Do sputum eosinophils and ECP relate to the severity of asthma?

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ABSTRACT: There is much evidence that eosinophils play an important role in bronchial epithelial damage in asthma by releasing cationic proteins. However, the extent to which eosinophil inflammation relates to indices of asthma severity in chronic stable asthma is still a matter of debate.

We studied 46 clinically stable patients with mild to severe chronic asthma (forced expiratory volume in one second (FEV₁) 50–126% of predicted value). The clinical severity of asthma was graded from 1 to 4 according to the Aas scoring system. Twelve normal subjects were also studied as controls. Induction of sputum was performed by hypertonic saline to determine differential cell count, and eosinophil cationic protein (ECP) by the so-called "plug technique". The concentration of ECP was measured by a fluorimmunoassay. Bronchial hyperresponsiveness was recorded by inhaling progressive concentrations of histamine, and the concentration that caused a 20% decrease in FEV₁ (PC₂₀) was calculated.

Sputum eosinophils (range 0–61%), sputum ECP (range 24–10,800 µg·L⁻¹) and serum ECP (range 4–61 µg·L⁻¹) were significantly greater in asthmatics than in normal subjects, and distinguished the most severe group with the highest Aas score from the others. Sputum eosinophils and sputum ECP were strongly related to each other. The relationships between sputum or serum ECP and PC₂₀ (range 0.016–7.5 mg·mL⁻¹), and between sputum ECP and FEV₁ were found to be weak.

In conclusion, sputum outcomes of eosinophil activation and serum eosinophilic cationic protein appear to be useful indicators of disease. They do not accurately reflect current clinical or functional indices of asthma severity in chronic stable patients, and might therefore provide complementary data disease monitoring.

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An extensive eosinophilic inflammation of the bronchi has recently been described in bronchial asthma, even with mild clinical or subclinical disease [1–3]. Activated eosinophils are able to release many cytotoxic proteins, including eosinophil cationic protein (ECP), which has a central role in bronchial epithelial damage [4, 5]. The role of blood eosinophils and serum ECP in monitoring disease activity in chronic asthma has not been conclusively defined, as there is conflicting evidence concerning their relationship to clinical severity of disease [6–10].

The use of spontaneously produced or induced sputum, proposed as a reliable and noninvasive method to characterize airway inflammation [11–14], appears promising for clinical survey and longitudinal studies with asthmatic patients. In line with previous studies showing that the degree of eosinophil activation is more important than the increase in number of eosinophils in reflecting an ongoing inflammation in asthma [15], VIRCHOW *et al.* [16] have shown that the level of sputum ECP, rather than the number of sputum eosinophils, correlates with lung function in asthma. However, that study relied upon a small number of asthmatic patients, in whom the

indices of asthma severity, such as clinical scores, duration of disease, and bronchial hyperresponsiveness, were not defined.

Therefore, we carried out the present study in a group of patients with clinically defined chronic stable asthma to verify whether and to what extent ECP and eosinophil differential counts in sputum and blood relate to the indices of asthma severity.

Materials and methods

Subjects

Forty six out-patients (30 males and 16 females) aged 17–73 (median 41) yrs with chronic bronchial asthma according to the criteria of the National Heart, Lung and Blood Institute (NHLBI) [17], and a group of normal subjects, participated in the study. Thirty patients were nonsmokers, 16 were exsmokers (with less than 10 pack-yrs). Informed consent was given by each subject and the study was approved by the local Ethics Committee.

Asthma was characterized by episodes of dyspnoea with wheezing, variable airflow limitation with reversible

obstruction ($\geq 20\%$ increase in FEV₁ after inhalation of 200 μg of fenoterol), positive response to inhalation challenge with histamine (provocation concentration producing a 20% fall in forced expiratory volume in one second (PC₂₀) $\leq 8 \text{ mg}\cdot\text{mL}^{-1}$). The clinical severity of asthma was graded from 1 to 4 according to the scoring system of Aas [18]. Before entering the study, 15 patients required daily inhaled corticosteroids and bronchodilators. Bronchodilators, but not corticosteroids, had been withheld for at least 12 h before each study. All subjects had been free from acute respiratory infections within the preceding 4 weeks and were stable at the time of study.

The normal subjects were an age-matched group: 12 healthy, lifelong nonsmokers, with negative skin-prick tests, aged 25–65 (median 34) yrs, without symptoms of asthma, and with normal bronchial responsiveness to histamine (PC₂₀ $\geq 16 \text{ mg}\cdot\text{mL}^{-1}$). Their pulmonary function was in the normal range (forced expiratory volume in one second (FEV₁) $>80\%$ of predicted, FEV₁/vital capacity (VC) $>70\%$).

