Short communication

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Major basic protein, but not eosinophil cationic protein or eosinophil protein X, is related to atopy in cystic fibrosis

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Increased eosinophil granule proteins have been described in serum and sputum samples of patients with cystic fibrosis (CF). It has been assumed that eosinophil degranulation is enhanced in atopic subjects – as in asthmatics. Since in CF no differences in eosinophil cationic protein (ECP), eosinophil protein X (EPX), and eosinophil peroxidase between atopic and nonatopic subjects have been detected, we investigated whether major basic protein (MBP) is increased in serum and sputum samples derived from atopic (n=14) compared with nonatopic CF subjects (n=26). In CF patients, high mean serum (sputum) levels of ECP 29.7 μg/l (2.7 mg/l), EPX 53.7 μg/l (7.9 mg/l), and MBP 984.6 μg/l but low sputum MBP levels (57.4 μg/l) were measured. In addition, in serum and in sputum samples, a significant correlation between MBP and ECP ($P<0.03$ and $P<0.0001$, respectively) or EPX ($P<0.05$ and $P<0.0004$, respectively) was detected. By subdivision of the patients into allergic and nonallergic subjects, significant differences were found for serum MBP values only (mean 1382.2 μg/l *vs* 770.5 μg/l; $P<0.0001$), but not for ECP or EPX serum levels or for eosinophil proteins in sputum. Although no differences between atopic and nonatopic CF patients in ECP and EPX were found, serum MBP levels were higher in patients sensitized to inhalant allergens than in nonsensitized subjects. These results indicate differential release of eosinophil granule proteins in peripheral blood from eosinophils, and they also indicate that MBP in serum likely is to be a better discriminator of atopy in CF.

Caused by mutations on chromosome 7 (1), cystic fibrosis (CF) is an autosomal recessive disease characterized by progressive pulmonary tissue injury due to chronic bacterial

infection and excessive immune-mediated inflammatory process (2). Recently, increased concentrations of eosinophil-derived granule proteins, such as eosinophil cationic protein (ECP), eosinophil protein X (EPX), and eosinophil peroxidase (EPO), have been detected in both sputum and serum samples of patients with CF (3, 4). In addition, serum levels of eosinophil granule proteins are significantly related to pulmonary function and disease severity in CF (4, 5). Due to the cytotoxic potential of these proteins, it has been assumed that the activated eosinophil, in addition to the neutrophil granulocyte, also participates in pulmonary tissue damage (3). However, in addition to bacterial infection, colonization with fungi, predominately *Aspergillus fumigatus*, is frequently observed in CF (6), commonly resulting in IgE-mediated sensitization to this fungus. Moreover, it has been demonstrated that *Aspergillus* sensitization in the presence of increased total serum IgE levels is associated with lower pulmonary function values in children with CF (7), emphasizing the role of IgE-mediated sensitization in CF. In contrast to bronchial asthma (8), eosinophil activity is not enhanced in atopic compared with nonatopic patients with CF, as shown by the lack of any relationship between the levels of ECP, EPX, or EPO and the atopic status of patients with CF (4). Therefore, other mechanisms of eosinophil activation in CF compared with bronchial asthma have been suggested (9). Despite this, however, it appears unlikely that IgE-mediated events do not contribute in CF to enhanced eosinophil degranulation as measured by ECP, EPX, and EPO (4).

Since there is some evidence that eosinophil-granule proteins are released differentially *in vitro* (10), we investigated whether serum and sputum levels of major basic protein (MBP) are influenced by additional IgE-mediated sensitization in patients with CF. To have a homogeneous cohort, all of the patients studied were in stable clinical condition, and all of them were chronically infected/colonized by *Pseudomonas aeruginosa*.

