7). ECP has been measured from plugs selected from the sputum sample (8) and from the supernatant part of the sputum (7). However, in non symptomatic patients, especially after treatment, plugs are not always available. Therefore, a method has been developed for determining ECP in the total sputum sample (9). The present study was designed to compare total

sputum ECP levels and serum ECP levels as markers of asthmatic inflammation in children. For this purpose, we compared ECP levels in 14 children with newly detected asthma and in ten non atopic healthy children. To monitor the effects of different treatments, ECP levels in the sputum and serum of the patients, were also examined after treatment with inhaled budesonide for 6 months and with inhaled nedocromil for the following 6 months.

Materials and methods

Patients

Fourteen consecutive prepubertal children with newly detected asthma were recruited in an open longitudinal study at the Outpatient Department for Allergic Diseases of the Skin and Allergy Hospital, Helsinki, Finland. Their median age was 9.1 years (range 7.0–11.2) and the median duration of asthma was 0.7 years (0.4–2.0). Six were boys and eight were girls. Twelve were atopic. The patients had not had any anti-inflammatory treatment for asthma before the study, and had no other atopic or other diseases or medications. The diagnosis of asthma was

Abbreviations: ECP: eosinophil cationic protein; U-EPX: urinary eosinophil protein X; FEV₁: forced expiratory volume in 1 second; PEF: peak expiratory flow; PD: provocative dose.

based on a positive history, combined with at least one of the following criteria: diurnal variation of $\geq 20\%$ in peak expiratory flow (PEF) or $\geq 15\%$ increase in forced expiratory volume in 1 second (FEV_1) in a bronchodilating test or $\geq 15\%$ decrease in FEV_1 during an exercise test.

Study protocol

The patients inhaled budesonide (Turbuhaler[®], Draco AB, Lund, Sweden), 800 $\mu\text{g}/\text{m}^2/\text{day}$ divided into two doses for the first month and 400 $\mu\text{g}/\text{m}^2/\text{day}$ as two doses for the next 5 months. During the following 6 months, they inhaled nedocromil (Tilade[®], Fisons LTD, UK) 4 mg three times a day via a Nebuhaler (Draco AB, Lund, Sweden). They were permitted to use beta-adrenergic agonists (Bricanyl[®], Draco AB, Lund, Sweden, 0.25 mg/dose) as needed. Two children had an exacerbation of asthma soon after the switch to nedocromil, and were therefore restarted on budesonide. They were excluded from the analysis at 12 months.

Subjective symptoms were evaluated, pulmonary function was assessed, and blood, urine, and sputum samples were taken before treatment at 1 month and at 6 and 12 months. The interval between the diagnosis of asthma and the first sputum sample before treatment was 1–3 days.

Methods

Symptoms of asthma were evaluated on a visual analogue scale (from 0 (none) to 10 (severe)) which the parents were asked to mark at each visit, describing the symptoms since last visit. Pulmonary function was assessed with a pneumotachograph (Vitalograph Compact, Vitalograph Ltd., Buckingham, U. K.). The results are expressed as percentages of the predicted values calculated for healthy Finnish children of the same height and sex. Bronchial hyperreactivity was assessed by a histamine challenge test (10). The results are expressed as the provocative dose of histamine for a decrease in FEV_1 of 15% (PD_{15}).

Blood was taken in gel tubes (SST, Becton-Dickinson, England) and allowed to clot at room temperature for 60 (+/-10) min. Serum was separated by centrifugation at $1350 \times g$ for 10 min and stored at -20°C till analyzed for ECP using a radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Blood eosinophils counted with a hematologic analyzer (H2, Technicon).

First morning urine was sampled and analyzed for eosinophil cationic protein X (EPX) with a radioimmunoassay research kit (Pharmacia Diagnostics, Uppsala, Sweden) and for creatinine, using a modification of Jaffe's method. The results are expressed as $\mu\text{g EPX}/\text{mmol creatinine}$.

Sputum was induced by inhalation of 4 ml of a 5% NaCl solution using an ultrasonic dosimetric nebulizer (Spira Ultra, Spira Ltd, Hämeenlinna, Finland). The patients were asked to cough during and after the inhalation. The sputum sample was considered adequate if, after ethanol fixation and Papanicolaou staining, macrophages could be detected by light microscopy. The presence of macrophages showed that we had succeeded in inducing sputum in 12/14 asthmatics at diagnosis and in all of them during treatment. To the sputum sample, an equal volume of 6.5 mmol/l dithiotreitol solution (Sputolysin[®]) was added, and the mixture was vortexed, incubated at room temperature for 15 min, and stored at -20°C till analyzed. The sample was treated with a detergent, N-cetyl-N,N,N-trimethyl ammonium bromide, before determination of ECP, with a fluorescent enzyme immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Sputum albumin and urea concentrations were determined with a Hitachi analyzer. Sputum results are expressed as sputum ECP ($\mu\text{g}/\text{l}$), as sputum ECP/albumin ($\mu\text{g}/\text{mg}$), and as sputum ECP/urea ($\mu\text{g}/\text{mmol}$).

