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Eosinophil cationic protein in sputum is dependent on temperature and time

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Eosinophil cationic protein in sputum is dependent on temperature and time. E. Grebski, Ch. Graf, G. Hinz, B. Wüthrich, T.C. Medici. ©ERS Journals Ltd 1998.

ABSTRACT: Eosinophil cationic protein (ECP) in sputum may be used to estimate the severity of bronchial inflammation and obstruction in asthmatics as well as to monitor asthma drug therapy. For this purpose, standardized processing of sputum is important. The aim of our study was to determine whether time and temperature influence the ECP concentration in the sputum of asthmatics.

The samples of induced sputum obtained from 12 patients with stable asthma were homogenized using ultrasonification, and centrifuged. Supernatants were evenly divided and stored for 1, 6, 24 or 72 h at either 4 or 25°C, then frozen at -80°C. The ECP concentrations were determined using fluoroimmunoassay and compared with the immediately frozen samples.

After storing at 4°C, the ECP levels at the four time points were 101.2, 96.0, 98.2 and 90.6% of the initial concentration, respectively. When sputum specimens were stored at 25°C, ECP levels decreased to 96.1, 94.4, 90.7 and 87.7%, respectively. The influence of time on ECP concentrations in sputa was statistically significant (p=0.02). A significant temperature effect was found when comparing the specimens stored at 4°C with those at 25°C (p=0.03). Looking at individual time points, a significant decrease in ECP concentration was only seen at 25°C after 24 and 72 h.

We conclude that eosinophilic cationic protein in the sputum of asthmatics decreases in a time- and temperature-dependent process. If sputa cannot be processed after obtaining the specimens, they should be stored in a refrigerator at 4°C, until eosinophilic cationic protein is measured.

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The eosinophil cationic protein (ECP) in sputum may be used to estimate the severity of bronchial inflammation and obstruction in asthmatics, as well as to monitor asthma drug therapy [1–3]. However, sputum ECP concentrations vary significantly in different studies, due to differences in collecting and processing sputum samples. Hence, standardized processing of sputum, *i.e.,* induction, homogenization and centrifugation, is important.

Little is known about the influence of storage conditions on the stability of ECP in sputum. Therefore, the aim of our study was to determine whether time and temperature influence ECP concentration in homogenized and centrifuged sputum specimens of asthmatics.

Materials and methods

Twelve adult subjects with stable mild-to-moderate asthma as defined by the American Thoracic Society [4] were randomly selected for the study. All patients gave informed written consent.

Clinical assessment involved a full history (including a smoking history and details of current and past medication) and auscultation of the heart and lungs. Skin-prick tests (Soluprick; ALK, Copenhagen, Denmark) with the most common perennial allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog and horse hair, wheat flour), histamine and diluent control were performed.

Measurements of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1) and peak expiratory flow (PEF) were performed according to American Thoracic Society criteria [5] using a wet spirometer (Pulmonary Function Lab 2400; SensorMedics, Bilthoven, the Netherlands).

Methacholine responsiveness was measured using a breath-activated dosimeter (Mefar "MB 3", Bovezzo, Italy), with subjects breathing from functional residual capacity to total lung capacity [6]. Subjects took 10 successive tidal breaths of normal saline followed by increasing cumulative doses of methacholine: 100, 250, 500, 1,000 and 2,000 μg. The results were expressed as the provocative dose giving a 20% fall in FEV_1, as obtained from the log dose-response curve by linear interpolation of the last two points (PD_{20}).

Venous blood samples were collected and eosinophils counted using an automated haematology analyser (H 2 Technicon Hemalog, NY, USA).

For ECP determinations, venous blood was taken with the use of 4 mL serum separating tube (SST) vacutainer tubes (Becton Dickinson, Meyran, France) and allowed to clot at stable room temperature for 60 min before centrifugation. The samples were then centrifuged at 1400×g for

10 min (Megafuge 1.0 R, Heraeus, Zurich, Switzerland) and the serum separated and frozen at -20°C.

Sputum processing

To induce sputum, subjects inhaled a sterile 3% saline solution from an ultrasonic nebulizer (Heyer Mono, Carl Heyer GmbH, Germany) for 10 min. The nebulizer generates particles with a mean diameter of 4.6 μm and has an output of 2 mL·min^{-1}. Subjects were encouraged to cough throughout the procedure and to expectorate all secretions raised (sputum and saliva) into a plastic container. The volume of the sample was measured. One gram of sputum was used to determine the total cell count with a standard haemocytometer. To that purpose, an equal volume of a 20% formalin solution was added to protect cells by fixation before liquefying the specimens by ultrasonification, as described by OPPENHEIMER *et al*. [7]. Two smears of the unfixed sputum were prepared and stained by the Papanicolaou method. A differential count of the cell types was performed, including all types of inflammatory and bronchial epithelial cells [8, 9]. Eosinophil percentage, as well as total cell number per millilitre, was determined.