Aas score

According to the Aas score, the clinical severity of asthma ranges from very mild forms (score 1) to incapacitating forms (score 5). Grading is based on: the number and duration of asthma episodes; the total duration of symptoms; the presence or absence of symptom-free intervals between attacks; and the requirement for medication over 1 yr [18]. In this study, clinical severity of asthma was graded from 1 (very mild asthma) to 4 (severe asthma): in 14 patients asthma was scored 1; in 11 it was scored 2; in 17 it was scored 3; and in 4 it was scored 4.

Diagnosis of immediate-type hypersensitivity

Allergy tests, including a battery of common aeroallergen extracts, were performed by the skin-prick method. Cutaneous positivity to allergen was defined by a wheal ($>3 \text{ mm}$) and erythema with pseudopods. Common aeroallergens included mixed graminaceous pollen, mixed composite pollen, *Parietaria officinalis*, olive pollen, *Alternaria*, *Aspergillus fumigatus*, *Cladosporium*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and cat and dog danders.

Lung function

Baseline pulmonary function testing was performed by measuring static and dynamic lung volumes using a water-sealed spirometer (Pulmonet Godart; Sensormedics Corp., Yorba Linda, CA, USA), as reported previously [19]. The normal values for lung volumes were those proposed by the European Coal and Steel Community [20].

Bronchial challenge

Each patient was administered a histamine aerosol inhalation test. Increasing concentrations of histamine-acid phosphate in normal phosphate buffered saline (pre-

pared by the University Hospital Pharmacy) were inhaled from a DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, PA, USA), driven at an airflow rate of 6 $\text{L}\cdot\text{min}^{-1}$, mean (SD) output 0.31 (0.03) $\text{mL}\cdot\text{min}^{-1}$, using the tidal breathing method. With this method, 4 mL of solution were placed in the nebulizer and inhalation continued during tidal breathing over 2 min. Histamine solution was stored at 4°C and nebulized at room temperature. Normal phosphate-buffered saline was inhaled first, followed at 5 min intervals by histamine in twofold increasing concentrations from 0.031 to 8 $\text{mg}\cdot\text{mL}^{-1}$. The test was withheld at the concentration of histamine which caused a decrease in FEV₁ $\geq 20\%$ from saline (provocative concentration). The PC₂₀ was determined from the log dose-response curve. Details of the technique have been described previously [21].

Induction and analysis of sputum

Induction of sputum was performed according to the method of PIN *et al.* [12]. Ten minutes after fenoterol inhalation (200 μg), hypertonic saline was nebulized with an ultrasonic nebulizer (Fisoneb; Fisons Corp., Rochester, NY, USA) and was inhaled for 5 min periods up to 20 min. The concentration of saline was increased at 10 min intervals from 3 to 4%. FEV₁ was measured every five minutes during inhalation of hypertonic saline solution. The sputum induction procedure did not cause troublesome symptoms, and the FEV₁ did not decrease by more than 20% in any subject. Every 5 min, subjects were asked to try to cough sputum into a Petri dish and to collect saliva in a separate container. Macroscopic characteristics of the sputum were recorded, and the quality of sputum sample was assessed, according to the method of PIN *et al.* [12], by the number of lower respiratory tract plugs and the extent of salivary contamination in cell counts.

Cytological analysis. Two to three plugs free of salivary contamination were suspended in dithiothreitol (DTT) solution (0.1%) and incubated for 30 min at 37°C for slide making. Cells were centrifuged at 1,500 rpm for 10 min and then resuspended in saline. Three sputum slides were then prepared for cytological examination by cytocentrifugation. Cells were air-dried and stained with May-Grünwald Giemsa stain. Cell differentials were determined by counting 200 nonsquamous cells on each sputum slide.

ECP measurement. The volume of the remaining portion of sputum samples was determined and an equal volume of dithiothreitol (0.1%) was added. The samples were mixed by vortex and incubated at 37°C for 20 min. The samples were then centrifuged at 1000 $\times$ g for 10 min. The supernatants were aspirated and frozen at -70°C for later ECP analysis.

