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A Cantin, D E Woods

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Research Article

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Protection by Antibiotics against Myeloperoxidase-dependent Cytotoxicity to Lung Epithelial Cells In Vitro

André Cantin and Donald E. Woods*

Unité de Recherche Pulmonaire, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4; and *Department of Microbiology and Infectious Diseases, Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1

Abstract

Myeloperoxidase, in the presence of noncytotoxic concentrations of H_2O_2 , was used to induce cytotoxicity to the lung epithelial cell line, AKD. When the cationic aminoglycosides, tobramycin and gentamicin were added to the cells in the presence of myeloperoxidase and H_2O_2 , cytotoxicity was completely inhibited. In addition, tobramycin prevented cytotoxicity induced by cystic fibrosis sputum and H_2O_2 . Protection against myeloperoxidase and H_2O_2 was also observed with the thioether-containing antibiotics, ticarcillin and ceftazidime, but at higher concentrations than with the aminoglycosides. Analysis of spectral properties, dimethylsulfoxide-mediated reduction, and ethyl acetate/NaCl partitioning, demonstrated that aminoglycosides converted HOCl to hydrophilic noncytotoxic chloramines, but were unable to prevent the oxidation of sulfhydryls and methionine by HOCl. In contrast, ticarcillin and ceftazidime were highly effective inhibitors of HOCl-mediated sulfhydryl and methionine oxidation. These results suggest that aminoglycosides protect lung epithelial cells against myeloperoxidase-dependent oxidant injury by binding to anionic cell surfaces and converting HOCl to hydrophilic noncytotoxic chloramines, whereas penicillins and cephalosporins are potent HOCl scavengers capable of protecting critical extracellular molecules against oxidation. (*J. Clin. Invest.* 1993;91:38–45.)
Key words: chloramines • cystic fibrosis • hypochlorite • myeloperoxidase • oxidants

Introduction

Chronic inhalation of antibiotics effective against *Pseudomonas aeruginosa* has been shown to reduce the number of hospital admissions and the rate of pulmonary function deterioration in patients with cystic fibrosis (CF).¹ The benefits of this therapy are observed despite evidence that *Pseudomonas aeruginosa* is rarely eradicated from the CF lung and that up to one third of patients receiving inhaled antibiotics develop resistant strains within their bronchial secretions (1–5).

Address reprint requests to Dr. Cantin, Pneumologie, CHUS, 3001 12e Ave Nord, Sherbrooke, QC, Canada J1H 5N4.

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1. Abbreviations used in this paper: ARDS, adult respiratory distress syndrome; CF, cystic fibrosis; EBSS, Earle's balanced salt solution; IPF, idiopathic pulmonary fibrosis.

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Bronchial secretions from CF patients contain bacteria, neutrophils, and their respective toxic products (6–8). Both *Pseudomonas* and activated neutrophils are capable of releasing significant amounts of hydrogen peroxide (H_2O_2) (9, 10). In addition, Mohammed et al. (11) have demonstrated that CF bronchial secretions have high concentrations of myeloperoxidase, a neutrophil-derived enzyme which, in the presence of H_2O_2 , converts chloride to toxic hypochlorous acid/hypochlorite anion ($HOCl/OCl^-$, but abbreviated as HOCl for simplicity). These studies suggest that CF airway cells are at risk of oxidant-mediated injury, and that essential molecules at the CF respiratory epithelial surface, such as α_1 -antitrypsin, may undergo oxidative inactivation. In this context, protection of airway epithelial cells and extracellular molecules against HOCl-mediated oxidant damage could conceivably be beneficial to CF patients.

HOCl is a strong oxidant which reacts readily with various chemical groups including primary amines, thiols and thioethers (12, 13). Aminoglycosides, such as gentamicin and tobramycin, have multiple primary amines, whereas ticarcillin and ceftazidime each contain two thioether groups, all of which may potentially react with HOCl (Fig. 1). These antibiotics are among some of the anti-*Pseudomonas aeruginosa* agents most commonly administered intravenously and by aerosolization to CF patients. We hypothesized that, in addition to their microbicidal activity, these antibiotics may have significant antioxidant properties. The present study was designed to determine whether tobramycin, gentamicin, ticarcillin, and ceftazidime can protect lung epithelial cells, methionine, and thiol groups in vitro against myeloperoxidase-derived oxidants.

Methods

Cytotoxicity assay. The cat lung epithelial cell line, AKD (CCL 151; American Type Culture Collection, Rockville, MD) (14) was used as the target cell for all cytotoxicity experiments, as previously described (15). The cells were seeded at a density of 50,000 cells per well in 24-well culture plates (Linbro Chemical Co., New Haven, CT) in 0.5 ml of Dulbecco's modified Eagle's medium (DME, Gibco Diagnostic Laboratories, Grand Island, NY) supplemented with 10% calf serum, in 10% CO_2 at 37°C. At confluence, the cells were labeled with sodium chromate (^{51}Cr , 22 mCi/nmol, 5 μCi per cell) overnight and subsequently washed three times with PBS. Earle's balanced salt solution (EBSS), 0.5 ml, was added to each well and the cells were incubated in the presence of either EBSS alone or various test conditions (see below) for 7 h in 5% CO_2 at 37°C. Maximum ^{51}Cr release was assessed by incubating the cells with 1% Triton-X (J.T. Baker Chemical Co., Phillipsburg, NJ). Spontaneous release of ^{51}Cr was determined in the presence of EBSS alone. At the end of the incubation period, the amount of ^{51}Cr released in the supernatant of each test condition was quantitated. ^{51}Cr release did not reflect cell detachment since centrifugation of samples prior to measurement of ^{51}Cr in the supernatant did not change the results. A cytotoxicity index was determined according to the following

