
Total eosinophil cationic protein levels in induced sputum as a marker of changes in eosinophilic inflammation in a patient with allergic asthma

Charlotte Barck, MD*; Joachim Lundahl, MD, PhD†; Gunilla Halldén, DrMedSC†; and Gunnar Bylin, MD, PhD*

Background: Counting eosinophils in induced sputum seems to be a better way to survey disease activity than indirect clinical assessments of airway inflammation, such as pulmonary function measurement. The conventional analysis of induced sputum, however, is time-consuming and requires skilled personnel, which have restricted its use.

Objective: To determine whether measuring eosinophil cationic protein (ECP) levels in the entire sputum sample could replace the method of counting eosinophils and measuring ECP levels in the supernatant only to establish a clinically more applicable method of studying the intraindividual changes in eosinophilic activity in induced sputum.

Methods: In 13 patients with mild, nonsymptomatic allergic asthma, sputum was induced before and 24 hours after allergen challenge. The entire sputum sample was diluted with 0.1% dithiothreitol, incubated, and divided into 2 parts. One part was processed according to the conventional method, and released ECP levels in the supernatant were measured. The second part was treated with a lysing reagent. Cell debris was separated, and total (intracellular and extracellular) ECP levels in the solution were measured.

Results: We found good correlation between total ECP levels in the entire sputum sample and released ECP levels in the supernatant before ($r = 0.97$) and 24 hours after ($r = 0.99$) allergen challenge ($P < .01$ for both). We also found a good correlation between the changes in total and released ECP levels ($r = 0.99$; $P < .01$).

Conclusions: Total ECP concentration seems to reflect the eosinophilic inflammatory changes in asthma and might be a useful tool in clinical practice.

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INTRODUCTION

Treatment of asthma should be directed primarily toward minimization of airway inflammation.¹ Induced sputum is a safe, valid, reliable, and noninvasive method for investigating airway inflammation in individuals with asthma. Using this method, it has been shown that the number of eosinophils often is increased in asthma.^{2,3} An increase in eosinophilic inflammation is assumed to precede deterioration in asthma.⁴ This assumption was corroborated when it was shown that a strategy to maintain a normal eosinophilic count in induced sputum greatly reduced the number of exacerbations of asthma.⁵

The induced sputum method has the potential to monitor the treatment of asthma, but some factors restrict its use in

clinical practice. Processing induced sputum and counting eosinophils are time-consuming and require skilled laboratory technicians. In addition, the processing has to be performed within hours, which further limits the number of patients that can be examined in parallel. These limitations, until now, have restricted its use mainly to research tasks. If an easier and more rapid method of detecting increased eosinophilic activity were available, it would probably improve the therapy control of asthma in clinical practice.

Attempts to simplify the method by measuring inflammatory markers, including eosinophilic cationic protein (ECP) levels, without separating cells and fluid phase, have been reported with good repeatability.⁶ However, fewer data are available regarding whether these methods are sufficiently sensitive to detect changes in eosinophilic activity, which can signal risks for exacerbations of asthma.

The aim of the present study was to assess a simplified method for evaluating changes in eosinophilic activity in sputum. We therefore induced increased eosinophilic activity using bronchial allergen challenge and measured ECP levels in the entire sputum sample before and after challenge. The entire sputum sample, including saliva and sputum, was used, and we compared this simplified method with the reference method. Our hypothesis was that the information on changes

* Division of Respiratory Medicine and Allergology, Karolinska Institutet, and Department of Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden.

† Department of Clinical Immunology, Karolinska University Hospital, Stockholm, Sweden.

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Table 1. Characteristics of the 13 Study Patients

Patient No.	Age, y	Smoking status	Skin prick test result, mm	Allergen inhaled	FEV ₁ at inclusion, L (% predicted)	Histamine PD _{SRaw} , μ g
1	27	Never	7.5	Timothy	3.34 (92)	172
2	38	Former	15.5	Timothy	4.14 (103)	66
3	34	Never	10.5	Birch	3.98 (85)	402
4	43	Former	6.5	Birch	3.57 (84)	129
5	30	Never	10.0	Birch	2.45 (75)	150
6	38	Never	14.5	Birch	3.96 (87)	74
7	29	Never	10.0	Timothy	4.23 (105)	142
8	37	Never	7.0	Birch	3.61 (82)	128
9	33	Never	8.0	Birch	3.87 (89)	549
10	41	Former	8.5	Birch	4.82 (116)	223
11	45	Current	7.5	Birch	4.57 (104)	147
12	31	Never	12.0	Birch	4.30 (101)	102
13	22	Never	8.5	Birch	4.09 (86)	326
Range	22–45	NA	6.5–15.5	NA	2.5–4.8	66–549

Abbreviations: FEV₁, forced expiratory volume in 1 second; NA, not applicable; histamine PD_{SRaw}, provocation dose of histamine that caused a 100% increase in specific airway resistance.

in eosinophilic activity yielded by ECP levels in the entire sputum sample is the same as that given by ECP levels in the sputum supernatant or by the number of eosinophils.