Controls

Morning blood, urine, and sputum samples were similarly taken and analyzed from 15 healthy children. Sputum induction was successful in ten of them, who served as controls in the study. They were 9.3 (6.5–10.2) years of age, and had no history of atopy or any evidence of specific serum IgE (negative Phadiatop Combi test, Pharmacia Diagnostics, Uppsala, Sweden). The pulmonary function of the ten controls was assessed with a pneumotachograph (Vitalograph Compact, Vitalograph Ltd., Buckingham, U. K.) resulting in a median (range) FEV_1 of 104 (89–112)%.

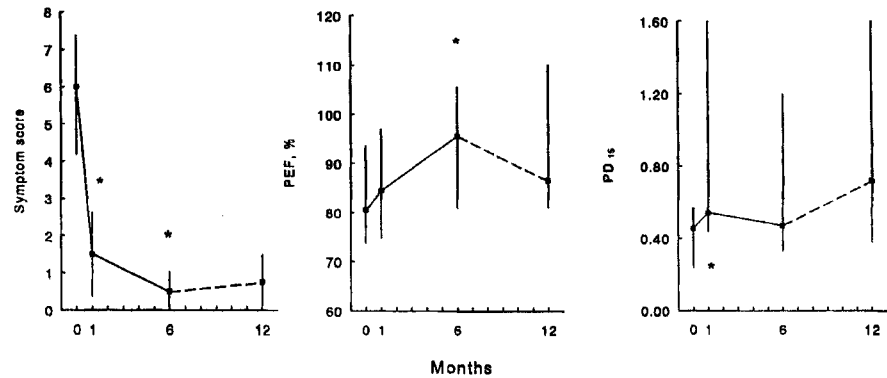
Statistics

The results for patients and controls were compared using the Mann-Whitney test. The effects of budesonide were evaluated by comparing the results for the intraindividual samples (at 0, 1, and 6 months) with the Friedman test (non parametric analysis of variation). Paired samples (two consecutive samples) were evaluated with the Wilcoxon signed rank test. The correlations were calculated using Spearman's rank correlation test.

Ethics

The study protocol was approved by the Ethics Committee of the Department. All parents and children gave their informed consent.

Fig. 1. Asthma symptom scores, peak expiratory flow (% of predicted) and PD₁₅ for histamine (medians and interquartile ranges) in 14 children with newly detected asthma before and after treatment for 1 and 6 months with inhaled budesonide (continuous line) and nedocromil for a further 6 months (dotted line). * = significant difference ($p < 0.05$ at least) compared with the baseline level.



Results

Symptoms and pulmonary function

Asthma symptom scores decreased ($p < 0.001$) and PEF increased ($p = 0.013$) during inhalation of budesonide, and did not change during treatment with nedocromil (Fig. 1). During the higher dose of budesonide, there was a significant increase in FEV₁, from a median (range) of 100 (70–117)% to 104 (87–127)% at 1 month ($p = 0.039$). FEV₁ was 100 (88–118)% at 6 months (ns) and 100 (79–116)% at 12 months (ns). PD₁₅ for histamine also increased during the higher dose of budesonide (Fig. 1), but did not change any further during either subsequent treatment.

Markers of inflammation

Patients vs healthy children (Table 1)

Sputum induction was successful in 12/14 asthmatic children at entry and in 10/15 controls. At entry, both serum and sputum ECP concentrations were higher in the patients than in the healthy children ($p = 0.038$ and $p = 0.018$, respectively). The difference in sputum ECP was more significant after adjustment for sputum albumin ($p = 0.001$). In the patients, sputum ECP/albumin correlated directly with serum ECP level ($r = 0.71$, $p < 0.05$). Blood eosinophil count and U-EPX were not significantly different in pa-

tients and controls. However, the blood eosinophil count correlated with serum ECP ($r = 0.60$, $p < 0.05$) and sputum ECP/albumin ($r = 0.67$, $p < 0.05$) in the patients. Serum ECP and U-EPX were also directly related in them ($r = 0.62$, $p < 0.05$).

