

The relationship of eosinophil granule proteins to ions in the sputum of patients with cystic fibrosis

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Summary

Background Increased sputum levels of eosinophil granule proteins have been reported despite normal eosinophil numbers in peripheral blood and in the lung in cystic fibrosis (CF). Mechanisms of eosinophil priming and activation are still unclear in CF.

Objective In the present study we investigated whether ion concentrations in the sputa of CF patients are related to eosinophil activity.

Methods We assessed concentrations of eosinophil cationic protein (ECP), eosinophil protein X (EPX), major basic protein (MBP) and ions (Na^+ , Cl^- , Ca^{2+} , Mg^{2+}) in sputum samples of 29 children with CF as well as in 10 controls with bronchial asthma.

Results Patients with CF demonstrated significantly higher levels of ECP, Na^+ , Cl^- and Ca^{2+} levels than asthmatics ($P < 0.04$, $P < 0.0001$, $P < 0.0001$, $P < 0.02$). No differences were seen between concentrations of EPX and Mg^{2+} in the two groups. In CF, eosinophil granule proteins correlated significantly with Ca^{2+} and Mg^{2+} concentrations (ECP, $P < 0.0001$, $r = 0.65$, $P < 0.0001$, $r = 0.66$; MBP, $P < 0.03$, $r = 0.41$, $P < 0.03$, $r = 0.42$), furthermore inversely with Cl^- concentrations (ECP, $P < 0.0003$, $r = -0.63$; EPX, $P < 0.02$, $r = -0.45$; MBP, $P < 0.03$, $r = -0.41$) but not with Na^+ levels. ECP, Na^+ and Cl^- were also correlated with lung function variables (FVC, $P < 0.04$, $r = -0.38$, $P < 0.02$, $r = 0.44$, $P < 0.03$, $r = 0.41$; FEV1, $P < 0.007$, $r = -0.49$, $P < 0.006$, $r = 0.5$, $P < 0.008$, $r = 0.48$; MEF50, $P < 0.003$, $r = -0.54$, NS, $P < 0.03$, $r = 0.42$; MEF25, $P < 0.039$, $r = -0.4$, $P < 0.005$, $r = 0.51$, $P < 0.05$, $r = 0.37$).

Conclusion Our results demonstrated a significant relationship of eosinophil degranulation and ions in CF, indicating that ion composition in CF sputa may be at least partly responsible for high levels of eosinophil products despite low eosinophil numbers.

Keywords: bronchial asthma, cystic fibrosis, sputum ions, eosinophil granule proteins, lung function

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Introduction

Cystic fibrosis is a fatal genetic disease caused by abnormalities in fluid and electrolyte transport in exocrine epithelia [1,2]. Alteration in CF transmembrane conductance regulator protein leads to an impermeability of the luminal surface to Cl^- [3]. The consequence is a defective Cl^- secretion, together with an excessive Na^+ absorption.

The faulty transepithelial ion transport leads to abnormal rheological properties of CF respiratory mucus [4]. In addition, the efficacy of mucociliary clearance is related to the water content and rheological properties of sputum [5,6]. It has been demonstrated that sputum Na^+ and Cl^- concentrations are related to sputum hydration and rheology and hence probably influence lung function parameters.

Recently, increased serum and sputum levels of eosinophil granule proteins (eosinophil cationic protein (ECP), eosinophil protein X (EPX) eosinophil peroxidase (EPO) and

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major basic protein [MBP]) despite normal eosinophil numbers in CF have been reported [7–9]. These proteins are accepted to be specific eosinophil products with cytotoxic properties, i.e. reduction of mucociliary clearance, epithelial cell injury, and promotion of fibrosis [10–13]. However, mechanisms of eosinophil priming and activation in CF remain unclear. Since *in vitro* studies demonstrated a significant influence of Mg^{2+} and Ca^{2+} on eosinophil degranulation [14], we examined concentrations of Na^+ and Cl^- , that are known to be dysregulated in CF, as well as Mg^{2+} and Ca^{2+} and looked for a relationship to eosinophil activation in sputum samples derived from CF patients. Since bronchial asthma is characterized by high levels of eosinophil granule proteins in both the lung and in serum but showing no abnormal ion transport, patients with bronchial asthma were used as controls.