METHODS

Patients

Thirteen patients with mild asthma, seasonal rhinitis, and allergy to birch or timothy pollen were entered into the study (Table 1). There were 11 men and 2 women aged 22 to 45 years. At a screening visit 1 month before the study started, allergy was confirmed by a positive skin prick test result (>3 mm), and airway hyperresponsiveness was confirmed by a positive histamine inhalation test result. Three patients were sensitized only to birch (patients 9–11), and the others were sensitized to birch, timothy, and animal dander. All patients used inhaled β_2 -agonist as needed, and 11 used inhaled corticosteroids during the pollen season. The minimum interval between corticosteroid use and the start of the study was 2 months, and none of the patients used any corticosteroids or other anti-inflammatory medications during the study, which was outside the pollen season. All patients were instructed not to use bronchodilators the morning before the study started.

This study was conducted in accordance with national and institutional guidelines for human studies. We obtained ethics approval from the ethics committee at Huddinge University Hospital before commencement of the study. Informed consent was obtained from all participants.

Study Design

Each patient visited the laboratory on 2 consecutive days. The morning of day 1, sputum was induced, and a blood sample was obtained. Directly afterward, an allergen bronchial provocation with birch or timothy allergen was performed. Specific airway resistance (SRaw), thoracic gas volume, and

forced expiratory volume in 1 second (FEV₁) were measured before and immediately after allergen inhalation. The FEV₁ was then registered every hour during the day. Symptoms were also recorded. The morning of day 2, the patient returned to the laboratory for FEV₁ measurement, blood sample collection, and sputum induction.

Allergen Bronchial Challenge

Allergen challenge was performed using a dosimeter jet nebulizer (Spira Elektro 2; Respiratory Care Center, Hameenlinna, Finland) with an adjustable aerosol delivery time according to a method described elsewhere.^{7–9} Standardized and freeze-dried birch or timothy allergen extracts (Aquagen; Alk, Copenhagen, Denmark) were diluted and used at a maximum of 4 concentrations: 1,000, 4,000, 16,000, and 64,000 SQ (standardized quality) allergen units $\times$ mL⁻¹. The nebulizer was set to nebulize for 0.5 second, giving an output of 7.1 μ L per breath. At each concentration, 2 and 4 breaths could be taken, and, if needed, 8 and 16 breaths could be taken at the highest concentration, providing doses from 14 to 7,040 SQ allergen units.

Both SRaw and thoracic gas volume were measured in a constant-volume whole-body plethysmograph (model 2000 TB; Cardio Pulmonary Instruments, Houston, TX) 15 minutes after each dose of allergen was given until a 100% increase in SRaw was reached.^{7,10,11} The PD_{SRaw} for allergen was calculated using linear interpolation on a logarithmic scale. The FEV₁ was measured using a portable computerized spirometer (Spirobank; MIR Srl, Rome, Italy) approximately 15 minutes after allergen bronchial challenge. The maximal decline in FEV₁ 3 to 10 hours after allergen challenge was used to measure the asthmatic reaction during the late phase. Throughout the day, and then the following morning, the patients kept a self-administered daily record of bronchodi-

lator medication use and symptoms from the bronchi, nose, and eyes.

Sputum Induction and Processing

Sputum was induced using a standardized method.¹² All the patients were pretreated with an inhaled β_2 -agonist (0.5 mg of terbutaline). Hypertonic saline solution was nebulized using an ultrasonic nebulizer (DeVilbiss 2000; DeVilbiss Co, Somerset, PA) with an output of 1.5 mL/min. Inhalation was performed at intervals of 7 minutes using increasing concentrations of saline solution (3%, 4%, and 5%). The FEV₁ was monitored before commencing and after every inhalation period. All the patients received all 3 concentrations of saline solution. After each inhalation interval, patients were advised to rinse their mouth with water and to blow their nose before trying to cough sputum into a sterile plastic container.