Sputum specimens were then homogenized with an ultrasonic Branson Sonifier (Sonic Power CO, Danbury, CT, USA) for 30 s at maximal output, before being centrifuged at 1400×g for 10 min at 4°C. One portion of supernatant was aspirated and frozen at -80°C. The rest was evenly divided into eight portions and stored for 1, 6, 24 or 72 h either at 4 or 25°C, and then frozen at -80°C. The ECP concentrations were determined using fluoroimmunoassay and compared with the immediately frozen samples.

ECP concentrations

Serum and sputum ECP concentrations were measured by an ECP fluoroenzyme immunoassay (FEIA) method (Pharmacia CAP system), according to the instructions of the manufacturer (Pharmacia Diagnostics AB, Uppsala, Sweden). Briefly, monoclonal antiECP antibodies covalently coupled to immunoCAP were allowed to react with patient samples and subsequently, after washing, with monoclonal antiECP antibodies containing fluorogenic labelling. The level of fluorescence after adding fluorogenic substrate was measured. The measuring range was 2–200 μg·L^{-1}. The serum samples remained undiluted, those of sputum were diluted in the ratio of 1:10, and each was assayed twice.

Statistical analysis

The mean values of the ECP measurements in duplicate constituted the input data for statistics. As ECP data show a skewed distribution, changes in per cent of initial values were used for statistical evaluation. Variance analysis with a repeated measures design was performed (univariate comparisons with mixed-model approach; Statistical Products and Service Solutions (SPSS) Advanced Statistics 6.1). Besides the factors: time and temperature, the inhalation of steroids was considered as an interindividual dichotomous variable. Significant results were confirmed with an analysis on log-transformed ECP values. The global significance level was defined with α=0.05 for two-sided tests. Since time and temperature were the primary parameters, the lower p-value of these results has to be compared with significance threshold α=0.025 (Bonferroni-Holm method) [10]. Further secondary comparisons at individual time points were done with t-test for paired observations. Descriptive data comprise geometric means with regard to ECP measurements and mean±SD for the other parameters.

Results

The characteristics of the study population and the results of the pulmonary function are shown in table 1. On average the patients collected 9.6 g of sputum over a period of 10 min, ranging 2.6–23.1 g.

ECP concentrations in the sputum of asthmatics after specimens storage are shown in table 2. After 0, 1, 6, 24 and 72 h at 4°C, the geometric mean of ECP was 320.5, 320.5, 305.2, 311.7, and 284.9 μg·L^{-1}. When sputum specimens were stored at 25°C, the geometric mean of ECP decreased from 320.5 to 306.1, 301.0, 286.9, and 277.8 μg·L^{-1}, respectively. A distinct and significant influence of time on ECP concentration in sputum was shown (p= 0.02). The analysis of temperature effect between the two series observed during 72 h demonstrated a significant difference (p=0.03). Further comparisons of the individual time points with the initial values are shown in table 3, where a significant lowering of ECP concentration is seen after 24 and 72 h, when storing the specimens at room temperature.

In patients treated with inhaled steroid, time-dependent decreases in ECP concentrations were somewhat clearer than in patients not receiving steroids. However, compari-

Table 1. – Characteristics of the 12 study patients

Patients	
Sex Females/Males	2/10
Age yrs	40.0±11.8
Smokers n	2
Therapy	
Inhaled steroid n	6
β-agonist n	11
Pulmonary function	
FVC % pred	109.4±12.3
FEV_1 % pred	87.7±20.1
FEV_1 %FVC	67.3±13.3
PEF % pred	88.2±22.0
PD_{20} μg methacholine	208 (25–2000)
Blood	
Eosinophils %	5.2±2.8
ECP μg·L^{-1}	14.6 (6.9–45.9)
Sputum	
Total cell count ×10^6·L^{-1}	2.13±1.52
Eosinophils %	14.0±19.4
Epithelial cells %	14.7±9.4
ECP μg·L^{-1}	320 (57–1728)

Values are absolute number, mean±SD or geometric mean with range in parenthesis. FVC: forced vital capacity; % pred: percentage of predicted value; FEV_1: forced expiratory volume in one second; PEF: peak expiratory flow; PD_{20}: provocative dose causing a 20% fall in FEV_1; ECP: eosinophil cationic protein.