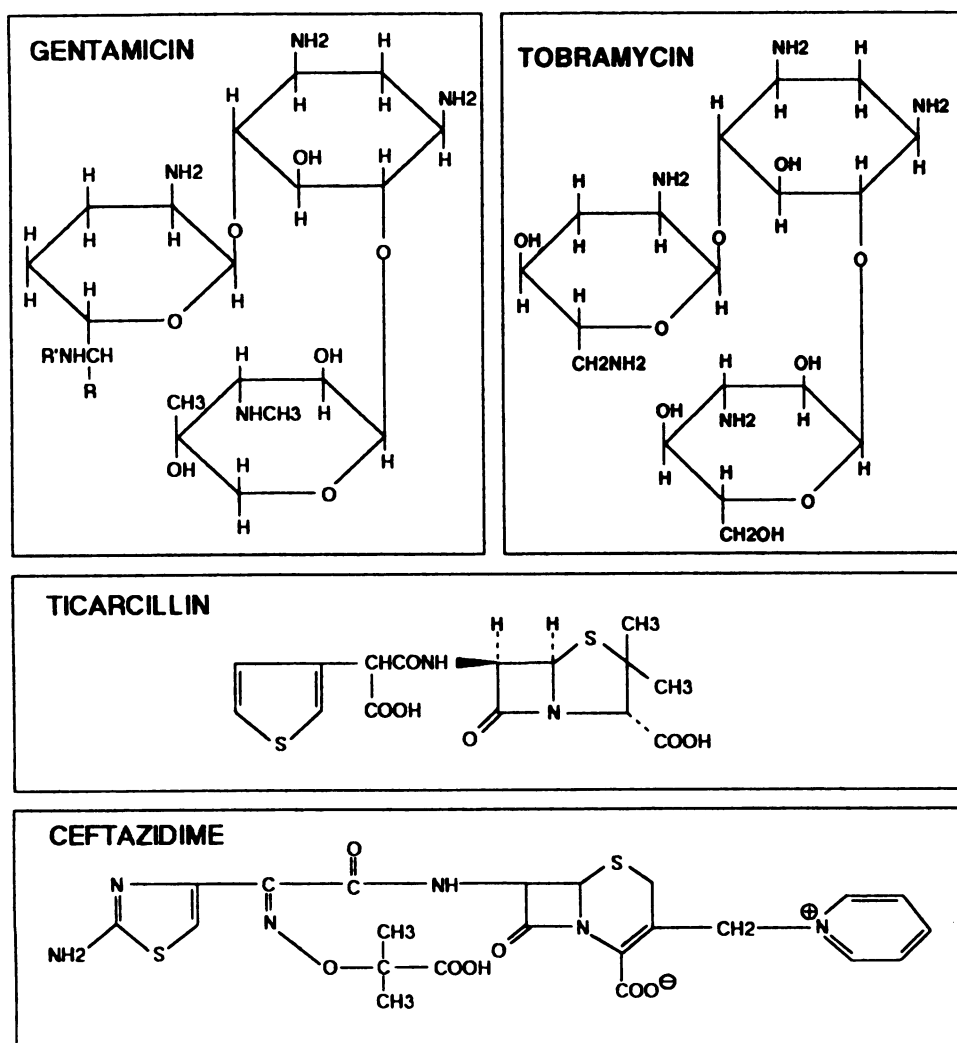


Figure 1. Chemical structures of gentamicin, tobramycin, ticarcillin, and ceftazidime. Both aminoglycosides have multiple primary amines (-NH₂). Ticarcillin and ceftazidime each have 2 thioether groups (-R-S-R-).

formula: cytotoxicity index = $(A - B)/(C - B) \times 100$, where *A* is dpm of the test sample, *B* is dpm of spontaneous release, and *C* is dpm of 1% Triton-X-treated cells.

Myeloperoxidase and HOCl-induced cytotoxicity. Myeloperoxidase was purified from granulocytes obtained by leukaphoresis of a patient with chronic myeloid leukemia, according to the methods of Matheson et al (16). The *A*₄₃₀/*A*₂₈₀ ratio (Reinheit Zahl purity number) of the purified material was 0.82, a value similar to the Reinheit Zahl number of 0.83 reported for 100% pure human myeloperoxidase (17). The labeled cells were incubated with various concentrations (0–5 µg/ml) of myeloperoxidase for 1 h, followed by the addition of 150 µM H₂O₂ to the culture media for 7 h in an atmosphere of 5% CO₂ at 37°C. The effect of incubation time of myeloperoxidase before the addition of H₂O₂, on cytotoxicity was assessed by adding 2.5 µg/ml myeloperoxidase to the cells and subsequently adding 150 µM H₂O₂ at 0–60 min after the addition of myeloperoxidase. The cells were then incubated for 7 h and the cytotoxicity index determined as described above. Based on the results of these experiments, all subsequent experiments were performed using the conditions that induced maximal cytotoxicity, i.e., 2.5 µg/ml myeloperoxidase added to the media 1 h before the addition of 150 µM H₂O₂.

To determine whether cytotoxicity was induced by myeloperoxidase-derived oxidants, cells were incubated with either (a) 2.5 µg/ml of myeloperoxidase, (b) 150 µM H₂O₂, (c) myeloperoxidase + H₂O₂, (d) heat-inactivated myeloperoxidase (100°C for 10 min) + H₂O₂, (e) myeloperoxidase + H₂O₂ + 100 U/ml of catalase, (f) myeloperoxi-

dase + H₂O₂ + 100 U/ml of heated catalase, (g) myeloperoxidase + H₂O₂ + 500 µM [GSH], glutathione, reduced or (h) myeloperoxidase + H₂O₂ + 500 µM methionine.

To determine whether antibiotics affected myeloperoxidase activity, 2.5 µg/ml of myeloperoxidase was incubated alone or with 200 µM of either tobramycin, gentamicin, ceftazidime, or ticarcillin in EBSS for 7 h, and myeloperoxidase activity was measured spectrophotometrically with the substrate *o*-dianisidine as previously described (18).

