

Measuring airway inflammation in asthma: Eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood

Emilio Pizzichini, MD,* Marcia M. M. Pizzichini, MD,* Ann Efthimiadis, MLT,
Jerry Dolovich, MD, and Frederick E. Hargreave, MD *Hamilton, Ontario, Canada*

Background: Airway eosinophilic inflammation is a characteristic feature of asthma. This can be assessed directly by measurement of eosinophils and eosinophilic cationic protein (ECP) in sputum or indirectly by measurement of the same markers in blood. We investigated the performance of these markers of airway eosinophilic inflammation in a population of patients with asthma compared with control subjects and the extent to which the markers differed.

Methods: In a cross-sectional study, subject characteristics were documented on day 1 and induced sputum and blood samples were obtained on day 2. Nineteen patients with asthma and 20 control subjects (10 healthy subjects and 10 smokers with nonobstructive bronchitis) were consecutively enrolled in the study. Sputum (selected from saliva) and blood samples were processed by persons blind to the clinical details. Results are presented as median values (minimum-maximum); differences were measured by Mann-Whitney U test. The accuracy of the tests (sensitivity and specificity) was measured by plotting the data in receiver-operating characteristic (ROC) curves and comparing the areas under the curve for each marker.

Results: Patients with asthma in comparison with control subjects had a higher proportion of sputum eosinophils (5.2% [0.2% to 93%] vs 0.3% [0% to 1.7%], $p < 0.001$), higher numbers of blood eosinophils ($350.0 \times 10^6/L$ [144.0 to $1520.0 \times 10^6/L$] vs $155.0 \times 10^6/L$ [34.0 to $426.0 \times 10^6/L$], $p = 0.003$), and higher levels of ECP in sputum ($1040.0 \mu g/L$ [76.8 to $32,000.0 \mu g/L$] vs $455.3 \mu g/L$ [54.4 to $1280.0 \mu g/L$], $p = 0.001$) but not in serum ($25.0 \mu g/L$ [5.6 to $52.4 \mu g/L$] vs $16.5 \mu g/L$ [3.3 to $36.0 \mu g/L$], $p = 0.08$). Markers of airway inflammation in induced sputum and blood samples were correlated with clinical and physiologic variables. The area under the ROC curve showed that eosinophils in sputum (0.90) are significantly more accurate markers than blood eosinophils (0.72) and serum ECP (0.67) ($p = 0.02$). Although the area under the ROC curve for sputum ECP was

greater than those for blood eosinophils and serum ECP, the differences could have occurred by chance ($p \geq 0.1$).

Conclusion: We conclude that the proportion of eosinophils in sputum is a more accurate marker of asthmatic airway inflammation than the proportions of blood eosinophils or serum ECP. (*J Allergy Clin Immunol* 1997;99:539-44.)

Key words: Induced sputum, eosinophils, eosinophilic cationic protein, airway inflammation, asthma

Airway inflammation is a major feature of airway diseases including asthma and chronic bronchitis. The evidence for the role of airway inflammation was highlighted by studies that examined bronchial tissue or bronchoalveolar lavage (BAL) fluid obtained by bronchoscopy.^{1,2} The bronchial mucosa from patients with asthma features a rich eosinophilic infiltrate, which is regarded as characteristic of asthma.³ BAL fluid in patients with asthma has a higher proportion of eosinophils and their activation marker, eosinophilic cationic protein (ECP), than that in healthy subjects.⁴ In contrast, BAL fluid in chronic bronchitis features a predominance of neutrophils.⁵ Bronchoscopic studies, however, are invasive, expensive, and restricted to use in patients with mild or moderate airflow limitation.

Another characteristic feature of asthma is the presence of peripheral blood eosinophilia.^{6,7} Because this correlates with the severity of symptoms, degree of airflow limitation,⁶⁻⁸ and airway responsiveness to methacholine⁹ or histamine,¹⁰ it has been suggested that blood eosinophils may be an indirect marker of airway inflammation in asthma.^{2,11} However, blood eosinophils are also increased in other conditions that may or may not be associated with asthma such as atopy,¹² rhinitis,¹³ and eczema.¹⁴ It has been increasingly suggested that the state of activation of blood eosinophils, as measured by serum ECP, may be a useful indirect marker of the airway inflammation in asthma.¹⁵

An increase in the proportion of eosinophils in sputum of patients with asthma was observed more than 100 years ago.¹⁶ However, in the past sputum examination was considered to be difficult and unreliable. A resurgence of interest in sputum examination to measure indices of airway inflammation in research has occurred in the past 7 years. Cell counts on cytopins and fluid phase measurements of induced or spontaneous sputum were found to be reproducible, responsive, and val-

From the Asthma Research Group, Departments of Medicine and Pediatrics, St. Joseph's Hospital and McMaster University, Hamilton, Ontario, Canada.