Materials and methods

Patients and control subjects

Twenty-nine children (16 male, 13 female; mean age $\pm$ SD 12.1 ± 3.5) with CF, were recruited (Table 1). The diagnosis of CF was confirmed by repeated pathological sweat-test results in all patients. The clinical condition was assessed by the Shwachman-Kulczycky score [15] without reviewing the X-ray, resulting in a maximum score of 75 points. The higher the score, the better is the clinical condition of the patients. Of the patients, 9 were atopic based on increased total serum-IgE antibodies (224 kU/L, 42–884) vs. nonatopic patients with CF (15 kU/L, 6–198) and the presence of specific IgE antibodies (positive results RAST class ≥ 2). None of the patients received inhaled or systemic steroids within a month before sputum sampling.

In addition, 10 patients with symptomatic atopic bronchial asthma (five females and five males; age 12.7 ± 3.4) were recruited (Table 1). Patients were defined as having asthma on the basis of clinical signs relieved by inhaled β_2 -agonists.

In addition reversible airway obstruction was detected by pulmonary function tests. Atopy was considered as a result of positive skin prick test to more than one common allergen, increased total serum IgE concentrations (425; 322–712), and the presence of specific IgE antibodies (RAST class ≥ 2). Patients were treated with various combinations of inhaled β_2 -agonists (salbutamol/terbutalin) and inhaled glucocorticoids (budenoside/fluticasone).

Sputum preparation

Sputum samples (1 g) were centrifuged at 2000 *g* for 20 min at 4 °C. After this procedure sputum supernatant was immediately frozen at –20 °C, as previously described [16]. Supernatant was used for analyses of eosinophil proteins. To determine ion levels, undiluted CF sputa were ultracentrifuged and supernatant was used for analyses. Regarding ECP no differences for levels in ultracentrifuged or normally centrifuged sputum were observed (median 2.38 mg/L vs median: 2.45 mg/L).

An aliquot of diluted sputum from each patient was used for cell differential counts and was stained with May-Grünwald-Giemsa stain. Before it has been shown by Trypan blue exclusion that the viability of cells in the samples was $> 65\%$. Also the percentage of squamous cells was less than 10% indicating that no notable salivary contamination was present.

Sputum measurements of ECP, EPX and MBP

Serum ECP and EPX concentrations were determined by using a double antibody radioimmunoassay (Pharmacia Upjohn AB, Uppsala, Sweden). ECP and EPX in the sample compete with a fixed amount of ^{125}I labelled ECP/EPX for the binding sites of specific antibodies [17]. The intra-assay coefficient of variation was less than 3% and the interassay coefficient of variation was below 11% for all assays.

For determination of MBP sputum samples were reduced

	CF (<i>n</i> = 29)	Bronchial asthma (<i>n</i> = 10)	<i>P</i> -value <
Age	12.6 (9.4–15.3)	12.6 (7.7–18.4)	NS
Sex (boys/girls)	16/13	5/5	–
Score (points)	64 (58–69)	–	–
Total serum IgE (kU/L)	24 (14–198)	425 (322–712)	0.001
FVC percentage predicted	85.7 (61–94.5)	96.74 (76.2–128.0)	0.02
FEV ₁ % predicted	55.9 (41.4–83.6)	85.25 (62.4–99.9)	0.03
MEF50% predicted	28.7 (20.9–58.4)	59.3 (41.2–83.2)	0.04
MEF25% predicted	18.8 (10.8–34.9)	42.9 (27.1–57.9)	NS

Results are presented as median (quartile 1 – quartile 3). FVC: forced vital capacity; FEV₁: forced expiratory volume in one second; MEF50/25%: maximum expiratory flow at 50/25% of vital capacity; ND: not done; NS: not significant.

Table 1. Characteristics of patients with cystic fibrosis and control subjects with bronchial asthma

and alkylated to recover full detection of MBP [18]. MBP concentrations were determined by a two site immuno-radiometric assay employing two monoclonal antibodies [19]. In brief, reduced and alkylated sputum samples as well as standards were placed in with capture antibody coated 96-well plates and incubated for two hours. After washing a 125-iodinated antibody was placed in the wells and incubated for one hour. Wells were washed and radiation counted in a gamma scintillation counter [18,19].

