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Catalase, myeloperoxidase and hydrogen peroxide in cystic fibrosis

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ABSTRACT: An oxidant-antioxidant imbalance with damaging consequences for the bronchial epithelium has been hypothesized in the airways of patients with cystic fibrosis (CF). It is based on the assumption that neutrophils entering the lumen of the infected airways undergo activation and release toxic oxygen metabolites and myeloperoxidase (MPO), an enzyme which transforms hydrogen peroxide (H_2O_2) into highly toxic oxygen metabolites. Our aims were to substantiate this hypothesis.

H_2O_2 levels were measured in breath condensates of 63 CF patients and 51 normal subjects. In CF sputum samples, activities and concentrations of MPO and catalase (CAT) were determined. MPO/H_2O_2-mediated cytotoxicity of CF sputum was measured in cell culture assays.

H_2O_2 levels were similar in CF patients and normal subjects (mean±SD 0.97±0.69 *versus* 1.11±0.78 μM; p=0.427). Concentrations and activities of CAT (0.31±0.18 μM; 105±69 units) and MPO (5.93±4.8 μM; 87.8±75 units) were detectable in 38 CF sputa. Addition of H_2O_2 to *in vitro* cells preincubated with CF sputum did not induce cytotoxicity even when CAT was removed from sputum. Sputum MPO together with H_2O_2 did not inactivate α_1-proteinase inhibitor. Preincubation of MPO with sulphated glycoconjugates or deoxyribonucleic acid (DNA) totally inhibited its cytotoxic effect.

In conclusion, catalase, sulphated glycoconjugates and deoxyribonucleic acid may prevent myeolperoxidase-mediated oxygen radical generation in cystic fibrosis sputum.
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In cystic fibrosis (CF), the most common autosomal recessive disorder of white populations, abnormal exocrine gland secretions lead to persisting endobronchial bacterial infections [1]. Consequently, large numbers of polymorphonuclear leukocytes (PMN) are recruited to the infected airways where PMN activation and lysosomal enzyme release occur [2]. In particular PMN-elastase (PMN-Ela) has been implicated in chronic lung destruction [2], the major cause of morbidity and mortality in CF, and, consequently, trials of aerosol therapy with a_1-proteinase inhibitor (α_1-PI, synonymous: α_1-antitrypsin) have been initiated [3].

It is not yet certain whether toxic oxygen metabolites produced by stimulated PMN also contribute to lung injury in CF, as has been suggested in other airway diseases [4–7]. However, indirect evidence may support this hypothesis; high sputum concentrations of extracellular myeloperoxidase (MPO), a PMN-derived enzyme which transforms hydrogen peroxide (H_2O_2) into highly reactive oxygen metabolites, have been detected in CF patients [8–11], and lung function has been inversely correlated with MPO levels [9–11]. In addition, increased lipid peroxidation [12], reduced free-radical-trapping capacity [13] and altered plasma antioxidant status [12–14] have been reported to occur in CF patients.

In this study, we have attempted to test this hypothesis by measuring H_2O_2 concentrations in breath condensates of CF patients, to determine in CF sputum samples activities and concentrations of MPO and catalase (CAT), an enzyme that detoxifies H_2O_2 to oxygen and water, and, also, to investigate a possible cytotoxic effect of CF sputum on cells and on α_1-PI *in vitro*.

Materials and methods

Patients

A total of 63 CF patients from six German CF clinics were studied. Patients (mean±SD (range) age 24±8 (8–37) yrs, males n=30; females n=33) had significantly increased sweat electrolyte levels [15], and were chronically infected by *Pseudomonas aeruginosa* (n=30) or other pathogens (n=33). All patients were receiving pancreatic enzyme replacement therapy, but no anti-inflammatory medication. All hospitalized patients (n=23) were being treated with parenteral antibiotics against *P. aeruginosa*. Of the hospitalized patients 10 suffered from acute exacerbations. Mean±SD maximal vital capacity (VC_{max}) (75±19%) and forced expiratory volume in one second (FEV_1) (69±25%) in the CF patients ranked below normal age-predicted values. A total of 54% of the patients had moderately reduced lung function and 46% had severely reduced lung function. Controls included 51 healthy, nonsmoking subjects (27±8 (12–61) yrs) and 23 patients with bronchial asthma (36±16 (12–61) yrs). Informed written consent was obtained from all individuals or their surrogates. The protocol was approved by the Ethics Committee of the University of Tübingen.