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Reprint requests: F. E. Hargreave, MD, Firestone Regional Chest and Allergy Unit, St. Joseph's Hospital, 50 Charlton Ave. East, Hamilton, Ontario, Canada, L8N 4A6.

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Abbreviations used

AUC: Area under the curve
BAL: Bronchoalveolar lavage
ECP: Eosinophilic cationic protein
ROC: Receiver-operating characteristic

id.^{17, 18} Similar to the findings of bronchial biopsies and BAL fluid examinations the sputum of patients with asthma is characterized by an increase in the proportion of eosinophils and levels of ECP, and this is not seen in the sputum of smokers with nonobstructive bronchitis or healthy control subjects.¹⁷

No study to date has compared the diagnostic accuracy of eosinophils in sputum and blood and ECP in sputum and serum in subjects with asthma compared with control subjects. We therefore sought to determine the performance of these markers of airway eosinophilic inflammation measured directly in sputum or indirectly in blood and the extent to which these markers were different in these two groups.

METHODS**Study design**

In a cross-sectional study 19 patients with asthma and 20 control subjects (10 healthy subjects and 10 smokers with nonobstructive bronchitis) were seen on 2 consecutive days at the same time of day. On the first day subject characteristics were documented and on the second day, venous blood and induced sputum samples were obtained. The blood and sputum samples were examined by persons blind to the clinical characteristics. Because there is no gold standard for asthma diagnosis, the study population was carefully characterized by conventional diagnostic procedures (symptoms and variable airflow limitation) and by a methacholine inhalation test. This construct was accepted as the gold standard of asthma for comparisons.

Subjects

Patients with asthma had symptoms suggestive of asthma plus either airway hyperresponsiveness to methacholine ($PC_{20} < 4$ mg/ml) if the FEV_1 was $\geq 70\%$ ($n = 16$) or an improvement in FEV_1 of $\geq 20\%$ after the administration of salbutamol if the FEV_1 was $< 70\%$ ($n = 3$). They were nonsmokers, in stable condition, who had recorded symptoms daily for 5 days in a diary card, and they had no changes in medication in the previous month. They withheld the use of a short-acting β -agonist inhaler for 6 hours before being seen.

The control subjects were matched for number, age, gender, and atopic status. They comprised healthy subjects ($n = 10$) and smokers with nonobstructive bronchitis ($n = 10$). The healthy subjects were free of symptoms, nonsmokers, had no history of asthma or other respiratory diseases, and had normal spirometry and airway responsiveness to methacholine ($PC_{20} > 16$ mg/ml). The subjects with nonobstructive bronchitis were current smokers and had a history of cough with sputum production. Asthma was excluded by normal findings on spirometry and normal methacholine airway responsiveness (Table I).

None of the subjects had symptoms of a respiratory infection

within 1 month. All subjects were able to produce sputum after induction with hypertonic saline solution. The study was approved by the hospital research committee and all subjects gave written informed consent.

Clinical methods

Clinical characteristics. Symptoms were graded on a modified Borg scale (range: 4 = worst to 36 = best) and recorded on a diary card twice a day for 5 days after the first visit.¹⁹ Subjects without symptoms had few or no symptoms with mean symptoms scores of > 30 and subjects with symptoms had scores of ≤ 30 . Asthma severity was judged as mild, moderate, or severe by the frequency and chronicity of symptoms, the presence of persistent airflow limitation, and the medication needed to maintain control of the disease.²⁰ Spirometry, methacholine inhalation tests, and allergy skin prick tests were done with standard procedures.²¹⁻²³

Blood samples. Venous blood was collected into tubes containing 5.0 ml ethylenediaminetetraacetic acid (K_3 Vacutainer BD, Rutherford, N.J.) before sputum induction, and a differential white blood cell count was obtained with use of a Coulter STKS instrument (Coulter Corp., Hialeah, Fla.). Serum was collected after blood coagulation for 1 hour at room temperature. It was centrifuged at 20°C at 1500 rpm for 10 minutes and stored at -20°C until analysis.

