INFECTION AND IMMUNITY, Mar. 1997, p. 1071–1076
0019-9567/97/$04.00+0
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Vol. 65, No. 3

Augmentation of Oxidant Injury to Human Pulmonary Epithelial Cells by the *Pseudomonas aeruginosa* Siderophore Pyochelin

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Received 22 July 1996/Returned for modification 19 September 1996/Accepted 30 December 1996

Pseudomonas aeruginosa **causes acute and chronic infections of the human lung, with resultant tissue injury. We have previously shown that iron bound to pyochelin, a siderophore secreted by the organism to acquire iron, is an efficient catalyst for hydroxyl radical ($HO^{\cdot}$) formation and augments injury to pulmonary artery endothelial cells resulting from their exposure to superoxide ($O_2^{-\cdot}$) and/or H_2O_2. Sources for $O_2^{-\cdot}$ and H_2O_2 included phorbol myristate acetate (PMA)-stimulated neutrophils and pyocyanin. Pyocyanin, another *P. aeruginosa* secretory product, undergoes cell-mediated redox, thereby forming $O_2^{-\cdot}$ and H_2O_2. In *P. aeruginosa* lung infections, damage to airway epithelial cells is probably more extensive than that to endothelial cells. Therefore, we examined whether ferripyochelin also augments oxidant-mediated damage to airway epithelial cells. A549 cells, a human type II alveolar epithelial cell line, was exposed to H_2O_2, PMA-stimulated neutrophils, or pyocyanin, and injury was determined by release of ^{51}Cr from prelabeled cells. Ferripyochelin significantly increased (>10-fold) oxidant-mediated cell injury regardless of whether H_2O_2, neutrophils, or pyocyanin was employed. Apo-pyochelin was not effective, and ferripyochelin was not toxic by itself at the concentrations employed. Spin trapping with α-(4-pyrridyl-1-oxide)-*N*-*t*-butyl-nitrone–ethanol confirmed the generation of $HO^{\cdot}$, and injury was decreased by a variety of antioxidants, including superoxide dismutase, catalase, and dimethylthiourea. These data are consistent with the hypothesis that the presence of ferripyochelin at sites of *P. aeruginosa* lung infection could contribute to tissue injury through its ability to promote $HO^{\cdot}$-mediated damage to airway epithelial cells.**

Formation of the hydroxyl radical ($HO^{\cdot}$) via the iron-catalyzed reaction between superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) (Haber-Weiss reaction) has been linked to numerous forms of in vitro and in vivo cell injury (18, 25). There is essentially no free iron in vivo, with most extracellular iron present being chelated to transferrin and related iron-binding proteins (18). Such iron complexes are not capable of acting as efficient catalysts of the Haber-Weiss reaction (7, 18, 37).

We have recently begun to examine the hypothesis that secretory products of the important bacterial pathogen *Pseudomonas aeruginosa* may contribute to the extensive tissue injury observed at sites of infection with this organism by promoting Haber-Weiss reaction-mediated $HO^{\cdot}$ production (3–6, 9, 24). During this work, we demonstrated that iron bound to pyochelin (i.e., ferripyochelin), a siderophore secreted by the organism as part of its means for acquiring extracellular iron, is an efficient catalyst of the Haber-Weiss reaction (9). Furthermore, ferripyochelin was found to markedly augment injury to pulmonary artery endothelial cells resulting from their exposure to $O_2^{-\cdot}/H_2O_2$ (5, 6). Sources for $O_2^{-\cdot}$ and H_2O_2 in these studies included phorbol myristate acetate (PMA)-stimulated neutrophils and pyocyanin (5, 6). The latter compound is another secretory product of *P. aeruginosa*, which undergoes cell-mediated redox cycling resulting in the continuous generation of $O_2^{-\cdot}$ and H_2O_2 (19, 20).