Antibiotic protection of lung cells against oxidant injury. The protective effects of the antibiotics against myeloperoxidase + H₂O₂-mediated cytotoxicity were determined by adding 0–200 µM of each antibiotic to the cells in the presence of 2.5 µg/ml myeloperoxidase for 1 h, followed by the addition of 150 µM H₂O₂ for 7 h as described above. In that HOCl is the major toxic species synthesized by myeloperoxidase and H₂O₂ at physiologic pH (19), similar assays were performed by replacing myeloperoxidase and H₂O₂ with 150 µM HOCl.

Cytotoxicity from CF sputum and H₂O₂. Sputum was obtained from two patients (aged 19 and 21 yr, nonsmokers) with cystic fibrosis, during an acute exacerbation of their lung disease. Neither was taking antibiotics at the time of sputum collection. The sputum was homogenized in 0.05 M potassium phosphate buffer, pH 7.0 (1:1 wt/vol), centrifuged 15,000 *g*, 15 min at 20°C and the supernatant was eluted from a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) with EBSS. The eluate was passed through a 0.22-µm filter and 0–250 µl was added to the ⁵¹Cr-labeled cells in the absence and presence of 200 µM tobramycin, and the final volume was adjusted to 500 µl with

EBSS. The cells were incubated for 1 h, and subsequently either EBSS or 150 μM H_2O_2 was added to the media for 7 h at 37°C, 5% CO_2 . The media from the cells was then harvested, centrifuged (500 g, 5 min) and the supernatant was collected to determine the cytotoxicity index. Each condition was evaluated in triplicate.

Thiol and methionine oxidation. To evaluate the capacity of the antibiotics to prevent HOCl-induced oxidation of thiol and methionine, 40 μM HOCl was incubated with 0–80 μM gentamicin, tobramycin, ticarcillin, or ceftazidime in EBSS for 15 min at 25°C. Subsequently, either 80 μM 5-thio-2-nitrobenzoic acid, prepared by adding 4 μl 2-mercaptoethanol to 1 mM dithiobis-nitrobenzoic acid as in Thomas et al. (20), or 100 μM methionine (Sigma Chemical Co., St. Louis, MO) was added to each sample for 15 min at 25°C. The residual concentration of 5-thio-2-nitrobenzoic acid was determined by measuring the absorbance of each solution at 412 nm, assuming an $\Sigma_M = 13,600$ (20).

The ratio of methionine to methionine + methionine sulfoxide was measured immediately after the incubation period by reverse-phase high-pressure liquid chromatography (HPLC), using a modification of the method described by Turnell and Cooper (21). Precolumn derivatization was performed by adding 20 μl sample to 20 μl *o*-phthalaldehyde reagent for 2 min. The *o*-phthalaldehyde reagent was composed of 5.5 mg *o*-phthalaldehyde dissolved in 1 ml of methanol to which was added 9 ml of 0.5 M potassium borate, pH 9.5, containing 40 μl of 2-mercaptoethanol. The analytical column was 150 \times 4.6 mm i.d. and prepacked with 5 μm Ultrasphere octadecyl silane (Beckman Instruments Inc., Palo Alto, CA). Solvent A was water and solvent B was methanol. The HPLC apparatus was a Beckman System Gold with a 166 detector set at 340 nm. The gradient, expressed as percentage of solvent B, was brought from 0 to 9% over 1 min, to 11% over 10 min, to 20% over 1 min, held at 20% for 2 min, and brought to 25% over 10 min. Methionine sulfoxide was detected at 8.5 min and methionine at 18.8 min.

Characterization of aminoglycoside N-Cl derivatives. The products of the reaction of aminoglycosides with HOCl were characterized by (a) analysis of their spectral properties, (b) their susceptibility to reduction by dimethylsulfoxide (DMSO), and (c) their lipophilicity.

Spectral properties of the aminoglycoside + HOCl product(s) were analyzed by incubating 500 μl of EBSS or 500 μl of either 400 μM gentamicin or tobramycin with 500 μl of 400 μM HOCl for 5 min at 25°C. The absorbance of each solution was measured at wavelengths 310–230 nm in a DU-7 scanning spectrophotometer (Beckman Instruments Inc.). Each sample was measured against a control sample containing either gentamicin or tobramycin in EBSS.

Since DMSO can reduce HOCl but not chloramines (20), the susceptibility of the aminoglycoside + HOCl reaction product(s) to reduction by DMSO was assessed. PBS solution, pH 7.4, (PBS), 200 μM tobramycin, or 200 μM gentamicin were incubated with either 40 μM HOCl or PBS for 15 min at 25°C. An equal volume of either PBS or 200 μM DMSO was then added to each sample for 5 min at 25°C.

Subsequently, 60 μM 5-thio-2-nitrobenzoic acid was added to each sample for 15 min at 25°C, and the amount of residual 5-thio-2-nitrobenzoic acid was determined from the absorbance of each sample at 412 nm as described above. All experiments were repeated five times.

The lipophilicity of N-Cl derivatives from the aminoglycoside + HOCl reaction was determined according to the method described by Thomas (22). Ethyl acetate was treated with 2.64 mM sodium borohydride and 0.1 M NaOH for 30 min to remove contaminating oxidants and acids, and washed extensively with EBSS. Ethyl acetate 3 ml, was added to a 3-ml aqueous sample composed of 200 μM tobramycin or gentamicin and 40 μM HOCl in EBSS. After phase separation, a 2-ml portion of the organic phase was mixed with 2 ml of EBSS, to which was added 400 μl of 600 μM 5-thio-2-nitrobenzoic acid (sample A). Similarly, 400 μl of 600 μM 5-thio-2-nitrobenzoic acid was added to the remaining portion of the combined aqueous and organic phases (sample B). The absorbance of the aqueous portions of each sample was measured at 412 nm and the concentration of oxidizing equivalents was determined using the extinction coefficient $\Sigma_M = 27,200$, since one -RNHCl derivative will oxidize two 5-thio-2-nitrobenzoic acid molecules (22). The concentration of oxidizing equivalents in the organic phase was calculated as 1.5 times that in sample A and the amount in the aqueous phase was calculated as that in sample B minus half that in sample A.