Table 2. – Influence of time and temperature on eosinophil cationic protein (ECP) concentration in sputum of asthmatics

Time h	ECP concentration			
	Temperature 4°C		Temperature 25°C	
	µg·L^{-1}	% of initial value	µg·L^{-1}	% of initial value
0	320.5 (57.3–1728)	100	320.5 (57.3–1728)	100
1	320.5 (54.2–1819)	101.2±17.2	306.1 (56.4–1803)	96.1±10.7
6	305.2 (58.1–1621)	96.0±12.4	301.0 (57.8–1667)	94.4±9.9
24	311.7 (58.8–1617)	98.2±15.2	286.9 (52.9–1757)	90.7±14.8
72	284.9 (53.9–1727)	90.6±17.9	277.8 (52.7–1616)	87.7±13.9

Values are geometric mean with range in parentheses, or mean±SD.

Table 3. – Change of eosinophil cationic protein (ECP) concentration in per cent of baseline in the sputum of asthmatics during 1, 6, 24 and 72 h

Time h	ECP concentration					
	Temperature 4°C			Temperature 25°C		
	Mean %	95% CI	p-value^{+}	Mean %	95% CI	p-value^{+}
1	1.2	(-9.7,+12.0)	0.82	-3.9	(-11.0,+3.2)	0.25
6	-4.0	(-11.1,+3.1)	0.24	-5.6	(-11.9,+0.7)	0.07
24	-1.8	(-9.0,+5.5)	0.60	-9.3	(-17.7,-0.9)	0.03
72	-9.4	(-19.8,+1.0)	0.07	-12.3	(-21.0,-3.5)	0.01

95% CI: 95% confidence interval of mean. $^{+}$: paired t-test.

son of these small subgroups did not reach statistical significance.

Discussion

In this study, we examined the stability of ECP of sputum specimens over time at room temperature and at 4°C. Our principal finding is that ECP in sputum is dependent on time and temperature, *i.e.* ECP concentrations decrease during storing and this effect is mainly seen at room temperature. Looking at the individual time points, the decrease of ECP concentrations in sputa reached statistical significance after 24 and 72 h of storing at 25°C. When specimens were stored at 4°C, there was a trend towards lower values only.

Little is known about the influence of storage conditions on ECP concentrations in sputum. Motojima *et al.* [11] examined the effect of 3 and 6 h of storage at 4°C in five sputum specimens. They found a statistically insignificant decrease of ECP after 3 h from 994±0.43 ng·mL^{-1} (geometric mean±log SD) to 891±0.3 ng·mL^{-1} and after 6 h from 1,208±0.4 ng·mL^{-1} to 875±.2 ng·mL^{-1}. However, to prevent degradation of ECP concentration in sputum, the authors recommended the addition of protamine sulphate (1/10 of sputum volume) after centrifugation [12].

In contrast to this unique observation, there are more data about the influence of time and temperature on ECP in serum. Reimert *et al.* [13] demonstrated a time- and temperature-dependent spontaneous release of ECP *in vitro*, resulting in an increase of ECP concentration. The greatest amount was released within the first 4 h of incubation. In a study similar to ours, Wantke *et al.* [14] found ECP levels in serum of asthmatics to decrease by on average 13% after 24 h storage at room temperature. The difference was not significant, but a statistical test of small power was used.

Contrary to other investigators, we homogenized sputum by ultrasonification. Using this method, the amount of ECP measured most likely reflects the ECP liberated by active secretion as well as from ultrasonically destroyed eosinophils. The reason why we choose ultrasonification was the fact that mechanical homogenization of sputum using VirTris homogenization, Ten Broeck glass homogenization or Branson ultrasonification had not been shown to destroy either proteins, peptides or enzymes released from inflammatory cells into sputum [15], unlike chemical agents. Whether dithiothreitol which splits S-S bonds of bronchial mucins as well as other proteins including immunoglobulin A [16] and which is widely used to homogenize sputum, destroys proteins is unknown so far. Given these properties, dithiothreitol may well have a detrimental effect on ECP or other molecules present in sputa of asthmatics.

Eosinophil cationic protein in the sputa of asthmatics decreased in a time- and temperature-dependent process. If the sputa cannot be processed after obtaining the specimens, they should be stored in a refrigerator at 4°C, until eosinophil cationic protein is measured. In order to achieve reliable and comparable results, the specimens should be collected and processed in a standardized way.

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