Breath collection and H_2O_2 quantification

Breath condensates were collected from 63 CF patients, 51 normal subjects and 23 patients with bronchial asthma. All participants breathed at a normal rate and tidal volume and expired for 15 min into a cooled (2–4°C) sterilized glass tube (Vetter, Ammerbuch, Germany) held at an angle of roughly 45°. Condensate (2–4 mL) was collected in plastic tubes and immediately frozen to -70°C. Condensate H_2O_2 was assayed by the p-hydroxyphenylacetic acid (pHPA)/horseradish peroxidase method [16]. Measurements (excitation 288 nm; emission 405 nm) were carried out in a quartz microcuvette (Type 109.009 F-QS; Perkin-Elmer, Überlingen, Germany) inserted in a thermostatic (37°C) cuvette holder of a LS-50 fluorimeter (Perkin-Elmer): 500 µL condensate, 50 µL pHPA (1.5 mM; Sigma-Aldrich, Deisenhofen, Germany) and 50 µL horseradish peroxidase (1 mg·mL^{-1}; Sigma-Aldrich), suspended in distilled water and prewarmed to 37°C, were added at 10 s intervals, mixed immediately with a magnetic stirrer inserted in the cuvette, and the reaction followed for 40 s. H_2O_2 was quantitated using a standard curve from 0–10 µM and specified in condensates by quenching $(pHPA)_2$ formation by addition of 100 ng purified CAT (Sigma-Aldrich) prior to adding horseradish peroxidase, and, in addition, by adding 10 µg rabbit anti-human CAT immunoglobulin (Ig)G (Athens Research Inc, Athens, GA, USA). The minimal detectable H_2O_2 level was 0.01 µM.

Cystic fibrosis sputum preparation

Sputum samples from 38 CF patients were collected and immediately frozen at -70°C. Supernatants were prepared by dilution of the sputum 1:1 (volume/volume (v/v)) with sterile saline. The mixture was homogenized (Unimag ZX, Vetter) and centrifuged at 1,000 revolutions per minute (rpm) at 4°C for 10 min. From nine sputum supernatants (3 mL) CAT was quantitatively removed by adsorption with 0.5 mL of Protein A Sepharose Cl-4B (Pharmacia, Freiburg, Germany) coupled with 2.2 mg of rabbit anti-human CAT IgG (Athens Research Inc) according to the method of the supplier, before the addition of the supernatant to the cells. Heat-inactivation of sputum supernatants was accomplished by heating the 1:10 diluted supernatants for 30 min at 80°C .

Enzyme and total protein quantification

CAT and MPO concentrations in 38 CF sputum supernatants and plasma samples were determined by solid phase radioimmunoassays [8] using purified human CAT (Sigma-Aldrich) or MPO (Wieslab, Lund, Sweden) and rabbit antibodies to human CAT (Athens Research Inc.) or MPO (Merck, Darmstadt, Germany). PMN-Ela concentrations in CF sputum supernatants and in plasma samples from CF patients and healthy individuals were measured using an enzyme linked immunosorbent assay (PMN-Elastase-Test, Merck). MPO activities in sputum supernatants were determined photometrically according to the method of KLEBANOFF [17], following the MPO catalyzed oxidation of guaiacol to tetraguaiacol by H_2O_2. Enzyme units for MPO were determined using the formula:

$$U = (\Delta OD \cdot 4 \cdot V_t \cdot \text{dilution factor})/(L \cdot \varepsilon_{470} \cdot \Delta t \cdot V_s)$$

where ΔOD=density change, V_t= total volume (mL), L= light path (1 cm), ε_{470} = extinction coefficient for tetraguaiacol: 26.6 mM^{-1}·cm^{-1}, Δt=time of measurement in min, V_s= sample volume in mL.

CAT activities in sputum supernatants were determined photometrically according to the method of AEBI [18] and BERGMEYER [19], following the decomposition of H_2O_2 by CAT at 240 nm. CAT units were determined using the Lambert-Beer law:

$$\Delta OD = \Delta c \cdot \varepsilon \cdot L$$

for the calculation of the reaction speed

$$k = 2.3/30\ s \cdot \log \Delta c.$$

One IU was defined as

$$13 \cdot k/6.39 \cdot 10^{-3} \cdot s$$

where $\Delta c = [H_2O_2]$ change, ε_{240} = extinction coefficient for H_2O_2: 0.0039 mM^{-1}·mm^{-1}. The minimal detectable levels of CAT, MPO and PMN-Ela in the radioimmunoassays and the enzyme linked immunosorbent assay ranged 5–10 ng·mL^{-1}, whereas the lower detection level in the enzyme activity assays for the three enzymes was 0.5 U. Protein concentrations in CF sputum specimens were determined using the Pierce assay (BCA protein assay reagent; Pierce, Rockford, IL, USA).