Sputum measurements of ions

Na^+ , Cl^- , Ca^{2+} and Mg^{2+} concentrations in sputum samples (undiluted sputum) were determined by using Ektachem clinical chemistry slides (Johnson & Johnson, Clinical Diagnostics, Rochester, New York, USA). Ion concentrations are measured by direct potentiometry. In brief, the potential difference poised between two electrodes is proportional to the ion concentration in the sample. Variability for ion measurement in serum was low in all cases (sodium: below 2%; chloride: below 1.5%; calcium: below 4%; magnesium: below 15%). Detection limits were 1 mmol/L for sodium, 50 mmol/L for chloride, 0.2 mg/dL for magnesium and 1 mg/dL for calcium. Standard curves were linear in all cases (sodium: intercept: 0.00, slope: 0.99; chloride: intercept: 1.81, slope: 0.98; calcium: intercept: 0.05, slope: 0.99; magnesium: intercept: 0.00, slope: 0.99) for serum.

Lung function

Pulmonary function tests were performed in all patients with CF and asthma. Forced vital capacity (FVC), forced expiratory volume in one second (FEV_1) and maximum expiratory flow at 50% of vital capacity (MEF50%) and at 25% of vital capacity (MEF25%) were recorded in form of a maximum flow-volume curve (Masterlab, Jaeger, Würzburg, Germany) according to American Thoracic Society (ATS) standards [20]. The best of three efforts was used for calculations. Results were expressed as percent predicted, based on accepted reference standards referring to Zapletal [21].

Statistical analysis

Results are presented as median (quartile 1 – quartile 3) unless mentioned otherwise. Differences between the groups were examined by the *t*-test. Coefficients of correlation were obtained by means of the Pearson correlation procedure. The level of significance was considered at the fifth percentile ($P < 0.05$).

Results

Concentrations of ECP, EPX and MBP

In patients with cystic fibrosis significantly higher sputum ECP concentrations compared with our asthmatic patients were observed (Fig. 2, Table 2). In contrast no differences of EPX concentrations between the two groups were observed. However, in bigger cohorts no differences between patients with CF and bronchial asthma have been reported. MBP levels were not analysed in asthmatics, but were a hundred fold lower in CF patients (Table 2) than it has been reported of patients with bronchial asthma. Atopy did not influence eosinophil protein or electrolyte levels in CF.

Concentrations of Na^+ , Cl^- , Ca^{2+} and Mg^{2+}

In patients with CF Na^+ , Cl^- and Ca^{2+} concentrations were significantly different to patients with bronchial asthma ($P < 0.0001$, $P < 0.0001$; $P < 0.017$; Table 2). Mg^{2+} concentrations did not differ between the groups (Table 2).

Correlations

A significant relationship between eosinophil proteins and Cl^- (ECP, $r = -0.63$, $P < 0.0003$; EPX, $r = -0.45$, $P < 0.02$; MBP, $r = -0.41$, $P < 0.03$), Ca^{2+} (ECP, $r = 0.65$, $P < 0.0001$; MBP, $r = 0.41$, $P < 0.03$) and Mg^{2+} (ECP, $r = 0.66$; $P < 0.0001$; MBP, $r = 0.42$, $P < 0.03$) but not to Na^+ levels was observed (Table 3, Fig. 1). In patients with CF variables of pulmonary function were significantly correlated with sputum ECP levels (FVC, $r = -0.38$, $P < 0.04$; FEV_1 , $r = -0.49$; $P < 0.007$; MEF 50, $r = -0.54$, $P < 0.003$; MEF25, $r = -0.39$, $P < 0.04$), but not with levels of EPX or MBP. No relationship to the severity of CF as determined by the Shwachman-Kulczycki score was seen [15]. In addition, levels of Cl^- and Na^+ also correlated significantly with lung function parameters (Table 3), whereas concentrations of Ca^{2+} and Mg^{2+} did not show any relationship. No correlation of ions to the severity of CF as determined by the Shwachman-Kulczycki score was observed [15]. In patients with bronchial asthma, a significant correlation between eosinophil proteins and Na^+ (ECP, $r = -0.74$, $P < 0.02$; EPX, $r = -0.79$, $P < 0.007$) but not to Cl^- , Ca^{2+} , Mg^{2+} or variables of pulmonary function was observed.

Discussion

The findings of this study demonstrated very high levels of

	CF (n = 29)	Bronchial asthma (n = 10)	P-value <
ECP (mg/L)	2.7 (2.34–3.53)	2.2 (1.78–2.83)	0.04
EPX (mg/L)	7.98 (6.99–9.18)	8.06 (7.04–8.28)	NS
MBP (µg/L)	47.1 (25.9–101.1)	ND	–
Na ⁺ (mmol/L)	135 (133–139)	32 (30–42)	0.0001
Cl [−] (mmol/L)	80 (74–83)	37 (28–48)	0.0001
Ca ²⁺ (mmol/L)	0.7 (0.5–1.2)	0.3 (0.2–0.5)	0.02
Mg ²⁺ (mmol/L)	1.2 (0.9–1.3)	1.1 (1–1.1)	NS

Results are presented as median (quartile 1 – quartile 3). ND: not done; NS: not significant.