Based on these studies, we suggested that ferripyochelin-catalyzed $HO^{\cdot}$ generation from neutrophil- or pyocyanin-derived $O_2^{-\cdot}/H_2O_2$ could contribute to microvasculature injury (5, 6) which occurs as a consequence of pulmonary infections with *P. aeruginosa* (12, 14). In such infections, damage to airway epithelial cells is probably more extensive than that to endothelial cells (12, 14). Epithelial cells are generally less susceptible to oxidant-mediated injury than endothelial cells (30, 31). Therefore, we chose to examine whether ferripyochelin exhibited a capacity to augment oxidant-mediated damage to airway epithelial cells similar to that which we had previously observed with endothelial cells.

(Portions of this work were presented in abstract form at the November 1995 meeting of the Oxygen Society in Pasadena, Calif.)

MATERIALS AND METHODS

Culture of human lung epithelial cells. The type II alveolar cell-like A549 human lung carcinoma cell line (22) was maintained in continuous culture in Dulbecco's modification of Eagle's medium (The University of Iowa Cancer Center, Iowa City, Iowa) supplemented with 10% heat-inactivated fetal calf serum. For all experiments described, A549 cells from stock culture were seeded at 2.5×10^4 to 5×10^4 cells/well into either 24- or 48-well plates. Cells were incubated in the above-described medium at 5% CO_2 at 37°C until they were 80% confluent (usually 72 h).

Isolation of human neutrophils. Leukocytes were separated from the erythrocyte component of the peripheral venous blood of healthy human donors by dextran sedimentation as previously described (2). A purified (>98%) population of neutrophils was then prepared by centrifugation of the leukocyte fraction through a Ficoll-Hypaque gradient (2). Neutrophils were washed, resuspended in Hanks' balanced salt solution (HBSS), and placed on ice until they were added to the experimental system.

Preparation of pyochelin and pyocyanin. Pyocyanin was purified to uniformity by isolating the compound from culture medium supernatant of *P. aeruginosa* PA01 (ATCC 15692; American Type Culture Collection, Rockville, Md.) as previously described (10). This procedure consists of an initial series of chloroform extractions, which are then followed by sequential extractions with acid and

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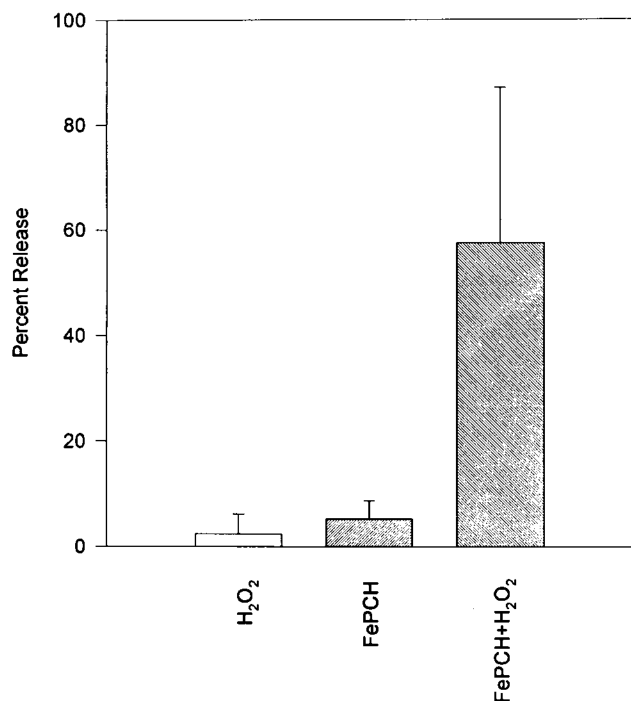

FIG. 1. ^{51}Cr release (means ± standard errors of the means [error bars], n = 5) following exposure of A549 cell monolayers to H_2O_2 (2.5 mM) and ferripyochelin (FePCH with 10 μM Fe) alone or in combination. No effect on cell injury was seen with the use of apopyochelin instead of ferripyochelin.

neutral water. After five separations, the pyocyanin was crystallized and dried under vacuum. For all experiments reported herein, pyocyanin was suspended in H_2O prior to use.