The whole sputum sample was diluted with an equal volume of 0.1% dithiothreitol (Sputolysin; CalbioChem, La Jolla, CA), and the mixture was incubated for 20 minutes on a roller mixer at room temperature. The mixture was thereafter divided into 2 aliquots to compare 2 methods of sputum processing. The first aliquot was processed according to the method described by Fahy et al,¹³ slightly modified. The sputum was centrifuged (at 800g for 10 minutes), and the cell-free supernatant was collected and stored at -70°C for analysis of ECP. These ECP values are referred to as released ECP. The cell fraction was subjected to cytocentrifugation, and cell slides were prepared for May-Grünwald-Giemsa staining; a minimum of 200 nonsquamous cells were counted. Eosinophil counts are expressed as a percentage of the total nonsquamous cell count.

The second aliquot was treated with N-cetyl-N,N,N-trimethylammonium bromide (CTAB) according to a previously reported method.¹⁴ Treatment with the lysing reagent CTAB was performed to bring intracellular and extracellular ECP into the solution. An equal volume of 0.4% CTAB was added to the sputum sample, followed by incubation on a roller mixer at room temperature for 60 minutes. After centrifugation (at 800g for 10 minutes) to yield a cell- and debris-free whole-sputum supernatant, the sample was stored at -70°C for ECP analysis. These ECP values are referred to as total ECP.

Measurement of ECP Levels in Sputum and Cells in Blood

The concentrations of ECP in the entire sputum sample and in the sputum supernatant were determined by using a fluoroimmunoassay (Pharmacia ECP CAP System FEIA; Pharmacia & Upjohn, Uppsala, Sweden), with a detection limit greater than 2 $\mu\text{g/L}$. Control experiments, using samples with known levels of ECP, demonstrated that neither dithiothreitol nor CTAB has any significant effects on the determination of ECP levels. The total number of leukocytes and the differential cell count were measured using routine chemical analysis.

Statistical Analysis

Data are presented as mean $\pm$ SD or median (25th and 75th percentiles), with $P < .05$ taken to indicate statistical significance. For nonnormally distributed variables, the Wilcoxon nonparametric signed rank test for paired observations was used to compare data on cells and inflammatory markers in sputum and blood before and after allergen challenge. Correlations were performed using the Pearson or Spearman test. All analyses were performed using statistical software (SPSS version 11.0; SPSS Inc, Chicago, IL; and STATISTICA version 7.0; StatSoft Inc, Tulsa, OK).

RESULTS

Lung Function and Eosinophil Counts in Blood

After challenge with birch or timothy allergen, all patients developed an immediate increase in SRaw (Table 2). The median provocation dose of allergen that caused a 100% increase in SRaw was 175 SQ units. The mean decrease in FEV₁ during the early phase was 13%, and the mean maximal decline during the late phase was 16%. Seven of the 13 patients had a late asthmatic reaction (FEV₁ decline $>15\%$). Eosinophil counts in blood increased by approximately 50% after allergen challenge ($P = .002$) (Table 3).

Sputum ECP Levels and Percentages of Eosinophils Before and After Allergen Challenge

Before allergen challenge, total ECP levels were higher than released ECP levels ($P = .001$) (Table 4 and Fig 1A). The 2 were highly correlated ($r = 0.97$; $P < .01$). There was no significant correlation between total or released ECP levels and the percentage of eosinophils in sputum before allergen challenge. After allergen challenge, total ECP levels were significantly higher than released ECP levels ($P = .001$) and were highly correlated with released ECP levels ($r = 0.99$; $P < .01$) (Table 4 and Fig 1B). There was no significant correlation between total or released ECP levels and the percentage of eosinophils in sputum after allergen challenge.

Allergen-Induced Changes in Sputum ECP Levels, Eosinophil Counts, and Lung Function

Allergen challenge augmented total and released ECP levels approximately 14-fold (Table 4 and Fig 2). The increase in total ECP levels 24 hours after allergen challenge was highly correlated with the corresponding increase in released ECP levels ($r = 0.99$; $P < .01$). There was a significant but moderate correlation between the changes in total and released ECP on the one hand and the change in the percentage of eosinophils on the other hand ($r = 0.66$; $P = .014$; and $r = 0.59$; $P = .04$, respectively).