ECP and EPX but low levels of MBP in sputum samples from patients with cystic fibrosis. Furthermore, a strong relationship between eosinophil granule proteins and Cl[−] as an indirect marker of sputum water content and rheology was observed. In addition, ECP and MBP were correlated to Ca²⁺ and Mg²⁺. In contrast to bronchial asthma, in CF high levels of eosinophil proteins in the sera and sputa of the patients are associated with a normal eosinophil count in peripheral blood and in the lung [22]. Previously, we were able to show, that eosinophils in CF have an increased propensity to release their granule proteins. Furthermore, other inflammatory cytokines than in bronchial asthma have been demonstrated in the airways of CF patients that may play a role in eosinophil priming and activation [23]. However, there may be other mechanisms involved in degranulation of eosinophils in CF respiratory tract. It has been shown *in vitro* that increasing concentrations of either Ca²⁺ or Mg²⁺ stimulated the release of ECP from eosinophils [14]. In the present study we have demonstrated, that sputum concentrations of eosinophil proteins were significantly correlated with levels of Ca²⁺ and Mg²⁺ in the sputa. Although a simple correlation does not prove any causality, it could be speculated, that high Mg²⁺ and Ca²⁺ concentrations in the sputum contribute to eosinophil degranulation and to the high levels of eosinophil proteins in the sputum of patients with CF. In contrast to Mg²⁺

concentrations, Ca²⁺ concentrations in CF patients were significantly higher than in asthmatics. The elevated Ca²⁺ levels appear to be caused by increased concentrations of calcium regulating substances as calcitonin and calmodulin in the sputum that may be an expression of the genetic defect found in CF [24–26]. There are no studies reporting about any influence of chloride on eosinophil degranulation. Nevertheless we found a strong inverse relationship between Cl[−] concentrations and levels of eosinophil proteins. Nevertheless, it has been reported, that eosinophil proteins are capable to directly influence airway secretions through influencing both Cl[−] secretion and mucus production *in vitro* [27,28]. First, it has been shown in an animal model that human eosinophil granule MBP selectively increased net Cl[−] secretion across the short circuited canine tracheal epithelium without significantly affecting net Na⁺ transport [28]. The strongest effect showed MBP, ECP played a minor role, EPX had no effect on Cl[−] secretion. In contrast to these results, Cl[−] concentrations in CF sputa were lower in patients with high concentrations of eosinophil proteins. Indeed, in our study levels of MBP in patients with CF, that showed the strongest influence on Cl[−] secretion, were more than a 100-fold lower than it has been reported by numerous studies in asthmatic patients whereas levels of ECP and EPX were much higher. Furthermore, we found significant correlations between concentrations of Cl[−] and

Table 2. Concentrations of eosinophil cationic protein (ECP), eosinophil protein X (EPX), major basic protein (MBP) and ions in the sputum of children with CF and subjects with bronchial asthma

Table 3. Correlation between ions and variables of pulmonary function in children with cystic fibrosis

	Cl [−]	Na ⁺	Ca ²⁺	Mg ²⁺
FVC percentage pred.	r = 0.41; P < 0.03	r = 0.44; P < 0.02	NS	NS
FEV ₁ % pred.	r = 0.48; P < 0.008	r = 0.5; P < 0.006	NS	NS
MEF50% pred.	r = 0.42; P < 0.03	NS	NS	NS
MEF25% pred.	r = 0.37; P < 0.05	r = 0.51; P < 0.005	NS	NS
ECP (mg/L)	r = − 0.63; P < 0.0003	NS	r = 0.65; P < 0.0001	r = 0.66; P < 0.0001
EPX (mg/L)	r = − 0.45; P < 0.02	NS	NS	NS
MBP (µg/L)	r = − 0.41; P < 0.03	NS	r = 0.41; P < 0.03	r = 0.42; P < 0.03

FVC: forced vital capacity; FEV₁: forced expiratory volume in one second; MEF50/25%: maximum expiratory flow at 50/25% of vital capacity; NS: not significant.