Pyochelin was also prepared from culture supernatant of *P. aeruginosa* PA01. By previously described techniques (11), pyochelin was purified to uniformity in its iron-free (apo) form through dichloromethane extraction and thin-layer chromatography. Iron chelates of pyochelin (ferripyochelin) were then prepared by adding $FeCl_3$ to a solution of pyochelin in 100% ethanol at pH 5.0. Iron was added at a molar ratio such that 20% of the pyochelin remained iron free to be certain that all iron was chelated. Routinely negative control experiments were performed with apopyochelin as a means to confirm that all effects of ferripyochelin observed were related to the ferripyochelin and not to another contaminating factor (e.g., lipopolysaccharides [LPS]) in the preparation. In addition, analysis of pyocyanin and pyochelin preparations for LPS or protein prepared by the above-described methods has consistently revealed no evidence of such contaminants.

Epithelial cell injury. Oxidant-mediated damage to monolayers of A549 cells was quantitated as the release of ^{51}Cr from cells whose intracellular contents had been previously labeled with the compound by using a minor modification of the method that we had previously employed for the study of pulmonary artery endothelial cells (5, 6). Monolayers of A549 cells which had been cultured in microtiter plates were incubated in the presence of 0.5 μCi of ^{51}Cr (sodium chromate, 5 mCi; Amersham Co., Arlington Heights, Ill.) per well for 18 h at 37°C. After the desired period of incubation, cells were washed three times, and then 1 ml of HBSS was added to the cell monolayer along with the desired experimental compounds. The cell suspensions were incubated for the desired time period (37°C), the supernatant was removed, and the amount of radioactivity was determined with a gamma counter. For wells containing pyochelin, that compound was allowed to preincubate for 30 min prior to the addition of the oxidant sources. All experimental conditions were analyzed in triplicate. Spontaneous release of ^{51}Cr was determined at the end of the time period at which oxidant injury was assessed. Maximal ^{51}Cr release was quantitated by exposing the cells to 10% Triton X-100 in order to achieve 100% lysis and then determining the radioactivity in the supernatant. Experiments performed with pyochelin resulted in exposure of the monolayer to up to 4% ethanol, which did not result in any increase in ^{51}Cr release above background when added independently of pyochelin.

The result of each experiment was expressed as the mean specific ^{51}Cr release from triplicate wells of A549 cells, with specific ^{51}Cr release calculated as [(test well ^{51}Cr cpm − spontaneous release ^{51}Cr cpm)/(maximum release ^{51}Cr cpm − spontaneous release ^{51}Cr cpm)] × 100.

Spin trapping. Adherent A549 cells were placed in HBSS containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and either 100 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Sigma Chemical Co., St. Louis, Mo.), or 10 mM α-(4-pyrridyl-1-oxide)-*N*-*t*-butyl-nitrone (4-POBN; Sigma)–170 mM ethanol (Aaper Chemical, Shelbeysville, Ky.). After the addition of other desired reaction components the supernatant was transferred to an electron paramagnetic resonance (EPR) quartz flat cell which was in turn placed into the cavity of an EPR spectrometer (model ESP300; Brüker, Karlsrühe, Germany). The resulting EPR spectrum at 25°C was then obtained after 15 min with a microwave power of 20 mW, a modulation frequency of 100 kHz with a modulation amplitude of 0.501 G, a time constant of 322.68 ms, and a gain of 4×10^5.

Other reagents. Bovine liver catalase was obtained from Calbiochem, La Jolla, Calif. Human erythrocyte CuZn superoxide dismutase (SOD), phorbol myristate acetate (PMA), and dimethyl sulfoxide (DMSO) were purchased from Sigma, whereas dimethyl thiourea (DMTU) was acquired from Aldrich Chemical Co., Milwaukee, Wis.

Statistical analysis of data. Data was analyzed by analysis of variance with Epistat software; results were considered significant at a P value of <0.05.