Effects of treatment

During the period of budesonide inhalations the blood eosinophil count decreased ($p = 0.040$, Fig. 2). There was no further change during treatment with nedocromil. The s-ECP concentration did not change significantly (Fig. 2). Neither did U-EPX change significantly, the medians (range) per mmol of creatinine being 61.6 (11.8–319.9) μg before treatment, 51.2 (10.4–119) μg at 1 month, 29 (8.8–180) μg at 6 months, and 58 (12.9–248) μg at 12 months.

Sputum ECP decreased during inhaled budesonide ($p = 0.028$), the medians (range) being 592 (18–3992) $\mu\text{g/l}$ before treatment, 254 (24–1420) $\mu\text{g/l}$ at 1 month, and 82.5 (7–1252) $\mu\text{g/l}$ at 6 months. There was no further change during nedocromil, the median at 12 months being 101 (25–508) $\mu\text{g/l}$. After adjustment for sputum albumin (Fig. 2), the decrease in sputum ECP was even more significant ($p = 0.017$). After adjustment with sputum urea, sputum ECP was 150 (5–1535) $\mu\text{g/mmol}$ urea before

Table 1. Markers of inflammation at baseline in serum ($n = 14$), in urine ($n = 14$) and in sputum ($n = 12$) of the patients and in ten healthy non-atopic controls. Medians (ranges) are given. The significances between the groups are indicated

	Patients	Healthy controls	p
Blood eosinophils, $10^9/\text{l}$	0.21 (0.04–1.23)	0.14 (0.07–0.37)	>0.05
Serum ECP, $\mu\text{g/l}$	7.7 (3–131)	5.6 (2.9–9.8)	0.038
Sputum ECP, $\mu\text{g/l}$	591 (18–3992)	140 (22–556)	0.018
Sputum ECP/urea, $\mu\text{g/mmol}$	149.7 (5.0–1535)	46.0 (13.9–213.8)	0.010
Sputum ECP/albumin, $\mu\text{g/mg}$	45.2 (3.0–154.7)	3.2 (0.5–8.8)	0.001
Urine EPX, $\mu\text{g/mmol}$ creatinine	61.6 (11.8–319.9)	81.0 (16.4–118.4)	>0.05

ECP = eosinophil cationic protein.

EPX = eosinophil protein X.