There was no correlation between sputum ECP (total or released) levels and the percentage of eosinophils on the one hand and FEV₁ on the other hand before allergen challenge, during the early or late phase. This was also true for the 7 patients with a late-phase reaction.

Table 2. Allergen Bronchial Challenge Lung Function Data

Patient No.	FEV ₁ , L					Allergen PD _{SRaw} SQ units
	Before allergen challenge	Early phase*	Early phase % change	Late phase†	Late phase % change	
1	3.35	2.90	-13	2.92	-13	1,117
2	4.20	3.53	-16	3.04	-28	847
3	3.84	3.69	-4	3.01	-22	194
4	3.65	3.57	-2	3.72	2	972
5	3.36	2.32	-31	2.59	-23	152
6	4.28	3.71	-13	3.85	-10	46
7	4.07	3.43	-16	3.30	-19	175
8	3.83	3.29	-14	2.95	-23	84
9	3.76	3.41	-9	3.24	-14	222
10	4.61	4.07	-12	4.24	-8	90
11	4.93	4.52	-8	3.63	-26	175
12	4.12	3.74	-9	4.20	2	17
13	4.37	3.33	-24	3.30	-25	33
	4.03 ± 0.46‡	3.50 ± 0.53‡	-13§	3.38 ± 0.51‡	-16§	175 (65-535)¶

Abbreviations: Allergen PD_{SRaw}, provocation dose of allergen that caused a 100% increase in specific airway resistance; FEV₁, forced expiratory volume in 1 second.

*Fifteen minutes after allergen challenge.

†Three to 10 hours after allergen challenge (minimum value).

‡Data presented as mean ± SD.

§Data presented as mean.

¶Data presented as median (interquartile range).

Table 3. Eosinophil Counts in Blood Before and 24 Hours After Allergen Challenge*

Patient No.	Eosinophil count, ×10 ⁹ /L	
	Before allergen challenge	After allergen challenge
1	0.11	0.21
2	0.52	0.68
3	0.16	0.23
4	0.25	0.34
5	0.11	0.17
6	0.07	0.13
7	0.12	0.21
8	0.17	0.36
9	0.29	0.52
10	0.11	0.25
11	0.28	0.33
12	0.11	0.17
13	0.51	0.47
Median (interquartile range)	0.16 (0.11-0.29)	0.25 (0.19-0.42)†

*The Wilcoxon paired signed rank test was used for comparisons before and after allergen challenge.

†*P* < .01 vs before allergen challenge.

DISCUSSION

The results of this study demonstrate that the total ECP concentration processed from the entire expectorate, consisting of sputum plus saliva, is a suitable marker to survey the intraindividual changes in eosinophilic inflammation in pa-

tients with allergic asthma. These findings showed a significant correlation between total and released ECP concentrations before and 24 hours after allergen challenge. There was also a high correlation between the allergen-induced changes in total and released ECP levels. These ECP changes were moderately correlated with the changes in eosinophil counts.

Induced sputum is a clinically valid and reliable noninvasive method for directly studying cells and inflammatory markers in the lower airway in people with asthma.^{2,15} In the conventional sputum processing method, total and differential cell counts are determined after homogenization and centrifugation of selected sputum plugs, and various biochemical markers in the supernatant, such as released ECP, are analyzed.^{16,17} An alternative method was introduced by Fahy and coworkers¹³ and in 't Veen et al,¹⁵ who used the entire sputum sample and omitted the plug selection step. To further facilitate application of the sputum method, we modified the approach by Fahy and coworkers¹³ and treated the entire sputum sample with a mucolytic agent followed by a lysing reagent to release intracellular and extracellular ECP into the solution.¹⁴

The total ECP method described herein includes fewer centrifugation steps than the conventional sputum process, and because the method assesses the released and the cellular stored pool of ECP, the determination is presumably less sensitive for ex vivo modulation, which may affect the ratio between released and intracellular stored ECP. It is well established that cell handling procedures can induce cell modulation and subsequent release of intracellular stored

Table 4. Levels of ECP and Percentages of Eosinophils in Sputum Before and 24 Hours After Allergen Challenge*