RESULTS

Ferripyochelin enhances hydrogen peroxide-mediated epithelial cell injury. Previous work from other laboratories suggests that epithelial cells are more resistant to oxidant-mediated injury than endothelial cells are (30, 31). Consistent with these observations, preliminary experiments indicated that H_2O_2 concentrations in the range of 5 mM were required before significant injury (specific ^{51}Cr release) could be detected in A549 cells (data not shown). This result can be compared with previous results from our laboratory in which only 50 μM H_2O_2 was needed to produce similar damage to porcine pulmonary artery endothelial cells (5).

Having established the concentration of H_2O_2 which results in minimal epithelial cell injury, we examined the ability of ferripyochelin to augment such injury. As shown in Fig. 1, 10 μM iron added as ferripyochelin resulted in a statistically significant increase in H_2O_2-mediated injury ($P < 0.01$, $n = 5$). This injury occurred at multiple H_2O_2 concentrations (Fig. 2). In contrast, pyochelin to which iron had not been added (apopyochelin) failed to increase H_2O_2-mediated damage ($P >$

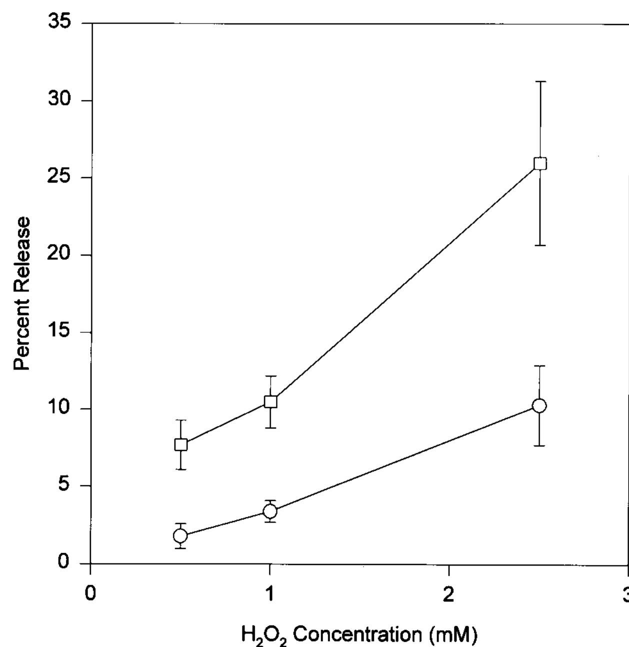

FIG. 2. ^{51}Cr release (means ± standard errors of the means [error bars], n = 3) following exposure of A549 monolayers to various concentrations of H_2O_2 either alone (○) or in the presence of 5 μM ferripyochelin (□).

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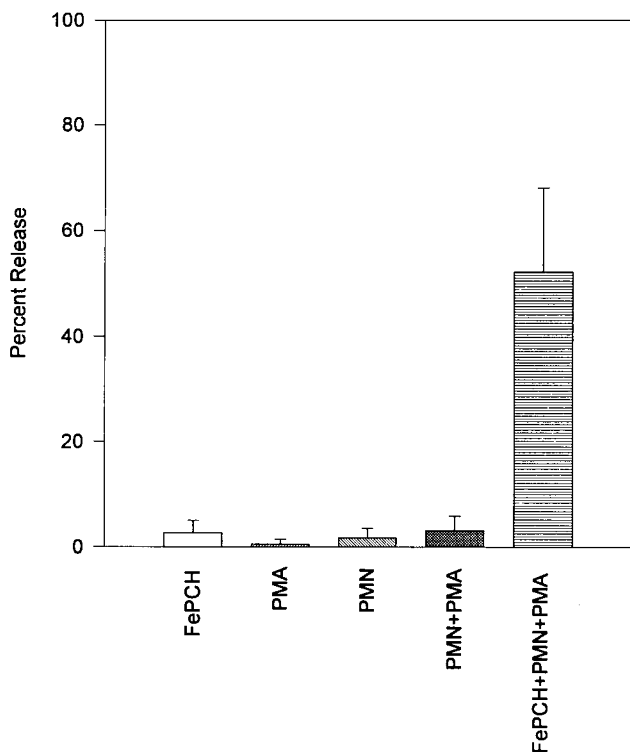