Statistical analysis. All data are expressed as the arithmetic mean \pm SEM. Analysis of variance was performed using the F test, and the Scheffé test was applied to the data when appropriate (23). A *P* value < 0.05 was considered significant.

Results

Myeloperoxidase-mediated cytotoxicity. The dose-response assay of myeloperoxidase-induced cytotoxicity to the epithelial cells, in the presence of 150 μM H_2O_2 , is shown in fig. 2 A. Maximal cytotoxicity was observed at the myeloperoxidase concentration of 2.5 $\mu\text{g}/\text{ml}$, a concentration used in all subsequent myeloperoxidase-dependent cytotoxicity assays. The effects of preincubation time of myeloperoxidase with the cells before the addition of H_2O_2 , are shown in Fig. 2 B. When myeloperoxidase was added to the cells simultaneously with H_2O_2 , the cytotoxicity index was relatively low (cytotoxicity index at 0 min = 12.8 \pm 3.3%); however, when myeloperoxidase was allowed to incubate with the cells for various periods of time before the addition of H_2O_2 , a time-dependent enhancement of cytotoxicity was clearly seen (cytotoxicity index at 1 h = 63.0 \pm 2.5%, *P* < 0.001 compared to 0 min). All subsequent experiments were performed with a 1 h preincubation of the cells and myeloperoxidase before the addition of H_2O_2 .

Evidence that the myeloperoxidase-dependent injury was mediated by oxidants, likely HOCl, is presented in Table I.

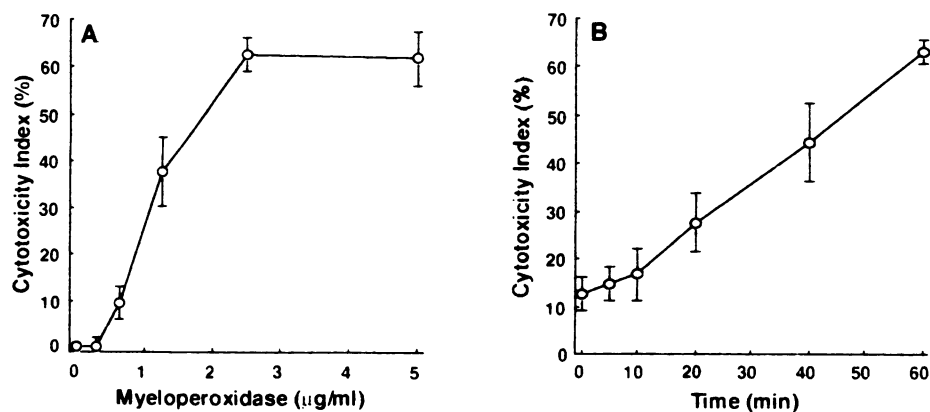


Figure 2. Cytotoxicity assay for evaluating lung epithelial cell injury induced by the MPO + H_2O_2 system. (A) ^{51}Cr -labeled AKD cells were incubated 1 h at 37°C with various concentrations of myeloperoxidase (MPO). Subsequently, H_2O_2 (150 μM) was added to the culture medium and the cells were incubated for 7 h, at 37°C before the cytotoxicity index was determined. (B) Effect of myeloperoxidase incubation time before H_2O_2 addition, on the cytotoxicity index. Each point represents the mean \pm SEM of triplicate cultures.

Table 1. Characterization of the Myeloperoxidase-H₂O₂ Cytotoxicity System

Additions	Cytotoxicity index
	%
MPO alone	0.1±0.3*
H ₂ O ₂ alone	0.3±0.5*
MPO + H ₂ O ₂	69.2±1.2
HiMPO + H ₂ O ₂	-0.6±0.4*
MPO + H ₂ O ₂ + catalase 100 U/ml	-1.9±0.9*
MPO + H ₂ O ₂ + Hi catalase 100 U/ml	67.7±3.6
MPO + H ₂ O ₂ + GSH 500 μM	-1.6±0.7*
MPO + H ₂ O ₂ + methionine 500 μM	-4.8±0.5*

Abbreviations: MPO, 2.5 μg/ml myeloperoxidase; H₂O₂, 150 μM hydrogen peroxide; Hi, heat-inactivated (100°C for 10 min); GSH, glutathione. Values represent the mean±SEM of triplicate assays performed on at least two separate occasions. * *P* < 0.001 compared to MPO + H₂O₂.

Neither myeloperoxidase nor H₂O₂ alone induced cytotoxicity. However, the co-incubation of both agents consistently induced cytotoxicity. Complete inhibition of cytotoxicity was observed in the presence of catalase (but not heat-inactivated catalase), and in the presence of heat-inactivated myeloperoxidase, thus further demonstrating the dependence of cytotoxicity on both H₂O₂ and myeloperoxidase. Both glutathione, an antioxidant capable scavenging both H₂O₂ and HOCl, and methionine, an HOCl scavenger without effect on H₂O₂-mediated cytotoxicity, protected the cells, implicating HOCl as the dominant cytotoxic species.

Antibiotics prevent myeloperoxidase and HOCl-dependent cytotoxicity. All four antibiotics tested provided a dose-dependent protection of the lung epithelial cells against myeloperoxidase/H₂O₂-related cytotoxicity (fig. 3 A). The antibiotics did not inhibit myeloperoxidase activity (myeloperoxidase alone, 47.1±2.0 U/ml; +tobramycin, 46.3±1.0 U/ml; +gentamicin, 47.5±1.1 U/ml; +ceftazidime, 45.3±2.0 U/ml; +ticarcillin, 44.6±0.8 U/ml, *n* = 6, *P* > 0.05 all comparisons). The most efficient antibiotics were tobramycin and gentamicin, which at 50 μM, decreased the cytotoxicity index from 66.8±3.3% to 0±2% and from 69.6±6.7% to 9.3±8.9%, respectively (*P*

< 0.01, both comparisons). The concentration of tobramycin necessary to provide a reduction of 50% in the cytotoxicity index was consistently 40–50% less than that of gentamicin. Ticarcillin and ceftazidime were also protective but only at much higher concentrations than those of the aminoglycosides (200 μM ticarcillin decreased cytotoxicity index from 61.3±5.2% to 27.1±2.4%, 200 μM ceftazidime decreased the cytotoxicity index from 62.8±2.8% to 14.1±1.8%, *P* < 0.01 both comparisons). In contrast, the protective effects of ticarcillin and ceftazidime against HOCl-induced cytotoxicity were observed within the same concentration range as those of the aminoglycosides (fig. 3 B).