ECP was assessed both in sputum and blood by a fluoroimmunoassay (CAP ECP FEIA; Kabi Pharmacia, Pharmacia Diagnostics AB, Uppsala, Sweden). Sputum specimens needed to be diluted 1:20 in 48% of cases and 1:80 in 16%. Anti-ECP, covalently coupled to immunoCAP, reacted with the ECP in the specimens. After washing, enzyme-labelled antibodies against ECP were added to form a complex. After incubation, unbound enzyme anti-ECP

was washed away and the bound complex was then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate was measured in Fluoro-Count 96 (Kabi Pharmacia). The sensitivity of this technique is $<0.5 \mu\text{g}\cdot\text{L}^{-1}$.

Serum ECP

Blood was collected using silicone-containing tubes (4 mL Vacutainer hemogard SST tubes; Becton Dickinson Vacutainer Systems Europe, Meylan, France) for serum separation. After clotting for 60–120 min at room temperature (22–24°C), serum samples were centrifuged at the same temperature at $1,000\times g$ for 15 min and aliquoted and frozen at -20°C for subsequent assay of ECP using the same technique (CAP ECP FEIA; Kabi Pharmacia).

ECP was determined in duplicate both in serum and in sputum. The normal range for serum ECP was $2.8\text{--}9.0 \mu\text{g}\cdot\text{L}^{-1}$ and for sputum ECP $7.1\text{--}70.0 \mu\text{g}\cdot\text{L}^{-1}$.

Study design

The patients were examined on three occasions within a 6 day period. On day 1, a questionnaire on the subject's characteristics and clinical scores was completed. Baseline spirometry and skin-prick tests were also performed. On day 2, the histamine inhalation test was performed. On day 3 (at least 48 h after the histamine challenge), sputum induction and drawing for serum ECP were carried out. All of the sputum from normal subjects and asthmatic patients was collected as induced sputum.

Ten patients and five normal subjects were asked to produce sputum on a second occasion within 6 days.

In a complementary study carried out in eight subjects (three mild asthmatics, two moderate asthmatics, and three normals), the sputum induction and the drawing for serum ECP were performed 24 before, and 24 and 48 h after the histamine challenge.

Statistical analysis

The repeatability of duplicate measurements of cell profile and ECP was assessed by the coefficient of repeatability (CoR). ECP concentrations were log-transformed before analysis, because differences between the two measurements were proportional to their mean value, and CoR was expressed as antilog [22]. The effects of histamine challenge on sputum eosinophils and ECP levels were assessed by two-way analysis of variance (ANOVA). Regression analysis was performed by Spearman's rank correlation coefficient. The significance of the differences between groups was assessed by Kruskal-Wallis analysis of variance, and Mann-Whitney U-test when appropriate. Bonferroni's adjustment (0.05/n test) for multiple testing was used. A p-value less than 0.05 was considered to be significant.

Table 1. – Clinical and functional data

	Sex	Age	FEV ₁	FEV ₁ /VC	PC ₂₀	Serum ECP	Blood eosinophils	Skin tests
	M/F	yrs	% pred	%	mg·mL ⁻¹	μg·L ⁻¹	cells·mm ⁻³	+/-
Asthma (n=46)	30/16	41 (17–73)	86*** (50–126)	70*** (44–112)	0.65‡ (0.016–7.5)	14.8*** (3.5–60.6)	290* (4–1484)	34/12
Normal (n=12)	5/7	34 (25–65)	108 (96–125)	80 (72–95)	≥16	5.9 (2.8–9.0)	100 (46–260)	0/12

Values are presented as median and range in parenthesis. ‡: geometric mean. M: male; F: female; FEV₁: forced expiratory volume in one second; VC: vital capacity; PC₂₀: provocative concentration of histamine causing a 20% fall in FEV₁; ECP: eosinophil cationic protein; % pred: percentage of predicted value; +: positive; -: negative. *: p<0.05; ***: p<0.001, compared to normal subjects.

Results

Clinical data

Table 1 summarizes the clinical and functional characteristics of the sample. Asthmatic patients showed significantly lower FEV₁ (% pred) and FEV₁/VC ratios than normal subjects (p<0.001 for both). In particular, eight patients exhibited mild-to-moderate airway obstruction (FEV₁ 50–66% pred). Bronchial hyperresponsiveness (BHR) to histamine ranged from mild to severe (PC₂₀ 0.016–7.5 mg·mL⁻¹): it was mild in nine patients (2–8 mg·mL⁻¹); moderate in 27 patients (0.250–2.000 mg·mL⁻¹); and severe in eight patients (0.016–0.250 mg·mL⁻¹). In three patients, the bronchial challenge with histamine was not performed owing to a previous history of Quincke's oedema. Skin-prick tests were positive in 34 patients.