FIG. 3. ^{51}Cr release (means ± standard errors of the means [error bars], n = 3) in A549 cell monolayers exposed to PMA (100 ng/ml)-stimulated neutrophils (PMN) (10^7/ml) in the presence or absence of ferripyochelin (FePCH with 10 μM Fe). Also shown is the effect of parallel exposure of monolayers to PMA alone, FePCH alone, or unstimulated neutrophils. No effect on cell injury was seen with the use of apopyochelin instead of ferripyochelin.

0.05, $n = 5$) (Fig. 1). These data suggest the involvement of an iron-dependent oxidant and also eliminate the involvement of either the ethanol vehicle or contaminating LPS in the process. In addition, the inclusion of ferripyochelin in the absence of H_2O_2 failed to result in detectable ^{51}Cr release above the spontaneous rate (Fig. 1), unless the iron concentration was increased to >25 μM (data not shown).

Ferripyochelin also increases neutrophil- and pyocyanin-mediated epithelial cell injury. Having demonstrated that ferripyochelin enhances H_2O_2-mediated epithelial cell injury, we assessed the ability of this iron chelate to augment injury resulting from exposure of epithelial cells to two forms of oxidant stress with greater relevance to *P. aeruginosa* lung infections: (i) stimulated human neutrophils and (ii) the redox active *P. aeruginosa* secretory product pyocyanin. Consistent with our earlier findings with endothelial cells (5, 6), ferripyochelin enhanced damage to A549 cells induced by either PMA-stimulated human neutrophils (Fig. 3) ($P < 0.05$, $n = 3$) or pyocyanin (Fig. 3) ($P < 0.001$, $n = 5$). Once again the process was iron dependent, as apopyochelin had no effect (Fig. 3 and 4) ($P > 0.05$) and ferripyochelin by itself yielded no consistent injury at the concentrations employed (≤80 μM). In the case of pyocyanin, cell injury increased in proportion to the concentration of ferripyochelin present (Fig. 5). This result could not be adequately tested in the neutrophil system because of deleterious effects of the pyochelin vehicle (ethanol) on neutrophil function.

Role of hydroxyl radical in ferripyochelin enhancement of oxidant-mediated epithelial cell injury. The above data indicated that regardless of the source of the oxidant, the ability of pyochelin to enhance epithelial cell injury was dependent on its iron chelation status. This finding suggested that the ability of ferripyochelin to catalyze the formation of $HO^\cdot$ was responsible for the enhanced cell injury. In order to test this hypothesis we first confirmed that $HO^\cdot$ was generated under the conditions of enhanced injury. When A549 cells were exposed to either H_2O_2, PMA-stimulated neutrophils, or pyocyanin in the presence of ferripyochelin and a spin trapping system comprised of 4-POBN and ethanol, EPR spectra indicating the spin trapping of $HO^\cdot$ were obtained (Fig. 6). In contrast, $HO^\cdot$ formation was not observed in the absence of ferripyochelin (Fig. 6). Using the spin trap DMPO, we were also able to confirm that exposure of A549 cells to pyocyanin resulted in the production of superoxide. This was manifested as formation of the DMPO spin adduct DMPO-OH, whose production was inhibited by SOD (Fig. 7).

Having confirmed that $HO^\cdot$ was produced as a consequence of the presence of ferripyochelin, we next utilized various oxidant scavengers and other inhibitors of Haber-Weiss-mediated $HO^\cdot$ production to provide additional evidence that $HO^\cdot$ formation contributed to cell injury. These consisted of SOD (consumes $O_2^{-\cdot}$), catalase (destroys H_2O_2), dimethylthiourea (DMTU) (scavenges H_2O_2 and $HO^\cdot$) and DMSO (scavenges $HO^\cdot$). As shown in Table 1, only catalase provided protection from H_2O_2-ferripyochelin-associated injury. SOD, DMTU, and DMSO had no effect. In contrast, SOD, catalase, and DMTU, but not DMSO, limited A549 damage resulting from exposure to pyocyanin and ferripyochelin (Table 1). SOD and DMTU, but not catalase, protected from neutrophil ferripyochelin-mediated injury. In cases in which SOD or catalase exhibited a protective effect, this effect was lost when heat-inactivated (100°C for 5 min) SOD or catalase was substituted (data not shown).