Tobramycin and CF sputum-H₂O₂ cytotoxicity. Sterile-filtered sputum from two CF patients added to the labeled cells at concentrations as high as 250 μl/ml in EBSS did not induce significant cytotoxicity. H₂O₂ alone was not cytotoxic, but in the presence of CF sputum, the cytotoxicity index clearly increased (cytotoxicity index 24% and 37% at 250 μl/ml) (Fig. 4). However, the addition of 200 μM tobramycin to the cells before the addition H₂O₂ completely inhibited cytotoxicity at all concentrations of sputum from both patients.

Antibiotic HOCl scavenging properties. HOCl-mediated sulfhydryl oxidation was evidenced by the decrease in 5-thio-2-nitrobenzoic acid concentration from 78.6±0.18 to 26.8±1.6 μM in the presence of 40 μM HOCl. Neither tobramycin nor gentamicin prevented 5-thio-2-nitrobenzoic acid oxidation by HOCl and at 20 μM aminoglycoside, the concentration of 5-thio-2-nitrobenzoic acid was even decreased compared to HOCl alone, an effect attributable to the greater stability of choramines than of HOCl (20 μM tobramycin + HOCl, 5-thio-2-nitrobenzoic acid = 17.6±0.1 μM; 20 μM gentamicin + HOCl, 5-thio-2-nitrobenzoic acid = 18.8±0.1 μM, *P* < 0.01, both compared to HOCl alone) (Fig. 5 A). An opposite effect was observed when either ticarcillin or ceftazidime was added to HOCl in the presence of 5-thio-2-nitrobenzoic acid, with marked protection against thiol oxidation (25 μM ticarcillin + HOCl, 5-thio-2-nitrobenzoic acid = 70.0±2.6 μM; 20 μM ceftazidime + HOCl, 5-thio-2-nitrobenzoic acid = 69.4±0.1 μM, *P* < 0.001 compared to HOCl alone).

Similarly, ticarcillin and ceftazidime, at concentrations of < 10 μM efficiently protected methionine from HOCl-dependent oxidation, whereas both tobramycin and gentamicin, even at concentrations as high as 40 μM, were unable to completely block the formation of methionine sulfoxide (Fig. 5 B).

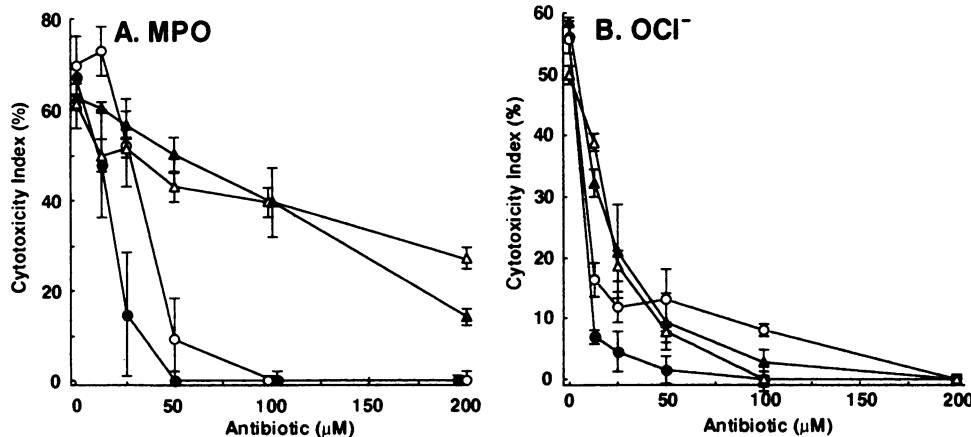


Figure 3. Comparison of cytoprotective properties of tobramycin (●), gentamicin (○), ticarcillin (Δ), and ceftazidime (▲) against oxidant injury. ⁵¹Cr-labeled AKD cells were incubated 1 h with various concentrations of the antibiotics in the presence of either (A) 2.5 μg/ml myeloperoxidase or (B) media alone. Subsequently, either (A) 150 μM H₂O₂ or (B) 150 μM HOCl was added to the culture media and the cells were incubated 7 h at 37°C to determine the cytotoxicity index. Each point represents the mean±SEM of at least three experiments performed in triplicate.

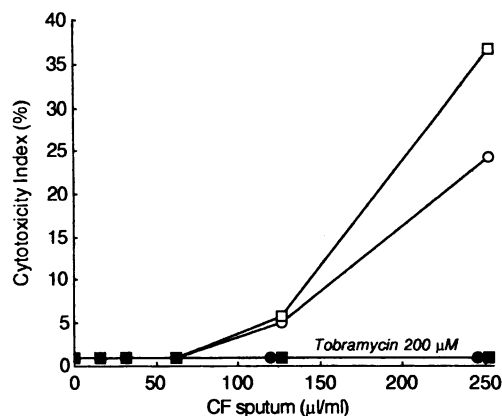


Figure 4. Protection of lung epithelial cells by tobramycin against H_2O_2 and sputum from two patients with cystic fibrosis. ^{51}Cr -labeled cells were incubated with various concentrations of the treated sputum in the presence (\bullet , \blacksquare) or absence (\circ , \square) of 200 μM tobramycin. After 1 h incubation, 150 μM H_2O_2 was added to the media and the cells were incubated for 7 h. At the end of the incubation, the supernatants were centrifuged, and the cytotoxicity index was determined from the ^{51}Cr measured in the culture supernatant. Incubation of sputum in the absence of H_2O_2 did not induce cytotoxicity. Each point represents triplicate cultures.