Cytotoxicity assays

A total of 1×10^5 Chinese Hamster Ovary (CHO) cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) ACC 110) or human lung carcinoma A-549 cells (DSM ACC 107) in Dulbecco's minimal essential medium (DMEM) (Gibco, Eggenstein, Germany), supplemented with 10% foetal calf serum, 1% sodium py-ruvate, 1% nonessential amino acids, and 50 µg·mL^{-1} gentamycin, were seeded for 48 h at 37°C and 5% CO_2 into 96 well flat bottom microtitre plates (Becton Dickinson, Lincoln Park, NJ, USA), washed and then incubated with buffer alone or in various test conditions for 5 h [20]. Cytotoxicity was assessed with 0.025% trypan blue. After 30 min the percentage of blue coloured cells was determined microscopically using a 0.5 mm grid attached to the bottom of each well. A minimum of 1,000 cells were counted in each well.

In addition, human nasal epithelial (HNE) cells were used as primary cell culture system [21]. Nasal polyps were obtained from CF patients undergoing polypectomy. After repeated washings, the polyps were digested with 0.1% pronase (Sigma-Aldrich) in DMEM and Ham's F12 1:1 medium (Gibco) supplemented with penicillin (50 µg·mL^{-1}) (Gibco), streptomycin (50 µg·mL^{-1}) (Gibco) and nystatin (1,000 U·mL^{-1}) (Gibco) overnight at 4°C under continuous rotation. After three washes with culture medium the cells were incubated 1–2 days in DMEM: Ham's F12 1:1 with penicillin (50 µg·mL^{-1}), streptomycin (50 µg·mL^{-1}) and nystatin (1,000 U·mL^{-1}), ultoser G (2%) (Gibco), choleratoxin (10 ng·mL^{-1}) (Sigma-Aldrich), retinoic acid (10^{-7} M) (Sigma-Aldrich), at 37°C and 5% CO_2 under continuous rotation for vesicle formation. These 1–2 day old vesicles were used for monolayer culture. They adhered on a collagen coated membrane (Biocoat cell culture inserts; Becton Dickinson) and were grown out as a monolayer. The cells remained in monolayer culture for 10–14 days.

After this time the majority of the cells were not ciliated. For ciliogenesis, vesicles were maintained in a T-25 flask membrane (Becton Dickinson) as a suspension culture on a shaker for 1 week. Later, they were kept stationary in a T-25 flask at 37°C and 5% CO_2. After 4 weeks the vesicles developed cells with cilia with normal ultrastructure and a co-ordinated cilia beating, goblet cells and nonciliated cells. In suspension these cells remained as stable vesicles and spheroids for 3–5 months. Cilia function was assessed by measuring vesicle rpm.

Effect of H_2O_2, MPO and CF sputum supernatant on CHO, A-549 and HNE cells

For negative controls, cells were incubated with 200 µL of buffer or purified MPO (33 nM, 330 nM) or H_2O_2 (150 µM, 1500 µM) for 5 h. In other experiments, cells were preincubated with 100 µL MPO (66 nM, 660 nM) for 1 h before 100 µL of H_2O_2 (300 µM, 3,000 µM) was added for an additional 4 h. In order to determine the minimal CAT concentration which inhibits the cytotoxicity of MPO/H_2O_2, 10 fold dilutions of CAT (0.024 nM–240 µM) were added to the cells before the addition of H_2O_2 and MPO. The potential cytotoxic effect of CF sputum, containing CAT and MPO or deprived of CAT, was tested by preincubating the different cell lines and primary cells with 100 µL diluted (1:10, 1:20) CF sputum supernatants for 1 h before 300 µM H_2O_2 were added for an additional 4 h. Additionally, 100 µL of heat-inactivated CF sputum super-natants, supplemented with 33 µM MPO and 150 µM H_2O_2 was used in CHO cell cytotoxicity assays. Since sputum supernatants contain serine proteinases, including PMN-Ela, which lead to the destruction of the cell monolayer, the 1:10 diluted supernatants were mixed with α_1-PI (0.2 mg/100 µL sputum) for 30 min before the cell experiments were carried out.

Effect of negatively charged substances on the cytotoxicity of the MPO/H_2O_2 system

In order to test the effect of negatively charged substances on the cytotoxic effect of the MPO/H_2O_2 system, 66 nM purified MPO was preincubated for 30 min at 37°C with 1, 10, 100, or 1,000 µg·mL^{-1} heparan sulphate (Sigma-Aldrich) or chondroitin sulphate (Sigma-Aldrich) or fish sperm deoxyribonucleic acid (DNA) (Serva, Heidelberg, Germany). One hundred microlitres of the mixtures were added to the cells for 1 h before 100 µL of 300 µM H_2O_2 was added for an additional 4 h.