Patient No.	Total ECP, $\mu\text{g/L}$		Released ECP, $\mu\text{g/L}$		Eosinophils, %	
	Before allergen challenge	After allergen challenge	Before allergen challenge	After allergen challenge	Before allergen challenge	After allergen challenge
1	63.4	815	21.8	235	0	3
2	60.5	2,046	28.7	907	0	14
3	106	5,663	39.5	2,705	0	5
4	732	622	313	243	7	7
5	41.9	382	10.9	165	1	3
6	55.7	292	21	105	0	3
7	45.5	334	17.8	148	2	5
8	55.2	4,195	23.8	1,573	3	38
9	42.5	672	19.2	288	0	9
10	119	798	57.5	361	1.5	3
11	17.4	702	3.56	192	0.1	20
12	176	2,045	65.6	811	12	27
13	25.6	7,169	12.6	4,283	0.1	10
Median (IQR)	55.7 (42.2–112.5)	798 (502–3,120.5)†	21.8 (15.2–48.5)	288 (178.5–1,240.0)†	0.1 (0–2.5)	7 (3–17)†

Abbreviations: ECP, eosinophil cationic protein; IQR, interquartile range.

*The Wilcoxon paired signed rank test was used for comparisons before and after allergen challenge.

† $P < .01$ vs before allergen challenge.

antigens. The design of the total determination method—fewer centrifugation steps combined with being less prone to be affected by the cell handling procedure—will presumably make implementation of this method in outpatient hospital settings more feasible.

Our results show a high correlation between total and released ECP levels. This is in accordance with data from Metso and coworkers,⁶ who reported a significant, although lower, correlation coefficient between total ECP in sputum and released ECP. Whether the observed difference in correlation coefficients depends on differences in patient selection or the sputum processing procedures used can only be speculated on. For example, in the study by Metso et al.,⁶ as opposed to the present study, sputum plugs were selected for further analysis. The high correlation between total and released ECP levels suggests that total ECP can replace released ECP in baseline measurements, at least when analyzed in the entire sputum sample.

Whether a correlation between released ECP and the eosinophil count at baseline exists is a matter of debate. In the present study, we did not find a significant correlation between these 2 variables. This finding is supported by Fahy and coworkers¹³ and is in line with the concept that the ECP measured in the supernatant reflects degranulation and does not necessarily correlate well with sputum eosinophil counts. Other investigators^{6,18,19} have reported a moderate correlation between released ECP levels and eosinophil counts. This discrepancy may be due partly to patient selection and partly to the different methods used.^{6,18}

An essential aim of the present study was to evaluate whether our method detects increased eosinophilic activity in

asthmatic patients after exposure to allergen. Indeed, induced sputum collected 24 hours after allergen challenge had significantly higher total and released ECP levels and percentages of eosinophils than induced sputum collected at baseline. The magnitudes of the changes in released ECP levels and eosinophil counts are similar to those previously reported.²⁰ We also found a good correlation between the allergen-induced changes in total and released ECP levels, which suggests that total ECP can replace released ECP in the evaluation of changes in eosinophilic activity.

Our inferences on the relationships between total and released ECP levels before and after allergen challenge are drawn from patients with a mild form of allergic asthma. We chose these patients because they constitute most patients with allergic asthma. However, the relationship between total and released ECP levels after allergen challenge may not necessarily be the same in patients with more severe allergic asthma. The relationships between total and released ECP levels in varying stages of eosinophilic activity in patients with nonallergic asthma might also differ from what is true for allergic asthma.

Sputum eosinophil counts are a direct marker of airway inflammation, but the assessments are time-consuming. It has been shown that a treatment strategy directed at normalization of induced sputum eosinophil counts reduces asthma exacerbations.⁵ Because our data show that changes in total ECP levels correlate with changes in eosinophil counts, our simplified method of measuring ECP concentrations in the entire sputum sample may be a useful tool for monitoring the severity of asthma across time. However, prospective clinical