Material and methods

Patients and controls

Forty patients with CF (age: 12.5 ± 3.47 years; 22 girls and 18 boys) were recruited. The diagnosis was confirmed by repeated positive sweat test results and typical course of disease. All of them were in a stable clinical condition, meaning that none of the patients suffered from acute pulmonary exacerbation as defined previously (4). Disease severity was determined by means of the Shwachman–Kulczycki score (11), which in our setting (12) was limited to the maximum of 75 points (i.e., excluding radiography) (Table 1). Chronic infection/colonization by *P. aeruginosa* was proved by repeated sputum cultures in all patients.

In addition, in 14 patients with CF, the diagnosis of atopy was based on increased total serum IgE proteins (above the age-related normal values) (Table 1) and specific IgE antibodies against inhalant allergens (positive results class ≥2) (UniCAP, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Sensitization to *A. fumigatus* was diagnosed by the presence of specific IgE antibodies in serum (positive results of ≥class 2) and by skin prick test results in six patients, but in none of them were the criteria of allergic bronchopulmonary aspergillosis fulfilled (6).

Sputum preparation

Sputum samples were diluted immediately with phosphate-buffered saline (PBS), pH 7.4, in order to avoid cell damage, and were vortex mixed for 30 s, according to a previously published protocol (3, 9). The mixture was centrifuged, and the supernatant was immediately frozen at –20°C until analysis.

An aliquot of diluted sputum from each patient was used for cell differential counts and was stained with May-Grünwald-Giemsa. It has been previously shown by trypan blue exclusion that the viability of cells in samples was above 65%. Moreover, the percentage of squamous cells was less than 10%, indicating that no notable salivary contamination was present (13).

Table 1. Clinical data of patients with cystic fibrosis (*n*=40)

	Mean ± SD
Shwachman–Kulczycki score (points)	52 ± 10.9
FVC % predicted	89.2 ± 21.49
FEV_1 % predicted	79.5 ± 30.30
MEF_{50} % predicted	59.0 ± 37.98
MEF_{25} % predicted	46.6 ± 33.55
TLC % predicted	114.5 ± 14.68
RV/TLC % predicted	165.3 ± 61.35
Arterial P_{O_2} (kPa)	10.5 ± 1.62
Arterial P_{CO_2} (kPa)	5.1 ± 0.53
Total serum IgE (kU/l)	157 ± 230.4
C-reactive protein (mg/l)	9.0 ± 7.3
Blood neutrophil counts (cells/μl)	4505 ± 1042.7

Assays for eosinophil granule proteins and blood differentials

Eosinophils and neutrophils in peripheral blood were determined by automated counting (Sysmex NE-5500; Müller, Austria) with coefficients of variation of about 7% and 4.8%, respectively (3). ECP and EPX levels were measured by using specific and sensitive radioimmunoassays (Pharmacia Upjohn AB, Uppsala, Sweden). Briefly, ECP or EPX in the samples compete with a fixed amount of ^{125}I-labeled ECP/EPX for the binding sites of specific antibodies (12, 14). All measurements were done in duplicate. The interassay coefficient of variation for both assays was below 11% (3, 6).

For determination of MBP, both serum and sputum samples were reduced and alkylated to recover full detection of MBP (15). MBP concentrations were determined by a two-site immunoradiometric assay employing two monoclonal antibodies (16). In brief, reduced and alkylated serum/sputum samples, as well as standards, were placed in capture antibody-coated, 96-well plates and incubated for 2 h. After washing, ^{125}I-labeled antibody was placed in the wells and incubated for 1 hr. Wells were washed and counted in a gamma scintillation counter (15, 16).

Normal serum values for ECP in 87 healthy nonatopic children (age: 10.2 ± 4.5 years) were 9.2 ± 3.67 μg/l (range 3.4–16.2 μg/l) (8); for EPX in 38 healthy nonatopic subjects (age: 13.1 ± 4.26 years), 19.4 ± 5.87 μg/l (range 9.2–28.6 μg/l (4, 12); and for MBP in 105 healthy controls, 454 ± 90 μg/l (range 312–800 μg/l) (16).