Incubation of each of the four antibiotics at a concentration of 200 μM with 100 μM HOCl did not decrease the bactericidal activity of the antibiotics (data not shown).

Characterization of the HOCl-aminoglycoside reaction product. The maximal absorbance of HOCl alone was observed at a wavelength of 291 nm (Fig. 6). However, in the presence of either tobramycin or gentamicin, the maximal absorbance was shifted to a wavelength of 252 nm, as expected of chloramines derived from primary amines (20). Consistent with the knowledge that gentamicin has fewer primary amine groups than tobramycin (Fig. 1), the maximal absorbance at 252 nm of the gentamicin-HOCl solution was always lower than that of the tobramycin-HOCl solution.

Further evidence that chloramines were formed from the reaction of aminoglycosides and HOCl is shown in Fig. 7. Neither tobramycin nor gentamicin alone affected the 5-thio-2-nitrobenzoic acid concentration in a buffered solution (5-thio-2-nitrobenzoic acid alone = $63.2 \pm 0.1 \mu M$, + tobramycin = $62.4 \pm 0.2 \mu M$, + gentamicin = 62.6 ± 0.2 , $P > 0.05$ all comparisons). As expected, the addition of HOCl oxidized the 5-thio-2-nitrobenzoic acid solution as can be seen by the decrease in 5-thio-2-nitrobenzoic acid concentration, and the addition of aminoglycosides in the presence of HOCl further oxidized 5-thio-2-nitrobenzoic acid (+ HOCl, 5-thio-2-nitrobenzoic acid = $16.7 \pm 0.1 \mu M$; + tobramycin + HOCl, 5-thio-2-nitrobenzoic acid = $7.8 \pm 0.2 \mu M$; + gentamicin + HOCl, 5-thio-2-nitrobenzoic acid = $8.4 \pm 0.3 \mu M$; $P < 0.01$ tobramycin and gentamicin compared to HOCl alone). DMSO added to HOCl protected the 5-thio-2-nitrobenzoic acid against oxidation (+ HOCl + DMSO, 5-thio-2-nitrobenzoic acid = 59.9 ± 0.2 $P < 0.001$ compared to HOCl without DMSO), but was unable to protect 5-thio-2-nitrobenzoic acid against oxidation from HOCl in the presence of either tobramycin (5-thio-2-nitrobenzoic acid = $8.0 \pm 0.2 \mu M$, $P > 0.20$ vs. without DMSO) or gentamicin (5-thio-2-nitrobenzoic acid = $8.1 \pm 0.1 \mu M$, $P > 0.20$ vs. without DMSO).

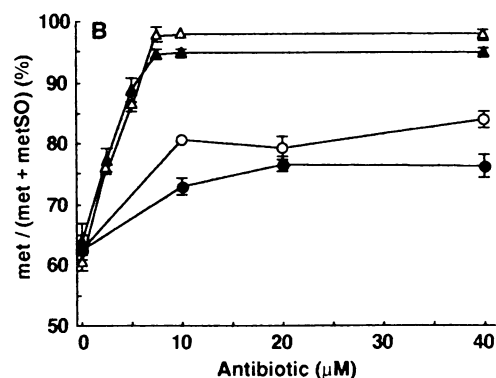
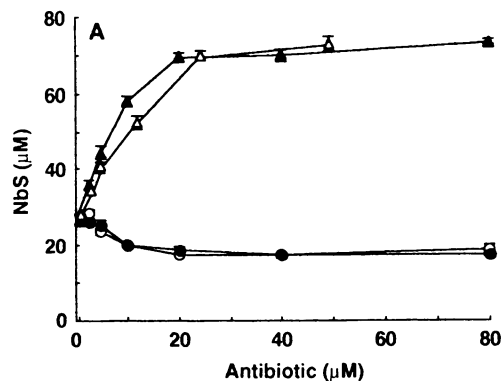


Figure 5. Ability of antibiotics to prevent HOCl-induced oxidation of sulfhydryl groups and methionine. Ticarcillin (Δ), ceftazidime (\blacktriangle), gentamicin (\circ), and tobramycin (\bullet) at different concentrations were incubated with 40 μM HOCl in EBSS for 15 min at 25°C before the addition of either 5-thio-2-nitrobenzoic acid, or methionine. (A) 5-thio-2-nitrobenzoic acid (80 μM) was added to the samples, for 15 min, and the absorbance of the solutions was measured at 412 nm to determine the concentration of residual 5-thio-2-nitrobenzoic acid (NbS). (B) Methionine (100 μM) was added to each sample, incubated for 15 min, and subsequently the amounts of methionine (*met*) and methionine sulfoxide (*metSO*) were determined by HPLC.

Partitioning of the aminoglycoside-HOCl reaction products between the organic and aqueous phases of an ethyl acetate/NaCl gradient revealed that nearly all of the oxidizing equivalents mobilized to the aqueous phase, thus confirming the hydrophilic nature of the oxidant products (Table II).

Discussion

Epithelial cells of the upper and lower respiratory tract are exposed to high concentrations of extracellular myeloperoxidase in diseases such as the adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF), and CF (11, 18, 24). The presence of oxidized α_1 -antitrypsin and of methionine sulfoxide in the bronchoalveolar lavage fluid of patients with ARDS and IPF, respectively, provides direct evidence of an excessive oxidant burden in vivo (24, 25). Furthermore, α_1 -antitrypsin is inactive in the airway secretions of CF patients, an observation that may, at least in part, be explained by a high oxidant burden (7, 26). The present study documents that the aminoglycosides, ticarcillin and ceftazidime, all efficient anti-*Pseudomonas aeruginosa* antibiotics commonly used in the treatment of CF lung infections, have cytoprotective