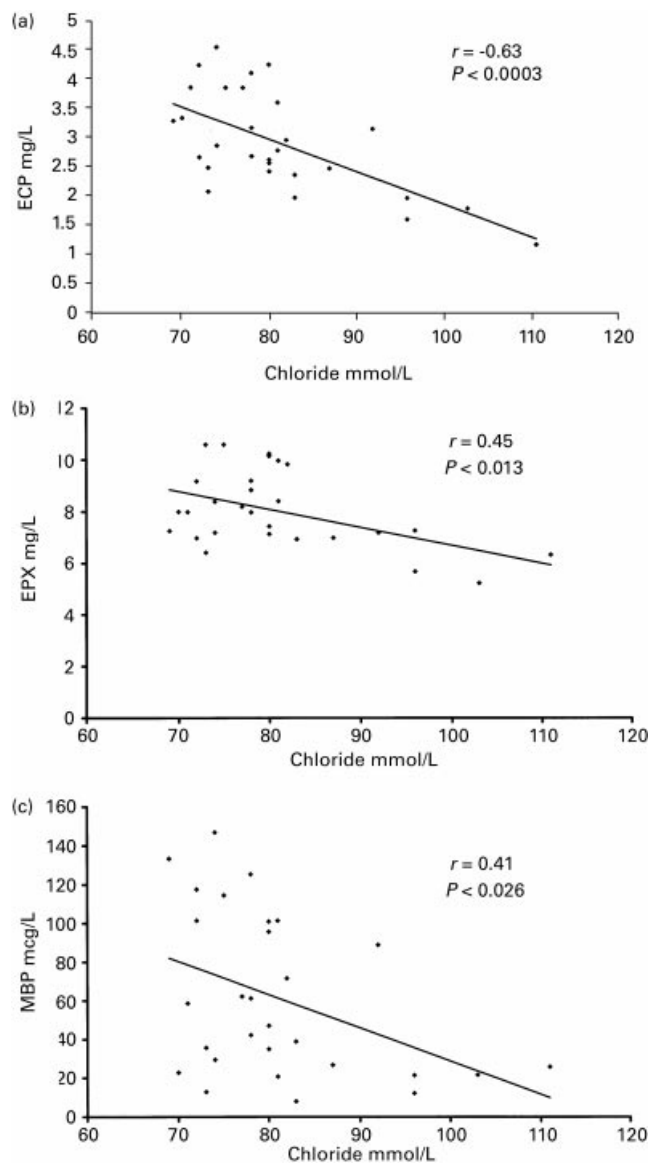


Fig. 1. Correlation between chloride and ECP (a), EPX (b) and MBP (c) in patients with CF

Na^+ and lung function parameters. Ion transport processes are essential for normal mucociliary clearance. As Cl^- and Na^+ secretions promote fluid movement from blood to airway lumen they might have a major influence on mucociliary clearance and cough mechanism. Additionally, ECP levels, but not concentrations of EPX or MBP were strongly correlated with lung function parameters. A possible explanation for that could be that eosinophils are activated during bacterial infection and this results in preferential release of ECP. In conclusion, we were able to demonstrate high sputum concentrations of ECP, EPX and MBP in patients with CF in a range that is highly cytotoxic *in vitro*, correlating positively with Ca^{2+} and Mg^{2+} that

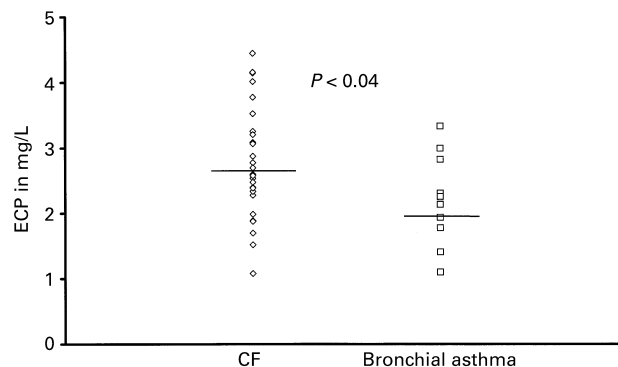


Fig. 2. Concentrations of ECP in patients with cystic fibrosis and bronchial asthma

may itself cause eosinophil degranulation and inversely with Cl^- concentrations and lung function parameters. To proof any causal relationship between eosinophil degranulation and ion sputum levels further *in vitro* studies are needed.

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