Pulmonary function test

Pulmonary function tests were performed by means of a whole-body plethysmograph (Masterlab, Jaeger, Germany). Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), and maximum expiratory flow at 50% and 25% of vital capacity (MEF_{50} and MEF_{25}) were recorded as a maximum flow-volume curve. Total lung capacity (TLC) and residual volume (RV) were also determined. The best of three efforts was used for analysis, according to the American Thoracic Society standard (17). The results are presented as percentage of predicted, according to accepted reference values (18).

Blood gas analysis

Arterial P_{O_2} and P_{CO_2} values were determined in arterialized ear-lobe capillary blood (Blood Gas Analyzer 1306 pH; Instrumentation Laboratory, USA).

Statistical analysis

Results are presented as mean ± SD unless mentioned otherwise. The coefficient of correlation was calculated by the Spearman ranks correlation procedure. The Mann–Whitney U test was used for differences between the groups. Probabilities of less than 5% were considered significant.

Results

Clinical characteristics

Detailed demographic data of the patients are summarized in Table 1. Subdivision into atopic (n=14) and nonatopic (n=26) subjects demonstrated for total serum IgE levels a significant difference (381 ± 261.4 kU/l [range 142–884 kU/l) *vs* 36 ± 16.7 kU/l [range 6–60 kU/l); $P<0.0001$). No significant differences for pulmonary function variables or other clinical parameters, such as Shwachman–Kulczycki score and blood gas analysis, were detected.

Eosinophil counts and eosinophil granule proteins in serum

Eosinophil counts were low in most of the patients with CF (234 ± 117.3 eosinophils/μl; range 80–520 eosinophils/μl). In addition, no significant differences were found between atopic and nonatopic patients (210 ± 114.6 *vs* 247 ± 118.9 eosinophils/μl). In contrast to blood eosinophil counts, increased ECP levels (29.7 ± 21.01 μg/l; range: 6.4–85 μg/l), EPX levels (53.7 ± 31.27 μg/l; range: 15.4–145.2 μg/l), and MBP levels (984.6 ± 370.55 μg/l; range: 412.2–1880.3 μg/l) were measured. In serum, MBP levels were significantly correlated with the levels of ECP (r=0.348, $P<0.03$) and EPX (r=0.321, $P<0.05$).

Moreover, serum MBP (Fig. 1), but not ECP and EPX concentrations, were significantly higher in atopic (34.8 ± 23.94 and 65.8 ± 26.36 μg/l, respectively) than in nonatopic patients (27.1 ± 19.71 and 47.1 ± 32.20 μg/l, respectively) with CF. However, levels of eosinophil granule proteins and blood eosinophil counts, except serum MBP concentrations, did not differ between *Aspergillus*-sensitized and nonsensitized patients (Table 2).

Eosinophil counts and eosinophil granule proteins in sputum

In 26 out of 40 patients with CF, no eosinophils were detected in sputum samples. In the remaining subjects,