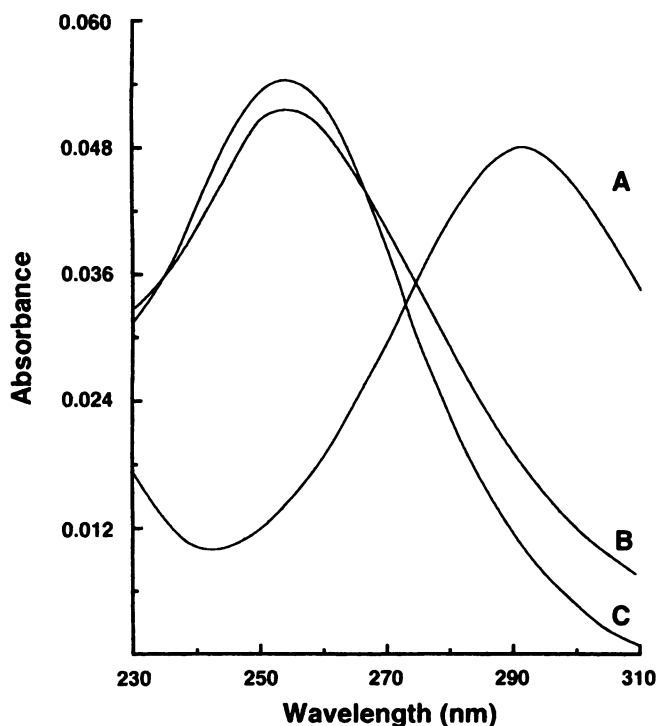


Figure 6. Absorbance spectra of 200 μM HOCl (A) alone, or in the presence of either (B) 200 μM gentamicin or (C) 200 μM tobramycin. All samples were incubated in EBSS at 25°C for 5 min before being scanned in the ultraviolet wavelength range. The reference cuvettes contained EBSS, gentamicin, and tobramycin, respectively.

tive properties against myeloperoxidase/ H_2O_2 -mediated lung epithelial cell injury. In addition, tobramycin completely protected lung epithelial cells *in vitro* against injury from CF bronchial secretions and H_2O_2 .

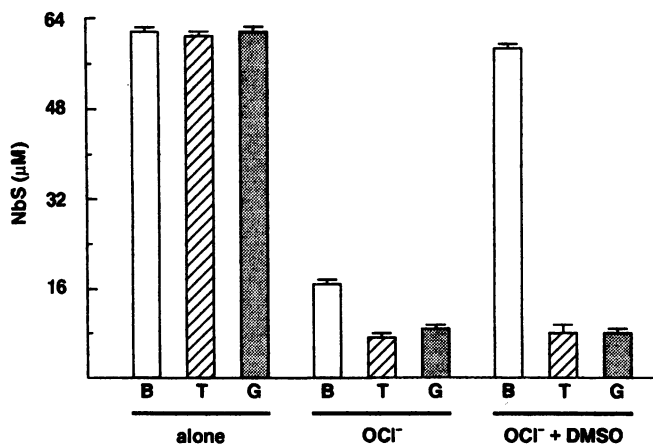


Figure 7. Evidence of chloramine formation from the reaction of HOCl with aminoglycosides. Phosphate buffered saline (B), 200 μM tobramycin (T), and 200 μM gentamicin (G) were incubated either alone or in the presence of 40 μM HOCl for 15 min. Subsequently, 200 μM dimethylsulfoxide (DMSO) was added to half of the samples containing HOCl, for 15 min, followed by the addition of 60 μM 5-thio-2-nitrobenzoic acid to all samples for 5 min. The residual 5-thio-2-nitrobenzoic acid was quantitated from the absorbance of each sample at a wavelength of 412 nm. Values represent the mean \pm SEM of five determinations.

Table II. Relative Hydrophilicity and Lipophilicity of Aminoglycoside Chloramines

Aminoglycoside	Chloramines	
	Aqueous phase	Organic phase
	μM	
Tobramycin	17.67 \pm 0.45 (97)*	0.47 \pm 0.18 (3)
Gentamicin	17.46 \pm 0.53 (92)	1.63 \pm 0.16 (8)

* Values represent the mean \pm SEM of five determinations. Numbers in parentheses represent percentage of total chloramines in both phases.

Myeloperoxidase and H_2O_2 added simultaneously to the lung epithelial cells caused little cytotoxicity, and it was found necessary to preincubate myeloperoxidase with the cells before the addition of H_2O_2 to induce maximal cytotoxicity. Myeloperoxidase is a highly cationic enzyme at physiologic pH, with a $\text{pI} > 10$ (27). Based on studies of myeloperoxidase-mediated glomerular injury, it has been suggested that myeloperoxidase, bound to anionic sites, reacts with H_2O_2 to induce epithelial cell cytotoxicity (28). The presence of anionic sites, particularly negatively charged phospholipids (29), in the membranes of normal epithelial cells raises the possibility that binding of myeloperoxidase to the epithelial cell surface through charge interaction is necessary to induce maximal cytotoxicity.

It is of interest that although the four antibiotics tested in the present study were of equivalent efficacy in preventing HOCl-induced cytotoxicity, the aminoglycosides were approximately fivefold more efficient than either ticarcillin or ceftazidime in preventing myeloperoxidase-mediated injury. None of the antibiotics were found to inhibit myeloperoxidase activity. Because of the cationic nature of the aminoglycosides (30), the likely explanation for this discrepancy is that the aminoglycosides bound to anionic cell surfaces as did myeloperoxidase and thus reacted with HOCl at its site of synthesis. In contrast, both ceftazidime and ticarcillin are organic anions at physiologic pH (31). Although ticarcillin and ceftazidime are potent HOCl scavengers, charge interactions may keep them away from areas of myeloperoxidase-dependent HOCl synthesis at anionic sites of the cell surface.

The mechanisms by which the aminoglycosides and the thioether-containing antibiotics, ticarcillin and ceftazidime, protected epithelial cells were different. Several lines of evidence suggest that the HOCl-aminoglycoside reaction resulted in the formation of chloramines. First, the reaction product was capable of oxidizing the thiol group of 5-thio-2-nitrobenzoic acid, as would be expected of chloramines (32, 33). Second, both aminoglycosides shifted the maximal absorbance wavelength of HOCl from 291 to 252 nm, a characteristic absorption peak for chloramines derived from primary amines (20). Third, whereas DMSO can reduce HOCl, it is unable to reduce chloramines (20). Because DMSO could reduce HOCl alone but not in the presence of either aminoglycoside, these results further support the conclusion that chloramines were synthesized.