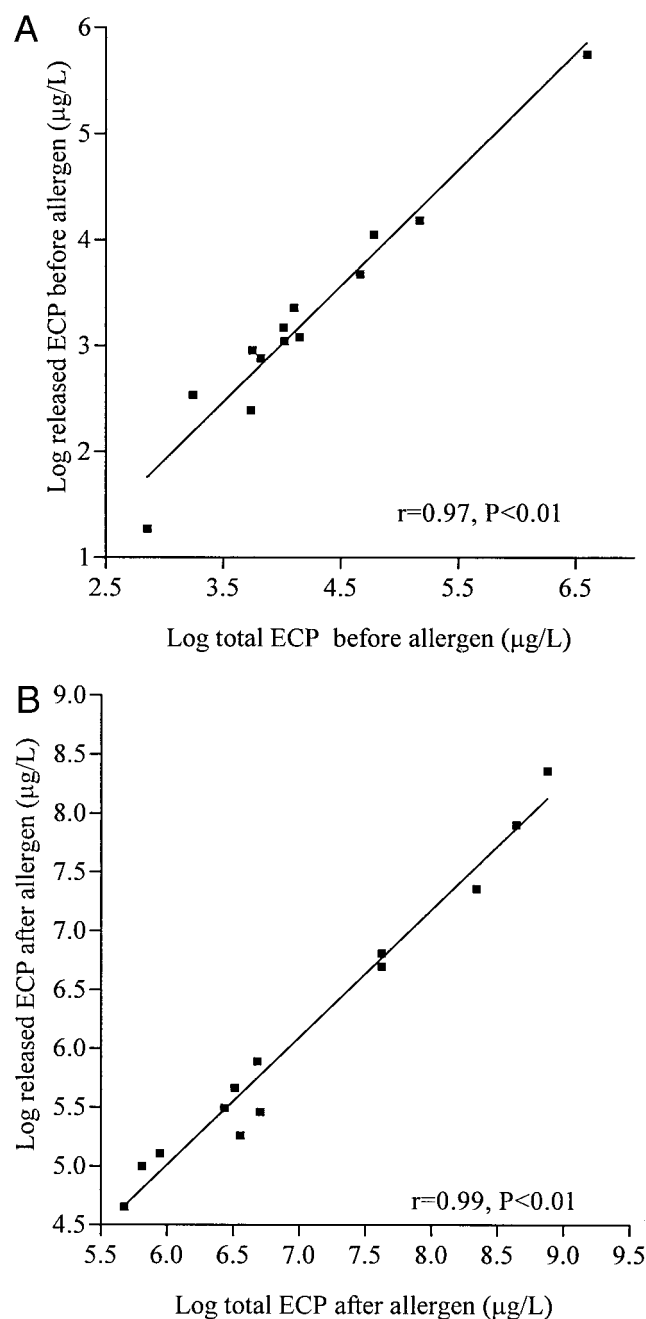


Figure 1. Correlation between total and released eosinophil cationic protein (ECP) levels in induced sputum before (A) and after (B) allergen challenge.

studies of patients with allergic and nonallergic asthma are necessary to support this assumption.

We propose that measuring total ECP levels in the entire sputum sample, reflecting intracellular and extracellular ECP, is suitable for recording changes in eosinophilic activity. Because the method is simplified compared with the refer-

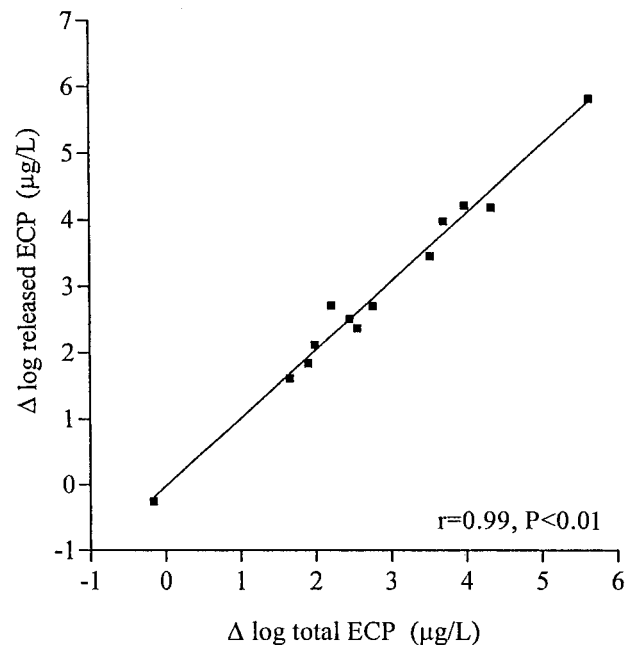


Figure 2. Correlation between changes in total and released eosinophil cationic protein (ECP) levels in induced sputum after allergen challenge.

ence method, its implementation in clinical practice, and in epidemiologic and environmental exposure studies, should be feasible. The method could be a useful tool for monitoring asthma severity and the effects of pharmacologic treatment.

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- Requests for reprints should be addressed to:*
Charlotte Barck, MD
Department of Respiratory Medicine and Allergy
Karolinska University Hospital Huddinge
S-141 86 Stockholm, Sweden
E-mail: charlotte.barck@medhs.ki.se
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