Sputum samples. Sputum was induced by the inhalation of aerosol doses of hypertonic saline solution at increasing concentrations (3%, 4%, and 5%) as previously described.¹⁷ The expectorate was collected into a sterile container and processed as soon as possible within 2 hours. Sputum was selected from saliva and treated with four volumes of dithiothreitol followed by four volumes of Dulbecco's phosphate buffered saline solution. The resulting suspension was filtered and centrifuged. The supernatant was aspirated and stored in Eppendorf tubes at -70°C for later assay of ECP. The cell pellet was resuspended in Dulbecco's phosphate buffered saline solution, cytopins were made and stained by the Wright method, and a differential cell count was done on 400 nucleated nonsquamous cells.

ECP concentration. The concentrations of ECP (in micrograms per liter) in the serum and thawed supernatant were determined by use of a sensitive radioimmunoassay with a lower limit of detection of $< 2 \mu\text{g/L}$ (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). The sputum results were adjusted for the dilution.

Data analysis

Descriptive statistics were used to summarize clinical and demographic characteristics of the sample. Results are expressed as median and minimum and maximum. PC_{20} data were log transformed and reported as geometric mean and geometric standard deviation. Differences between groups were analyzed by the Mann-Whitney U test. The correlation between variables was examined by Spearman's rank correlation coefficient; only correlations with an r_s value higher than 0.50 and significant at the 0.01 level were considered relevant. Because the variables had a continuous scale of values, where the selection of a cutoff point was arbitrary, we determined the diagnostic accuracy of sputum and blood eosinophils and ECP by generating a receiver-operating characteristic (ROC) curve^{24, 25} for each test. The areas under the curves (AUCs) were compared by the method of Hanley and McNeil.²⁶ Except for correlations, a p value of < 0.05 was considered statistically significant.

TABLE I. Characteristics of subjects

Characteristics	Patients with asthma	Control subjects	
		Healthy	Smokers
Subjects (n)	19	10	10
Age (yr)	44 (20-71)	37 (29-55)	30 (22-49)
Sex (m)	8	5	7
Smoking (ex)	0 (6)	1 (6)	10
Atopic*	13	4	5
FEV ₁ (% predicted)†	75 (57-97)	99 (85-116)	104 (94-118)
FEV ₁ /VC (%)	67 (44-84)	80 (70-96)	77 (70-89)
PC ₂₀ (mg/ml)‡	1.1 (0.1-4.0)	>16	>16
Receiving inhaled steroid§	13	—	—

Data expressed as median and minimum-maximum. VC, vital capacity.

*Atopic means the number of subjects with one or more positive allergy skin prick test results.

†FEV₁ predicted values from Crapo et al.³⁵ or previous best in the past 2 years.

‡Methacholine PC₂₀ geometric mean.

§Inhaled steroid was beclomethasone dipropionate or budesonide.

TABLE II. Median (maximum-minimum) values for markers of eosinophilic inflammation in control subjects

	Healthy	Smokers	p Value
Sputum eosinophils (%)	0.5 (0.5-1.7)	0.3 (0.0-1.0)	0.3
Sputum ECP (μg/L)	288 (104-1240)	352 (54-1280)	0.8
Blood eosinophils (× 10 ⁶ /L)	94 (34-426)	181 (97-322)	0.8
Blood ECP (μg/L)	13 (3.3-36.0)	20 (6.0-34.0)	0.3

TABLE III. Correlations between markers of eosinophilic inflammation in blood and sputum and clinical measurements

	Symptom scores*	FEV ₁ (% predicted)	FEV ₁ /VC (%)	PC ₂₀ (mg/ml)	Blood ECP (μg/L)	Sputum ECP (μg/L)
Sputum eosinophils	-0.68	-0.68	-0.50	-0.66	NS	0.89
Blood eosinophils	-0.56	-0.57	NS	-0.52	0.71	NS
Sputum ECP	-0.57	NS	-0.63	NS	NS	—
Blood ECP	NS	NS	NS	NS	—	NS

Correlations only with values of $r_s > 0.50$ and $p < 0.01$ are given. VC, vital capacity; NS, not significant.

*Only in subjects with asthma.