Chloramines, while retaining oxidant properties can be either cytotoxic or noncytotoxic, depending, in part, on their relative lipophilicity (32). In that the cell membrane is com-

posed primarily of lipids, the lipophilic chloramines are generally more cytotoxic than the hydrophilic ones. Consistent with the polar, hydrophilic properties of aminoglycosides (34), the present studies indicate that the aminoglycoside-HOCl chloramines were mostly hydrophilic, a characteristic that likely contributed to render the aminoglycoside/HOCl product innocuous. Since all aminoglycosides are rich in primary amines, other antibiotics of this class may have similar cytoprotective properties, but would not be expected to protect extracellular sulfhydryl and methionine groups against HOCl-mediated oxidation.

Although the thioether-containing antibiotics were less efficient than aminoglycosides at protecting lung epithelial cells against myeloperoxidase-mediated injury, they were much more effective in preventing HOCl-mediated oxidation of sulfhydryl groups and methionine. The anti-HOCl properties of ticarcillin and ceftazidime were identical in all assays, suggesting that similar mechanisms, such as thioether-mediated reduction of HOCl, may have been involved for each antibiotic. These observations likely apply to many other penicillins and cephalosporins, all of which contain thioether groups in their thiazolidine and dihydrothiazine rings (35). It is of interest that the protection of methionine against oxidation has been shown to prevent the inactivation of the major anti-elastase protein, α_1 -antitrypsin (36). The thioether antibiotics may therefore help protect this essential molecule at sites of bacterial infection such as the CF lung.

The concept that certain drugs may react with myeloperoxidase-derived oxidants, and in particular HOCl, is not new. It has been demonstrated that the sulfonamide antibiotics can decrease neutrophil candidacidal activity, likely by reacting with myeloperoxidase-derived HOCl (37). Kalyanaraman and Sohnle (38) also reported that foreign compounds, including many drugs, could react with products of the neutrophil myeloperoxidase system to generate toxic electrophilic molecules. Other investigators have demonstrated the scavenging of myeloperoxidase-derived HOCl by several anti-inflammatory drugs (39–41). However the observations from the present study differ in that they demonstrate the capacity of anti-*Pseudomonas aeruginosa* antibiotics to react with HOCl at a rate sufficient to protect lung epithelial cells and, for the thioether-containing antibiotics, thiol groups and methionine from myeloperoxidase-dependent oxidation. These properties may be of particular interest in the therapy of CF lung disease, and of other infectious diseases of the lung, an organ which has an unexpected sensitivity to damage from HOCl-derived oxidants (33).

The epithelial lining fluid of the normal lung contains many molecules that could potentially react with HOCl and act as endogenous antioxidants (15). If these naturally occurring antioxidants were always sufficient to prevent oxidation of essential lung molecules and epithelial cell death, then the antioxidant properties of antibiotics would be irrelevant. Evidence from the literature, such as methionine oxidation in IPF, and α_1 -antitrypsin oxidation in ARDS, suggests that the lung's naturally occurring antioxidants are not always sufficient to prevent oxidative damage and can be overwhelmed in neutrophilic inflammatory diseases (24, 25). These data would thus support the concept that antibiotic antioxidant properties may be clinically relevant. However, the current study does not confirm in vivo antioxidant efficacy, and further studies are needed.

The potential usefulness of these various classes of antibiotics as antioxidants may be modulated by several factors. First, significant antioxidant properties were observed at antibiotic concentrations of $\geq 25 \mu\text{M}$. These relatively high concentrations may be difficult to maintain in tissues involved in purulent infections. Second, although aerosolization of large doses of antibiotics to the respiratory tract in CF is safe and may be a rational approach to maintain high concentrations of antibiotics in the airways (1–5), it is not clear that the antibiotics will reach the epithelial surface in areas of severe inflammation. Aerosol distribution in the CF lung has been shown to be less efficient in patients with more severe lung disease (42). However, the current study raises the question of whether efficient delivery of aerosolized antibiotics to the young CF patient, before severe airway damage has occurred, could significantly delay destructive lung disease by providing effective antioxidant protection. Third, the potential problem of selecting resistant bacteria by routinely administering aerosolized antibiotics for their antioxidant properties must be considered. While this is an important consideration, experience with aerosolized antibiotics in CF to date, would indicate that resistance has not prevented this form of therapy from providing definite benefits to selected CF patients (1–5). Finally, inhibition of the toxicity of the myeloperoxidase system may decrease the bactericidal activity of neutrophils (37). However, this may not be a major problem, because (a) patients with a hereditary deficiency of myeloperoxidase are only slightly more susceptible to bacterial infections than are normal subjects (43), (b) CF patients receiving aerosolized antibiotics do not demonstrate more severe lung infections (1–5), and (c) the major pathogenic bacteria in the CF lung, the mucoid *Pseudomonas aeruginosa*, is already protected against myeloperoxidase-derived oxidants by an alginate coat that efficiently scavenges HOCl (44). Indeed, one may speculate that, if the mucoid *Pseudomonas aeruginosa* has adapted to its HOCl-rich environment by protecting itself against HOCl, then the CF airways may also benefit from anti-HOCl protection.

In summary, aminoglycosides convert HOCl to noncytotoxic chloramines and protect lung epithelial cells against myeloperoxidase-mediated oxidant injury. Thioether containing antibiotics, specifically ticarcillin and ceftazidime, but likely many other penicillins and cephalosporins, are potent HOCl scavengers and protect lung epithelial cells, sulfhydryl groups, and methionine from HOCl-mediated oxidation. These antioxidant properties of antibiotics may contribute to the protection of tissues involved in purulent infections, particularly the lung in patients with CF.

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