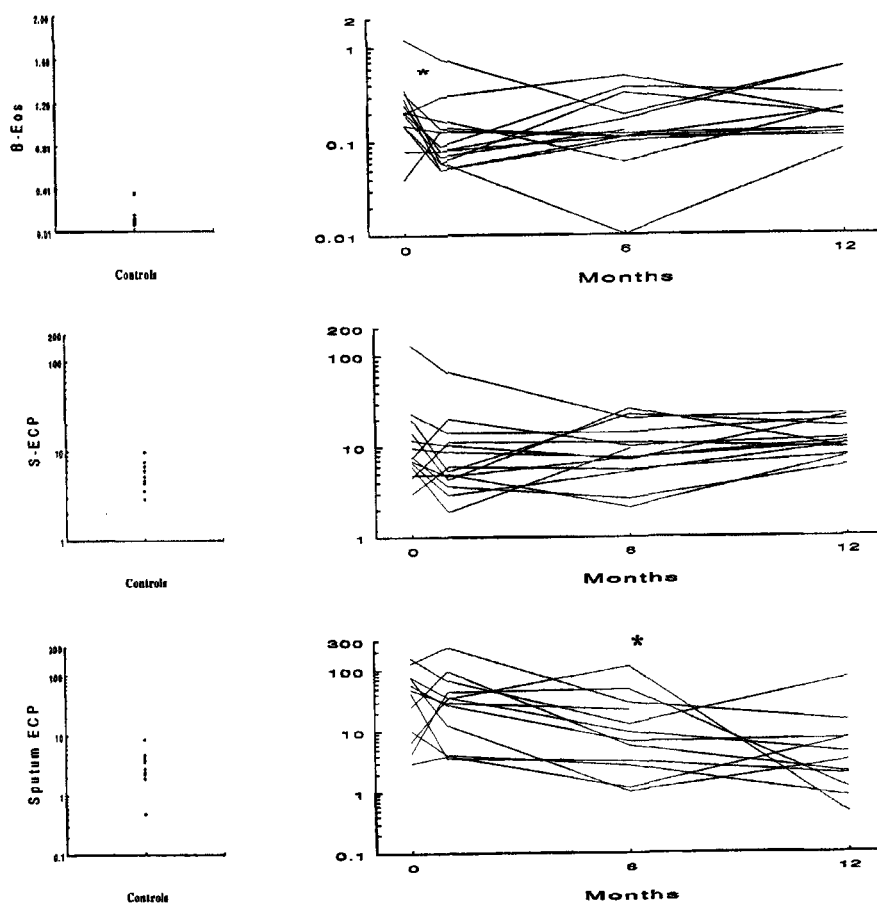


Fig. 2. Blood eosinophil count ($10^9/l$), and serum eosinophil cationic protein (ECP) ($\mu g/l$) and sputum ECP levels ($\mu g/mg$ sputum albumin) in ten healthy controls (left panels) and in 14 children with newly detected asthma (right panels) before and after treatment for 1 and 6 months with inhaled budesonide and nedocromil for a further 6 months. * = significant difference ($p < 0.05$ at least) compared with the baseline level.

treatment, 79.8 (6.6–710) $\mu g/mmol$ urea at 1 month, 31.2 (2.7–894) $\mu g/mmol$ urea at 6 months, and 36.0 (10.4–220) $\mu g/mmol$ urea at 12 months.

Sputum ECP concentration at baseline correlated inversely with the increase in FEV_1 during the first month of treatment ($r = -0.71$, $p < 0.05$). The decrease in sputum ECP ($\mu g/l$ and $\mu g/mmol$ urea) during the first month correlated with the decrease in the symptom score ($r = 0.69$, 0.54 , $p < 0.05$). At 1 month, sputum ECP levels ($\mu g/l$ and $\mu g/mmol$ urea) correlated inversely with FEV_1 ($r = -0.75$ and -0.70 , $p < 0.05$) and PEF values ($r = -0.59$ and -0.60 , $p < 0.05$). There were no other significant correlations.

Discussion

Whether serum ECP can be used for monitoring asthmatic inflammation is controversial (11, 12). In the present study, serum ECP levels were only slightly higher in the patients than in the healthy controls at entry, and did not change significantly during the treatments. But, in patients with more severe asthma, significant decreases in serum ECP have been observed during treatment (13–16), and

the levels have been reported to correlate with pulmonary function (17, 18). However, serum ECP may be increased in other inflammatory diseases besides asthma, e.g. in atopic dermatitis (19), and rheumatoid arthritis (20), and even in acute bacterial infections (21).

Cytologic and microbiologic examinations of sputum have long been used in the diagnosis of pulmonary diseases (22, 23). Sputum cell counts were shown to be reproducible in the short term (24), and eosinophil counts from sputum were found to correlate with pulmonary function in asthma (25). Fairly good agreement has been reported between the number of eosinophils counted in sputum, in bronchoalveolar lavage fluid, and in the submucosa of a bronchial biopsy (26). A recent study has demonstrated that sputum can provide even better quantitative information on asthmatic inflammation than bronchoalveolar lavage, the sputum being more concentrated than the lavage fluid (27). Biochemical markers of inflammation in sputum might improve the evaluation of asthma. This has been demonstrated for bronchoalveolar lavage fluid: no significant change was observed in cell counts, but there was a significant de-

crease in ECP levels after budesonide treatment (28). In the present study, at entry, sputum ECP levels were distinctly higher in the patients than in the healthy controls, and decreased during budesonide treatment. The levels were normal at 6 months, suggesting good control of asthmatic inflammation. Interestingly, the sputum ECP levels remained low during nedocromil treatment for 6 subsequent months in the 12/14 patients without exacerbation of asthma. A significant correlation has been demonstrated between sputum ECP and pulmonary function (5). In the present study, also, sputum ECP correlated inversely with the increase in FEV₁ during the first month on budesonide, suggesting that patients with higher sputum ECP at diagnosis responded less well to the treatment, possibly because of the more pronounced asthmatic inflammation.

In both patients and controls, the concentrations of sputum ECP covered a wide range in the present study. The sensitivity of the test was improved by adjusting sputum ECP especially in relation to sputum albumin and less so sputum urea, neither of which depends on the degree of dilution of the sputum (29). The use of albumin in the standardization is favored by the observation that albumin levels in bronchoalveolar lavage fluid are similar in asthmatic patients and in healthy controls (30). In contrast, the increased albumin levels observed in the sputum of asthmatics (7) might argue against it. Standardization of secretions from the respiratory tract still remains controversial.

In conclusion, the results of the present study suggest that sputum ECP levels might reflect the local inflammation in asthma more specifically than serum ECP, and that determination of total sputum ECP would be useful in the diagnosis and monitoring of asthmatic inflammation.

Acknowledgements

We thank the Finnish Anti-Tuberculosis Association Foundation, Helsinki, Finland, and Pharmacia, Uppsala, Sweden.

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