Capacity of α_1-PI to inhibit PMN-Ela in the presence or absence of MPO, CAT, H_2O_2, MPO/H_2O_2 or sputum

For controls, 200 µg of α_1-PI was incubated with buffer or MPO (33 nM) or H_2O_2 (150 µM) or MPO/H_2O_2 for 30 min at 37°C. Then PMN-Ela (Elastin Products, Owensville, Mo, USA) (0.3 nM) was added for 30 min at 37°C. In other experiments, PMN-Ela was added immediately to α_1-PI and MPO/H_2O_2. The total volume of the reaction mixtures was 1 mL. In order to investigate whether enzymatically active MPO present in CF sputum can inactivate added α_1-PI, seven sputum supernatants (diluted 1:20) containing CAT or nine supernatants quantitatively deprived of CAT were incubated with 200 µg of purified α_1- PI and buffer or H_2O_2 (150 µM) for 30 min at 37°C. Thereafter, PMN-Ela activity was determined in all samples using MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide (Bachem, Heidelberg, Germany) as specific chromogenic substrate [8]. One unit was defined as the release of 1 mole p-nitroanilide·min·mL^{-1} using ε_{410} = 8,800 M^{-1}cm^{-1}.

Statistical analysis

Statistical analysis was performed using SAS/STADT (User's Guide, Version 6, 4th edition 1990, SAS Institute, Cary, NC, USA). Raw data were checked for normality using the Shapiro-Wilks-test, thereafter significance was calculated using the Student's t-test or the Wilcoxon-test. Probability values less than 0.05 were considered significant. Correlations were calculated using the square of the Pearson correlation coefficient (r^2). Results are given as mean±SD.

Results

The H_2O_2 content of breath condensate was similar in 63 CF patients with moderately to severely reduced lung function and 51 normal subjects (0.97±0.69 *versus* 1.11± 0.78 µM; p=0.427) (fig. 1). In contrast, H_2O_2 levels from 23 patients with bronchial asthma (1.93±1.44 µM) were significantly increased (p<0.009) compared to controls. Adding CAT to condensates quenched the formation of the fluorescent $(pHPA)_2$ to baseline levels (12.4± 3.7%) and addition of CAT antibodies to these samples raised levels again to 87.7±7.1% revealing the fact that actually H_2O_2 was measured (data not shown).

From the 63 CF patients, 38 patients produced sputum. Protein concentrations in CF sputum specimens were 920±379 µg·mL^{-1}. Extracellular concentration of PMN-Ela, a marker of PMN activation, in the 38 CF sputum supernatants was 2.63±1.53 µM (fig. 2a) clearly demonstrating pronounced lung inflammation. Even PMN-Ela (5.43±5.77 nM) in 41 CF plasma samples was significantly higher (p<0.0001) than in 10 healthy individuals (1.33±0.53 nM) (fig. 2a).

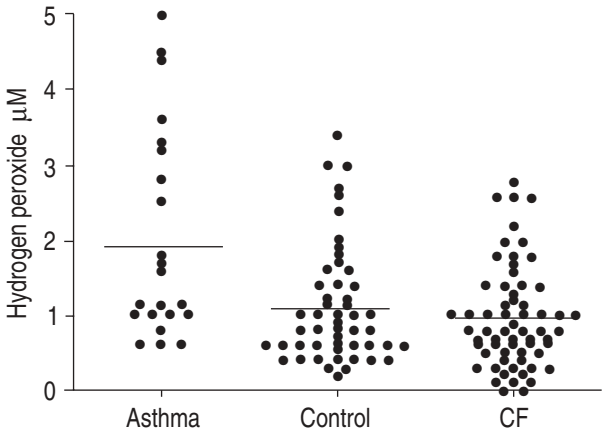

Fig. 1. – Hydrogen peroxide in breath condensates of 23 patients with bronchial asthma, 51 normal healthy controls and 63 patients with cystic fibrosis (CF). Horizontal bars represent mean values.