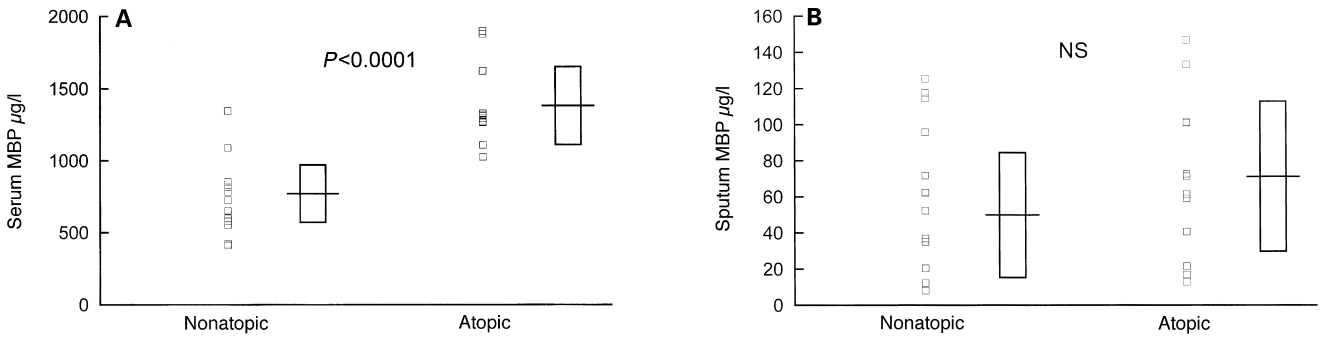

Figure 1. Differences in MBP concentrations (panel A, serum; panel B, sputum) in patients with CF subdivided into allergic and nonallergic subjects. Results are presented as individual values and as mean ± SD. Significant correlation between serum MBP and total serum IgE (r=0.717, P<0.0001) was found. Such relationship was not observed for other eosinophil granule proteins in serum or for eosinophil-derived proteins in sputum. In addition, MBP in either serum or sputum samples was not related to levels of specific IgE antibodies.

eosinophils were 1–12% of nonsquamous cells. Despite this, the neutrophil is the predominant cell in sputa derived from patients with CF (85 ± 8.7%). In addition, eosinophil numbers in sputum samples were, on average, slightly higher in atopic (3 ± 3.9%) than in nonatopic patients (0.5 ± 1.1%; P<0.05). Sensitization to *A. fumigatus*, however, resulted in no statistically significant differences for sputum eosinophil counts in atopic patients with CF (Table 2).

Moreover, high levels of ECP (2.7 ± 0.86 mg/l; range: 1.1–4.45 mg/l) and of EPX (7.9 ± 1.52 mg/l; range: 4.8–10.6 mg/l) but only small quantities of MBP (57.4 ± 38.10 µg/l; range: 8.0–146.9 µg/l) were detected. However, sputum concentrations of MBP were closely related to those of ECP (r=0.721, P<0.0001) and EPX (r=0.567, P<0.0004).

No differences in eosinophil granule protein levels in sputum between atopic (ECP 2.78 ± 0.85 mg/l and EPX 8.52 ± 1.16 mg/l) and nonatopic patients (ECP 2.65 ± 0.88 µg/l and EPX 7.63 ± 1.61 µg/l) with CF were observed (Fig. 1 shows the results for MBP). In addition, in atopic subjects, no influence on eosinophil activity by *A. fumigatus* sensitization was seen (Table 2).

Discussion

The present study has demonstrated high concentrations of ECP, EPX, and MBP in serum and of ECP and EPX in sputum derived from patients with CF. This finding supports previous results in patients with CF showing increased serum and sputum ECP levels (3, 4). Moreover, we have found that serum MBP levels, but not those for ECP and EPX, were significantly elevated in CF patients with sensitization to inhalant allergens, compared with nonsensitized subjects. This was not seen for eosinophil counts in peripheral blood.

The lack of blood and sputum eosinophilia does not exclude the participation of the eosinophil in the inflammatory process, as recently demonstrated, for example, in wheezing infants (19). The dissociation between eosinophil

Table 2. Influence of *A. fumigatus* sensitization on eosinophil counts and concentrations of eosinophil granule proteins in atopic patients with CF

	Aspergillus-positive (n=6)	*Aspergillus*-negative (n=8)	P value
Total serum IgE kU/l	324 ± 292.0	424 ± 247.1	NS
Blood eosinophils/µl	208 ± 12.7	211 ± 108.7	NS
Sputum eosinophils %	4 ± 3.7	2 ± 4.2	NS
Serum ECP µg/l	47.0 ± 27.04	25.6 ± 17.81	NS
Sputum ECP mg/l	2.6 ± 0.74	2.9 ± 0.93	NS
Serum EPX µg/l	71.2 ± 36.42	61.8 ± 17.32	NS
Sputum EPX mg/l	8.4 ± 1.59	8.6 ± 0.82	NS
Serum MBP µg/l	1610.9 ± 248.08	1210.7 ± 119.04	<0.005
Sputum MBP µg/l	78.7 ± 49.26	65.9 ± 37.43	NS