Sputum eosinophils and ECP

Table 2 shows the results of cytological and biochemical (ECP) sputum analysis. The differential count of eosinophils was significantly higher in asthmatic patients than in normal subjects (p<0.001). In asthmatics, there was also a trend for sputum neutrophils to increase (p=0.053); sputum macrophages were lower than in normals (p<0.002). The CoRs [22] of the differential counts were 9.8 for eosinophils and 14.2 for neutrophils. The CoR for sputum ECP was 1.3. In those subjects in whom sputum eosinophils, and sputum and serum ECP were evaluated 24 h before (A), and 24 h (B) and 48 h (C) after histamine challenge, the following median (range) values were observed: sputum eosinophils 0 (0–33) % in A; 0 (0–22) % in B; and 0 (0–28) % in C (F=2.03; p=0.17); sputum ECP 23 (8.3–6050) μg·L⁻¹ in A; 20.3 (6–4730) μg·L⁻¹ in B; and 21.8 (8.8–4452) μg·L⁻¹ in C (F=1.4; p=0.28); serum ECP was 12.4 (7.9–23) μg·L⁻¹ in A; 11.2 (9.2–16.8) μg·L⁻¹ in B; and 13 (7.9–18.2) μg·L⁻¹ in C (F=0.59; p=0.57).

Sputum ECP values (table 2) were greater in asthmatics than in normals (p<0.001). Figure 1 shows that eosinophil figures and ECP levels in sputum related

Table 2. – Sputum cellular and biochemical profile

	Eosinophils	Neutrophils	Macrophages	Lymphocytes	Sputum ECP
	%	%	%	%	μg·L ⁻¹
Asthma (n=46)	5.3*** (0–60.9)	17.7 (1.7–61)	66.2## (13.6–93)	1.4 (0–8)	528.5*** (24–10800)
Normal (n=12)	0 (0–2.2)	9.4 (0–25)	87.5 (0–97)	1.16 (0–2)	16.5 (7.1–70)

Values are presented as median and range in parenthesis. ECP: eosinophil cationic protein. ##: p<0.002; ***: p<0.001, compared to normal subjects.

Fig. 1. — Relationship between sputum eosinophil cationic protein (ECP) and sputum eosinophils in asthmatic patients. Δ : Aas score 1; $\bullet$: Aas score 2; $\circ$: Aas score 3; $\bullet$: Aas score 4.

strongly to each other ($r_s=0.82$; $p<0.0001$); the figure also shows that 55.5% of patients with no sputum eosinophils had sputum ECP above the range calculated for the normal control group ($<70 \mu\text{g}\cdot\text{L}^{-1}$). In figure 1, asthmatics were divided according to their clinical severity (Aas score): sputum eosinophils and sputum ECP were greater in each scoring group than in normals ($p<0.05$ to $p<0.016$). However, eosinophils only permitted the differentiation of group 4 from groups 1–3, whilst ECP differentiated group 4 from groups 1 and 2 ($p<0.05$ to $p<0.015$ for all comparisons).

Eosinophils and ECP related to clinical scores (Aas) ($r_s=0.40$; $p<0.008$ and $r_s=0.50$; $p<0.008$, respectively). ECP weakly related to FEV1 % pred ($r_s=-0.34$; $p<0.02$) and PC20 ($r_s=-0.40$; $p=0.01$), and eosinophils did not.

There were no differences in sputum eosinophils and ECP between treated and nontreated patients, or between allergic and nonallergic patients. In all subgroups sputum eosinophils and ECP related to each other ($p<0.005$ to $p<0.0001$). In addition, in all subgroups, but not in allergic patients, sputum eosinophils ($p<0.05$ to $p<0.0009$) and ECP ($p<0.05$ to $p<0.003$) related to clinical scores.

Blood eosinophils and serum ECP

Blood eosinophils were $>500 \text{ cells}\cdot\text{mm}^{-3}$ in eight of the 46 patients. Compared to normal subjects, serum ECP level was significantly higher in asthmatics as a group ($p<0.001$) (table 1), and in 1, 2 and 3 scoring groups. In contrast, no difference was found between scoring groups. Serum ECP was above the normal range in 32 patients (median 14.8 (range $3.5\text{--}60.6$) $\mu\text{g}\cdot\text{L}^{-1}$).

Blood eosinophils were not related to serum ECP or sputum eosinophils. Serum outcomes were not related to clinical scores or percentage predicted FEV1, while serum ECP was weakly related to PC20 ($r_s=-0.34$; $p=0.04$).