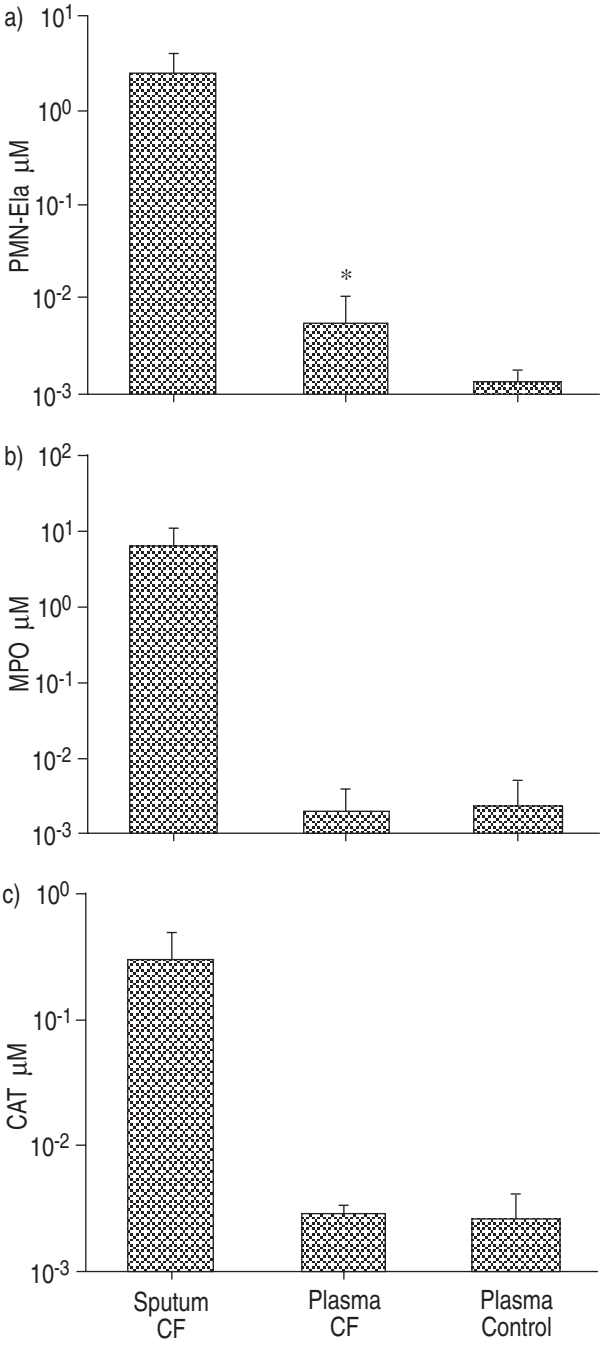

Fig. 2. – Sputum and plasma concentrations of: a) polymorphonuclear leucocyte elastase (PMN-Ela); b) myeloperoxidase (MPO) and; c) catalase (CAT). Bars represent mean±SD.

Sputum concentrations of MPO (5.93±4.8 µM) (fig. 2b) and CAT (0.31±0.18 µM) (fig. 2c) were detectable in 38 CF patients, suggesting that the low H_2O_2 levels in CF breath condensate are a result of the scavenging ability of these enzymes. Concentrations correlated with activities (CAT 105±69 U; MPO 87.8±75 U), giving high correlation coefficients (CAT: r^2=0.7354; MPO: r^2=0.5278). No significant differences in the concentrations or activities of H_2O_2, PMN-Ela, MPO or CAT were detected between CF patients who were hospitalized and receiving antibiotics *versus* clinically stable out-patients. Similarly, disease severity and the infectious status of the patients (*e.g.*, patients suffering from *P. aeruginosa* lung infection *versus* patients without *P. aeruginosa* infection) did not correlate with the measured parameters.

The CAT activities in CF sputa suggested that H_2O_2 in CF sputum undergoes CAT-induced detoxification rather than MPO-induced catalyzation to more toxic oxygen metabolites. Indeed, addition of H_2O_2 to CHO cells preincubated with CF sputum did not induce visible cytotoxicity (fig. 3), although the concentrations of active MPO in the diluted sputa were approximately 10 fold higher than those of purified MPO used for the CHO cell control experiments (fig. 3). As little as 0.24 nM CAT totally inhibited the cytotoxicity induced by 66 µM MPO/300 µM H_2O_2 supporting our notion that catalase is an important scavenger of MPO/H_2O_2 (fig. 3).

In contrast to CHO cells, HNE vesicles were much more resistant to the effect of purified MPO/H_2O_2 and did not show any cytotoxicity when incubated with 33 nM MPO/150 µM H_2O_2. Therefore, 10 fold higher concentrations (MPO: 330 nM/H_2O_2: 1,500 µM) were used which led to complete inhibition of cilia beating (data not shown). In contrast, cilia beating was not affected by incubating HNE vesicles with sputum specimens.

In order to investigate whether CF sputum supernatants contain other scavengers of reactive oxygen species, we removed CAT from CF sputum by affinity chromatography. Monitoring of CAT activity photometrically revealed that CAT was totally removed from the sputum material (<1 IU). Surprisingly, removal of CAT from sputum supernatants neither caused cytotoxicity nor impaired the cilia beat frequency of HNE cells (fig. 3). Although the MPO activity was reduced to approximately one third after CAT removal due to nonspecific adsorption and dilution, this activity was still much higher than that used for control experiments with purified MPO/H_2O_2. Also heat- inactivation of sputum supernatants did not result in visible cytotoxicity when purified MPO/H_2O_2 was added. These results