number in peripheral blood – which was within the normal range in most of the patients (3) – and the serum concentrations of eosinophil granule proteins in CF might be explained by the fact that blood eosinophils actively release their granule proteins during clot formation *in vitro*. The enhanced degranulation may reflect the fact that eosinophils are primed by eosinophil-active factors. Indeed, we have previously demonstrated that isolated blood eosinophils from patients with CF have an increased propensity to release ECP after stimulation with serum-treated Sephadex G-15 particles, indicating that the eosinophil must be primed (12). One putative principle involved in the priming of eosinophils is interleukin (IL)-5, as has been demonstrated *in vitro* and *in vivo* for bronchial asthma (20). In CF, however, studies on cytokines in sputum samples have shown that in most of the patients IL-5 could not be detected (9). Therefore, the other priming factors involved could be cytokines such as IL-3 and IL-8, since both were found to be increased in sputum samples from patients with CF, and they were markedly correlated with eosinophil activity (9). The lack of sputum eosinophilia in most of the patients might be due to the fact that after total degranulation the eosinophil cannot be identified by the usual staining procedure. However, the participation of activated eosinophils in the inflammatory process in the CF lung appears to be substantiated by the presence of very high sputum concentrations of ECP and EPX – higher than those needed for tissue injury *in vitro* – as shown by our group and others (3, 9, 21).

Surprisingly, the levels of MBP in sputum samples from patients with CF were low despite increased ECP and EPX values. It has been shown that patients with bronchial asthma had similar sputum ECP levels to those of subjects with CF (9, 22). In addition, sputum MBP concentrations higher than 100 μg/l have been described in bronchial asthma (23), whereas 32 of our patients with CF had values less than 100 μg/l. Moreover, these sputum concentrations of MPB were substantially lower than those in serum (range 412.2–1.880.3 μg/l; for comparison, in asthmatics, a mean concentration of 384 μg/l has been described [24]). The reason for this phenomenon might be either that MBP is degraded by the presence of proteolytic enzymes in the sputum of patients with CF, or that eosinophil granule proteins are released differentially in serum and sputum (10). Since the recovery rate for ECP and EPX is is 85–107% in sputum samples derived from patients with CF, it appears unlikely that among these proteins only MBP should be degraded. Nonetheless, there is some evidence from this study that eosinophil proteins are released differentially from the granules. This phenomenon is probably due to the fact that MBP is localized to the crystalline core of the eosinophil-specific granules whereas ECP, EPX, and eosinophil peroxidase are present within the amorphous granule matrix (25). A difference in solubilization rates between matrix and core may possibly account for the differential release of these proteins.

It has been demonstrated that sensitization to inhalant allergens, especially *A. fumigatus*, is associated with lower pulmonary function values in patients with CF (7), and the observation may be due to the presence of MBP, which is detected in higher serum concentrations in atopic than in nonatopic patients with CF, and in *Aspergillus*-sensitized than in nonsensitized subjects. Thus, our findings of increased serum MBP levels suggest that inhalant allergy contributes to enhanced eosinophil activity in CF, as observed in childhood asthma (8).

In conclusion, in serum samples obtained from patients with CF, high levels of MBP can be measured. In addition, blood eosinophils of allergic subjects with CF released higher amounts of MBP than those of nonallergic subjects. This was not observed for ECP, EPX, and EPO (4). Our results may also indicate that eosinophil granule proteins are released differentially in patients with CF. The mechanism of priming of the eosinophils is still unknown and needs further investigation. Nonetheless, despite low MBP concentrations in sputum, very high ECP and EPX levels were measured, concentrations higher than those needed for tissue injury *in vitro*.

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