RESULTS

Measurements in patients with asthma and control subjects

The median symptoms score was 30 (with a range of 14 to 36). Ten of the patients with asthma had few or no symptoms and 9 had symptoms. Six were considered to have mild asthma, 8 moderate asthma, and 5 severe asthma. Patients with asthma in comparison with control subjects had a higher proportion of sputum eosinophils (5.2% [0.2% to 93.0%] vs 0.3% [0% to 1.7%], $p < 0.001$), higher numbers of blood eosinophils [$350.0 \times 10^6/L$ [144.0 to 1520.0 $\times 10^6/L$] vs $155.0 \times 10^6/L$ [34.0 to 426.0 $\times 10^6/L$], $p = 0.003$], and higher levels of ECP in sputum (1040.0 μg/L [76.8 to 32,000.0 μg/L] vs 455.3 μg/L [54.4 to 1280.0 μg/L], $p = 0.001$) but not in serum (25.0 μg/L [5.6 to 52.4 μg/L] vs 16.5 μg/L [3.3 to 36.0 μg/L], $p = 0.08$). These markers did not differ within the

two subgroups of control subjects, healthy subjects and smokers with nonobstructive bronchitis (Table II).

Correlations between sputum or blood findings and clinical measurements in subjects with asthma

The strength of the correlations between sputum or blood findings and clinical measurements was higher for sputum than blood (Table III). The percentage of eosinophils in sputum was highly and inversely correlated with the symptoms score, FEV₁ value (expressed as a percentage of predicted or previous best), FEV₁/vital capacity value, and PC₂₀ of methacholine. The number of eosinophils in blood was also inversely correlated with symptoms score, FEV₁ value, and PC₂₀ of methacholine. Sputum ECP concentration was correlated with sputum

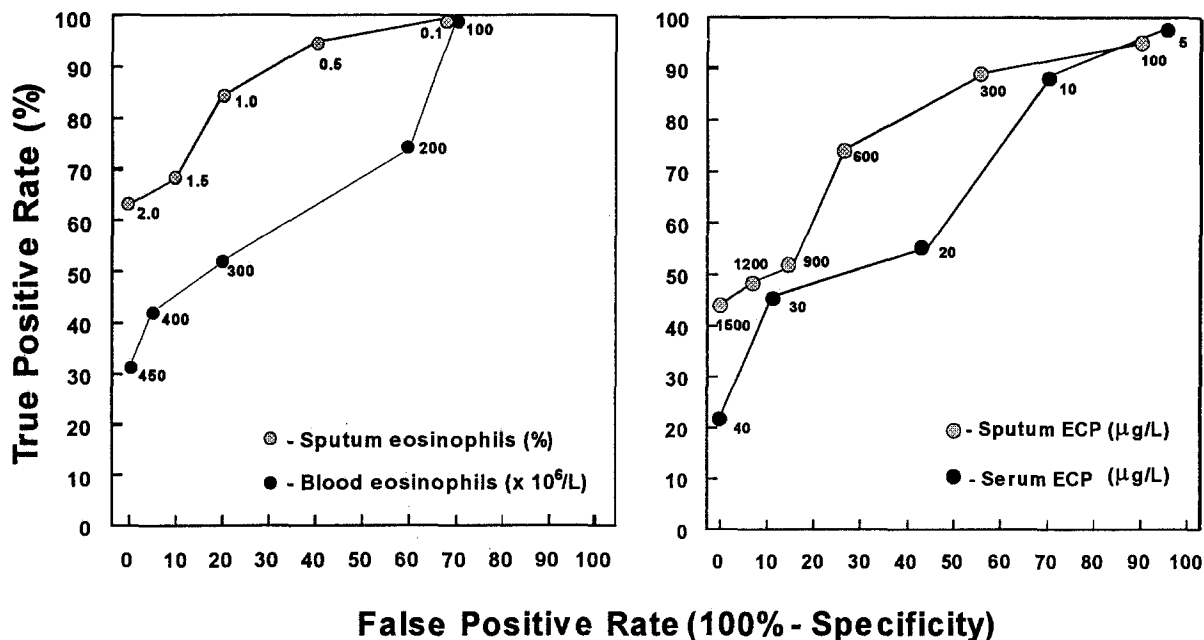


FIG. 1. ROC curves. Plots that lie farthest to the "northwest" represent more accurate values.

eosinophil concentration and inversely correlated with symptoms score and the FEV₁/vital capacity value. Serum ECP concentration correlated only with blood eosinophil concentration.

Accuracy of measurements to differentiate clinical groups

The ROC curves for eosinophils and ECP in sputum and blood were examined (Fig. 1). The AUC for each test revealed that the percentage of eosinophils in sputum (0.90) was more sensitive and specific than the determinations of blood eosinophils (0.72, $p = 0.02$) and serum ECP (0.67, $p = 0.02$) in the differentiation of patients with asthma from control subjects. Sputum eosinophils also performed better than sputum ECP but the AUCs were not significantly different (0.90 vs 0.80, $p = 0.2$). Although the AUC for sputum ECP was greater than those for blood eosinophils and serum ECP, the differences could have occurred by chance ($p \geq 0.1$). The AUCs for blood eosinophils and serum ECP were similar ($p = 0.4$).