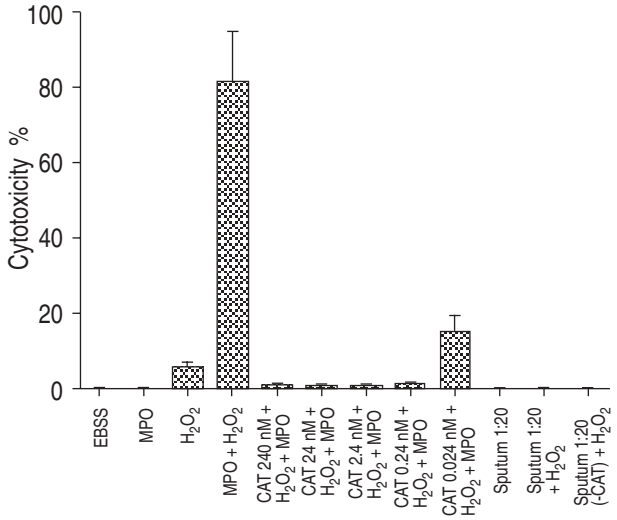

Fig. 3. – Effect of hydrogen peroxide (H_2O_2), myeloperoxidase (MPO) and cystic fibrosis (CF) sputum supernatants on chinese hamster ovary (CHO) cells. CHO cells were incubated with buffer (Earle's balanced salt solution (EBSS)) or different concentrations of purified MPO or H_2O_2 for 5 h. In other experiments, cells were preincubated with different MPO concentrations or diluted sputum supernatant for 1 h before H_2O_2 was added for an additional 4 h. Catalase (CAT) was quantitatively removed from the sputum supernatants of nine CF patients by immunoabsorption.

suggest that the active MPO present in CF sputa is prevented from reaching the surface of the CHO cell monolayer. Binding of MPO which is a highly cationic molecule to negatively charged substances present in sputum may explain this hypothesis.

We therefore preincubated purified MPO with the negatively charged substances heparan sulphate, chondroitin sulphate or fish sperm DNA. Indeed, this incubation totally inhibited the cytotoxic effect of the MPO in the presence of H_2O_2 in a dose-dependent manner (fig. 4). Also heat-inactivated sputum supernatants from seven CF patients which had been supplemented with MPO and H_2O_2 did not reveal a cytotoxic effect on CHO cells.

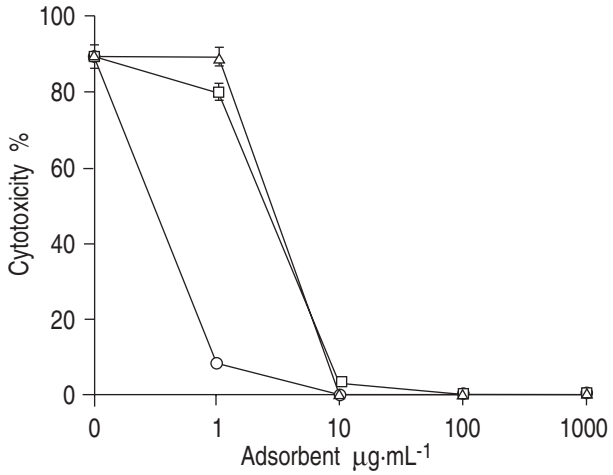

Fig. 4. – Effect of myeloperoxidase (MPO) on Chinese hamster ovary cell cytotoxicity. Purified MPO preincubated with -△- : fish sperm deoxyribonucleic acid; -□- : chondroitin sulphate; -○- : heparin sulphate. Values are presented as mean±SD of duplicate values of at least seven independent assays.

Table 1. – Capacity of α_1-proteinase inhibitor (α_1-PI) to inhibit polymorphonuclear neutrophil elastase (PMN-Ela), purified or present in sputum of cystic fibrosis (CF) patients, in the absence or presence of myeloperoxidase (MPO), catalase (CAT), hydrogen peroxide (H_2O_2) or MPO/H_2O_2

Incubations	After 30 min	PMN-Ela enzyme activity	
		mUnits±SD	%
-	PMN-Ela	0.87±0.04*	100.0
α_1-PI	PMN-Ela	0.07±0.01*	8.0
α_1-PI/MPO	PMN-Ela	0.02±0.002*	2.2
α_1-PI/H_2O_2	PMN-Ela	0.02±0.003*	2.2
α_1-PI/MPO/H_2O_2	PMN-Ela	0.88±0.05*	101.1
α_1-PI/MPO/H_2O_2/PMN-Ela	-	0.008±0.001*	0.9
Sputum		1.87±1.05+	100.0
Sputum/α_1-PI		0.06±0.06+	3.2
Sputum/α_1-PI/H_2O_2		0.05±0.06+	2.7
Sputum (-CAT)		0.43±0.26++	23.0
Sputum (-CAT)/α_1-PI/H_2O_2		0.01±0.01++	0.5

*: coefficients of variation ranged 4.6–15.0%. +: values represent the mean±SD of duplicate values of five independent assays for each sputum supernatant from seven CF patients. Coefficients of variation ranged 2.8–13.6% for each of the investigated supernatants. ++: values represent the mean±SD of duplicate values of five independent assays for each of the sputum supernatants devoid of CAT from nine CF patients. Coefficients of variation ranged 0–10.0 for each of the investigated sputum supernatants.