Discussion

Sputum eosinophils, and sputum and serum ECP were useful for differentiating asthmatic patients from normal subjects; however, their usefulness for differentiating clinical subgroups was limited. Sputum outcomes significantly related to each other. Sputum ECP but not

sputum eosinophils weakly related to baseline pulmonary function, as did the degree of bronchial hyperresponsiveness both to sputum and serum ECP.

Recent studies have proposed the analysis of induced sputum as a reliable and noninvasive method to investigate airway inflammation in asthmatic patients [11–14]. The results of the present investigation are in line with those of our previous study [21], and with the results of studies carried out either with biopsies [1–3] or bronchoalveolar lavage (BAL) [8], indicating the importance of eosinophilic airway inflammation in the pathogenesis of chronic stable asthma. The biochemical analysis of sputum also provides several indices of eosinophil activation, such as major basic protein (MBP) and ECP. ECP was measured in the entire sample of induced sputum and expressed as the level per millilitre of sputum supernatant. In an attempt to limit salivary contamination, we asked the patients to collect saliva in a separate container.

As in the study by FAHY *et al.* [13], in the present study the level of sputum ECP was lower in normal subjects than in asthmatics. The normal values of sputum ECP were similar in the two studies. On the contrary, sputum ECP from asthmatic patients showed a wider range and higher mean values than in the study by FAHY *et al.* [13]. This might be due to the different method of sputum induction and handling and, therefore, to a different relative dilution of sputum samples with saliva and saline. The possibility also exists that the two study groups were not clinically matched.

We did not analyse sputum gel phase, so we cannot exclude differences with respect to composition of sputum gel phase (SGP) and sputum sol phase (SSP). However, other authors have shown that the inclusion of SGP protein levels does not affect the variation of sputum protein analysis in patients with stable asthma and chronic obstructive pulmonary disease (COPD) [23].

The possibility that the inhalation of histamine may influence eosinophil and ECP levels is not supported by the present data, in line with previous results [24].

Some studies using sputum of asthmatics [12, 25] have shown a relationship between eosinophils on the one hand and baseline airway obstruction and BHR on the other. On the contrary, in other studies [16] eosinophil differential counts did not relate to functional data. Although the reasons for these discrepancies are likely to be complex, differences in clinical and functional characteristics of patients and a time lag between changes in inflammatory profile and measurement of functional parameters (FEV1, PC20) are not unlikely factors [26, 27]. In a recent study, sputum ECP, but not sputum eosinophils, related to FEV1 [16], so that sputum ECP was considered a more sensitive index than eosinophils in reflecting active airway inflammation, and thereby airway obstruction. The latter study [16], however, was based on a small sample of patients with a lack of clinical information: clinical scores, BHR and duration of disease. In addition, in the study by VIRCHOW *et al.* [16], the observation that some patients who were not treated with steroid exhibited a $<10\%$ FEV1 reversibility after β_2 -agonists does not seem to be consistent with the diagnosis of pure bronchial asthma. The present study, carried out in a well-defined asthmatic group, seems to indicate: firstly, that the activity of eosinophils, estimated by ECP levels in sputum, was highly related

to the number of eosinophils, even though in a small percentage of patients with no eosinophils the ECP level appeared to be higher than that calculated for the normal control group. Secondly, the relationship of neither of the two sputum outcomes with baseline airway obstruction was clinically meaningful. Thirdly, the data extend the results of previous studies, in that neither eosinophils nor ECP accurately reflect the clinical severity of the disease in patients with chronic stable asthma.

BHR is a cardinal feature of asthma and has been recognized to relate to severity of disease, frequency of exacerbation and the need for treatment [28]. In the present study, sputum eosinophils did not relate to, and sputum ECP was only weakly related to, the degree of BHR as assessed in terms of PC20. These results suggest that sputum expression of eosinophil airway inflammation is not identical to BHR and that other factors, such as airway remodelling, are involved in determining the level of BHR [29–31]. Thus, the sputum and the degree of BHR may provide complementary information in well-controlled asthmatic patients.

The role of blood eosinophil counts and serum ECP in monitoring disease activity in chronic asthma is not well defined [6–10]. In the present study, serum ECP was higher in asthmatic patients than in normal subjects, but neither of the two blood outcomes related to the clinical score or baseline pulmonary function.

In conclusion, although sputum eosinophils and eosinophilic cationic protein did not accurately discern disease severity subgroups, they provided a direct method for investigating airway inflammation. Repeated measurements may be useful for monitoring the inflammatory process and the response to therapy in the individual patient.

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