DISCUSSION

The results of this study suggest that examination of induced sputum samples for the proportion of eosinophils discriminates patients with asthma from control subjects better than determination of peripheral blood eosinophils and serum ECP. Markers of airway inflammation were correlated with the clinical and physiologic parameters with stronger correlations for eosinophils than for ECP. Blood eosinophil counts are a sensitive discriminant but much less specific. Induced sputum ECP examination performs better than serum ECP and eosinophil examination, but does not add clinically rel-

evant information to results of sputum eosinophil testing. Serum ECP levels are an insensitive and nonspecific index.

This study is the first comparison of diagnostic accuracy between eosinophil and ECP levels measured in induced sputum and peripheral blood samples. Because no test will be clinically useful if it is not discriminative,²⁵ the global assessment of the discriminatory power is an important step in the assessment of the construct validity of sputum measurements to determine eosinophilic airway inflammation in asthma. The study population was intentionally selected to comprise subjects whose clinical condition was clearly defined by conventional procedures for two reasons. First, we anticipated that a failure of the indices to discriminate patients with asthma from control subjects under these conditions would eliminate the future applicability of the indices to discriminate different conditions in clinical populations. Second, we do not have a gold standard for the diagnosis of eosinophilic inflammation in asthma to establish the sensitivity and specificity of each test. Therefore, for the purpose of this study, we accepted a constructed gold standard that included conventional parameters such as the presence of symptoms of variable airflow limitation, reversibility of airflow limitation after bronchodilator use,²⁷ and methacholine airway hyperresponsiveness.

One of the strengths of the study is that, by selecting consecutive subjects who met the entrance criteria, we obtained a group of patients in stable condition with asthma who had a range of symptoms, expiratory flow values, and inhaled steroid treatments, and these features reinforce the validity of our results. Another strength of this study is the method of sputum examination. We have shown that the method of sputum induc-

tion is successful in 80% of subjects who cannot produce sputum spontaneously^{28, 29} and does not influence the characteristics of the inflammation.³⁰ The method of processing sputum gives lower respiratory secretions almost uncontaminated by saliva,³¹ no adverse influence of treatment with dithiothreitol,³² excellent quality of cytopins to differentiate cell types,³³ and cell and fluid phase measurements that are highly repeatable between days in subjects in stable condition.¹⁷ As a result an instrument to monitor airway inflammation has been developed that shows good discriminative (repeatability and validity) and evaluative (responsiveness) properties. These features support the applicability of this method to assess and monitor lower airway inflammation.

The clinical implications of sputum eosinophilia need to be further evaluated. In the present study none of the healthy subjects or smokers with nonobstructive bronchitis had sputum eosinophil values >2.0%. All subjects with more than 2.0% of eosinophils in sputum were patients with asthma, and at this level of specificity the sensitivity of the test was 63.0%. In this study population the AUC for sputum eosinophils indicated patients with asthma have a probability 90% higher than control subjects to be seen with increased proportions of eosinophils. However, these results cannot be generalized to a random population. The significance of increased sputum eosinophils needs to be examined in such a population with correlations to symptoms, measurements of airway function, and, perhaps, treatment.

In this study we compared the discriminative properties of sputum and peripheral blood sample examinations. The examination of eosinophils and their activation markers in asthma is relevant because they are considered to be involved in the pathogenesis of asthma and therefore directly or indirectly through structural changes to be the cause of the symptoms and variable airflow obstruction. The findings of a better performance for the indices measured in sputum, of the presence of correlations with clinical/physiologic parameters, and of the better discriminative power of markers of inflammation in sputum compared with peripheral blood were probably a result of the direct measurement of airway inflammation with the use of sputum. It is well known that eosinophils are tissue inflammatory cells that arise from the bone marrow and are transported by the blood to the tissue of reaction, which, in the case of asthma, is the bronchial wall.³⁴ It is therefore not surprising that peripheral blood eosinophils and serum ECP are weaker and less-specific markers of the inflammatory events in the airways.

In conclusion, among a population of patients with asthma and control subjects, sputum examination for the proportion of eosinophils is a more accurate diagnostic test than measurement of peripheral blood eosinophils and serum ECP to identify airway eosinophilic inflammation. These findings have implications in the identification and monitoring of airway inflammation and suggest that when the available markers are used, a more

accurate picture of the process will be obtained by the examination of sputum.

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