Although not cytotoxic, sputum MPO may still be capable of oxidizing the methionine residue at amino acid position 358 (Met^{358}) in the active centre of the serine proteinase inhibitor α_1-PI, thereby inactivating the inhibitory capacity of α_1-PI towards PMN-Ela. However, when α_1-PI was incubated with seven sputum supernatants containing active PMN-Ela and active MPO, and H_2O_2 was added, the inhibitory capacity of α_1-PI towards PMN-Ela was not affected (table 1). These results were even obtained when nine sputum supernatants were rendered CAT-negative by totally absorbing CAT by affinity chromatography as described above, excluding that H_2O_2 was detoxified by CAT.

A plausible explanation for this surprising result was obtained from experiments using purified enzymes instead of CF sputum. Whereas, as expected, preincubation of purified α_1-PI with MPO/H_2O_2 for 30 min before addition of PMN-Ela rendered α_1-PI totally inactive with respect to PMN-Ela inhibition, immediate addition of PMN-Ela to the mixture of α_1-PI/MPO/H_2O_2 resulted in a complete retention of the inhibitory capacity of α_1-PI against PMN-Ela (table 1). These results suggest that MPO-induced oxidation/inactivation of α_1-PI is slower than complex formation between α_1-PI and PMN-Ela and that MPO-induced oxidation/inactivation of α_1-PI is inhibited when complexes have been formed.

Discussion

Reactive oxygen metabolites play a pivotal role in host defense during phagocytosis [22], as demonstrated by patients with chronic granulomatous disease, in whom defective reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase leads to recurrent, life-threatening bacterial infections [23]. However, these metabolites have also been implicated in the pathogenesis of many diseases, particularly ischaemia-reperfusion injury and phagocyte-dependent inflammatory damage [24]. Endobronchial inflammation as a result of bacterial infection is a hallmark of pathogenesis in patients with CF [2]. The hypothesis that PMN, the predominant cell population in the airway lumen, release reactive oxygen metabolites with damaging consequences for host cells and proteins has been widely discussed [25] without being substantiated sufficiently.

In vivo detection of oxygen radicals has been hampered by their short half-life times and high reactivities. Measurement of the relatively long-lived, uncharged and thus volatile H_2O_2 in breath condensates of patients with adult respiratory distress syndrome [4], bronchial asthma [5] and acute hypoxaemic respiratory failure [6] has been successfully used as a direct marker of PMN activation and airway inflammation. This noninvasive technique is considered a valuable tool in the assessment of airway inflammation, particularly in paediatric patients where bronchoalveolar lavage and endobronchial biopsy are unlikely to be used routinely.

Our results clearly show that H_2O_2 is not increased in breath condensates of CF patients compared to healthy controls. This finding is not due to methodological problems, since patients with bronchial asthma who served as controls in the present study revealed significantly increased H_2O_2 levels. Furthermore, the demonstration of elevated levels of PMN-Ela, an accepted marker of PMN activation, in CF plasma and sputum samples indicates that the low H_2O_2 levels in breath condensates cannot be

explained by low or absent airway inflammation within our cohort of CF patients. A more likely explanation is based on the presence of H_2O_2 scavengers in CF airways, particular-ly as we detected extracellular MPO and CAT in CF sputum. MPO is present in high amounts in PMN [17] which account for 80–90% of the cellular content of CF sputum. Most probably CAT in CF sputum is also derived from de-graded PMN. In contrast, human airways epithelial cells contain relatively low antioxidative enzyme levels and act-ivities [26] and CAT is not induced in these cells [27].

In other diseases of the respiratory tract PMN numbers may be orders of magnitude lower and, consequently, extracellular CAT levels may be orders of magnitude lower. Nevertheless, in such disorders, H_2O_2 levels may still be higher than in normal subjects, since H_2O_2 levels are derived from PMN stimulation rather than from PMN degradation. This hypothesis may explain, why H_2O_2 levels reach significantly higher values compared to normal subjects in patients with adult respiratory distress syndrome [4], bronchial asthma [5], acute hypoxaemic respiratory failure [6] and chronic obstructive pulmonary disease [28].

Here we also addressed the important issue of whether, in CF airways, toxification of H_2O_2 induced by MPO [17, 25] dominates detoxification induced by CAT or *vice versa*. Sputum CAT protected CHO, A-549 or HNE cells from the cytotoxic or ciliostatic effect of MPO/H_2O_2. *In vitro*, as little as 0.24 nM CAT protected CHO cells from 66 μM MPO. Since we determined in CF sputa mean values of 0.31 μM CAT and 5.93 μM MPO, a protective effect of CAT *in vivo* is highly probable.

Thus, our original conclusions [8], as those of others [7, 9–12, 25, 29], that reactive oxygen metabolites would play an important part in the pathogenesis of CF lung disease, may not reflect the situation in the CF airways adequately. The reported negative correlation of MPO levels and CF lung function [9–11] most probably reflects lung damage caused by PMN-derived lysosomal serine proteinases. In contrast to the proven proteinase-antiproteinase imbalance [2, 3, 8] we have found no evidence to support an oxidant-antioxidant imbalance in CF airspace secretions.

Our results do not rule out that reactions involving reactive oxygen species take place in the sputum of CF patients. DNA from decayed PMN may be oxidized to yield the major reaction product, 8-hydroxydeoxyguanosine, and, indeed, this compound has been detected in the urine of CF patients [30]. Likewise, lipids derived from decayed PMN in the sputum rather than from airways epithelial cells, may be oxidized. End products such as malondialdehyde may "escape" to the circulation where it is easily detectable [12]. Based on our results, however, the pathogenic importance of these reactions remains questionable.

MPO may also oxidize taurine to taurine chloramine which in turn may have pathogenic consequences [31]. However, catalase is known to inhibit taurine chloramine formation [31]. Nevertheless, catalase may not be present in very young children with CF in an acute stage of infection/inflammation when sputum is minimal or absent. Furthermore, in some parts of the airways activated PMN may be present, yet sputum may not be formed and, consequently, CAT not be released in sufficient amounts. In such situations, MPO/H_2O_2-mediated tissue damage may occur as it is thought to occur in patients with bronchial asthma. A similar investigation to ours in such a CF patient group would be required to test this hypothesis.

An alternative possibility which might explain our results of normal H_2O_2 values in CF exhaled breath condensates, is a functional defect of PMN at high densities in a chronic inflammatory situation. For instance, PMN do not generate superoxide anion radicals in the presence of neutrophil elastase [32], a situation which is present in CF as we and others have shown previously [2]. Furthermore, PETERS *et al.* [33] have shown that activation of adherent PMN led to a markedly attenuated release of superoxide anion per cell when neutrophils were activated at high density in comparison with cells activated at low density.

A further possibility is related to the fact that although reactive oxygen species are formed, scavengers other than CAT are present in the CF airways. For example, a high protein burden is present in the CF sputum which may exert scavenging effects for MPO-derived oxidants. We therefore absorbed CAT from CF sputum samples and tested the cytotoxicity of these samples on cell cultures *in vitro*. Indeed, sputum supernatants totally devoid of CAT did not induce cytotoxicity of CHO cells, of HNE cells, or of the cell line A549 when H_2O_2 was added. This suggests that MPO, although present and active in CF sputa, is prevented from reaching the cell surfaces. MPO is a highly cationic enzyme at physiological pH with an isoelectric point (pI) >10 [34] and thus may be complexed by negatively charged mucus glycoproteins (or mucins) such as chondroitin sulphate or heparan sulphate, secreted by goblet cells of the surface epithelium and by submucosal glands into the lumen of the CF airways. Indeed, preincubation of MPO with heparan sulphate or chondroitin sulphate totally inhibited the cytotoxic effect of the MPO in the presence of H_2O_2. These results point to a new protective effect of hypersecretion in the process of inflammation. In this context, it is interesting that PMN-Ela and cathepsin G are potent secretagogues [35].

Alternatively, MPO may be bound by DNA released from decayed PMN in the sputum material [36] and thus may be prevented from exerting toxic effects on epithelial cells.

Although not cytotoxic, sputum MPO may still be capable of oxidizing the methionine residue at amino acid position 358 (Met^{358}) in the active centre of the serine proteinase inhibitor α_1-PI, thereby inactivating the inhibitory capacity of α_1-PI towards elastase [37]. Our results clearly show that Met^{358} oxidation did not occur. Apparently, complexation of PMN-Ela by α_1-PI which renders Met^{358} inaccessible for MPO-induced oxidation is faster than MPO-induced oxidation of uncomplexed α_1-PI. This result may have considerable impact on trials of aerosol therapy with α_1-PI in CF: aerosolized α_1-PI may complex PMN-Ela despite the presence of high concentrations and activities of MPO in CF airways.

In summary, our findings reveal a major role for catalase, an enzyme which detoxifies hydrogen peroxide to oxygen and water, as a component of the extracellular antioxidant screen in the cystic fibrosis lung. Furthermore, we have shown that the potentially damaging enzyme, myeloperoxidase, is immobilized within the sputum matrix and is thus prevented from oxidising and inactivating the serine proteinase inhibitor α_1-proteinase inhibitor and from inducing cytotoxicity against lung epithelial cells. These findings have important implications for our understanding of the pathogenesis of cystic fibrosis and for the

development of novel therapeutic strategies to combat inflammatory damage in the cystic fibrosis lung.

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