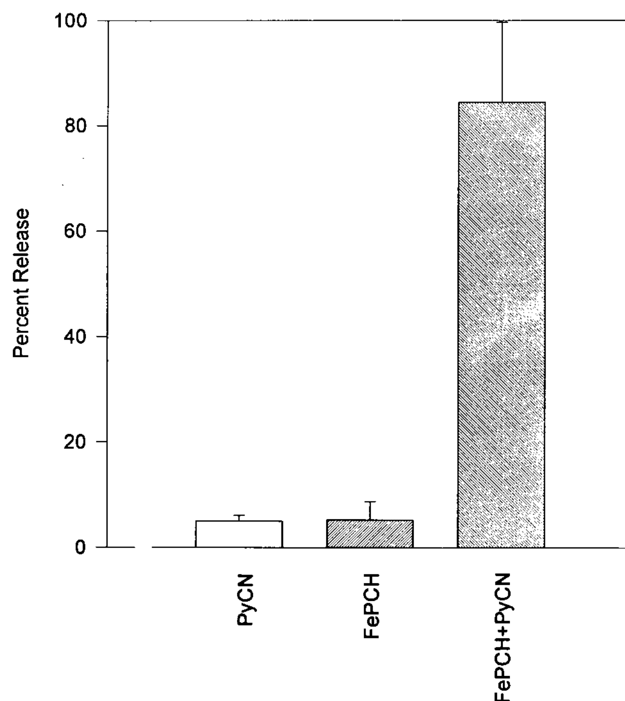

FIG. 4. ^{51}Cr release (means ± standard errors of the means [error bars], n = 5) following exposure of A549 cell monolayers to pyocyanin (80 μM) and ferripyochelin (FePCH with 10 μM Fe) alone and in combination. There was no effect on cell injury when apopyochelin was substituted for ferripyochelin.

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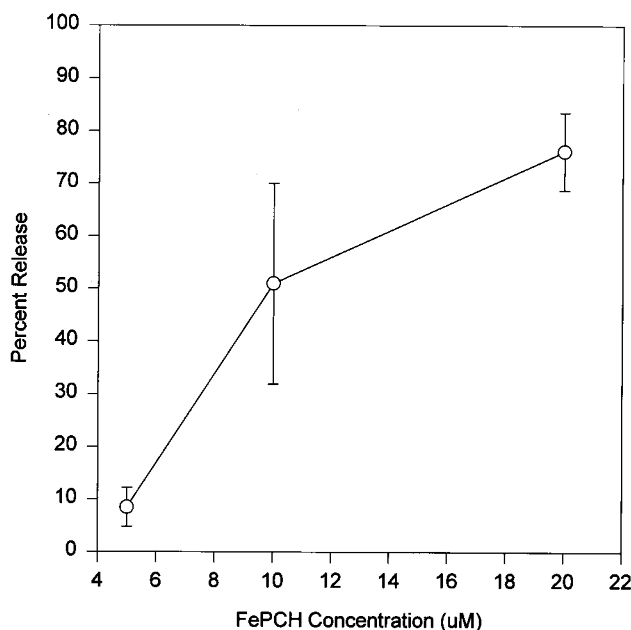

FIG. 5. ^{51}Cr release (means ± standard errors of the means [error bars], n = 3) following exposure of A549 cell monolayers exposed to 80 μM pyocyanin in the presence of different concentrations of ferripyochelin as well as the same ferripyochelin concentrations in the absence of pyocyanin. Pyocyanin at this concentration does not result in significant ^{51}Cr release above that of controls (see Fig. 4).

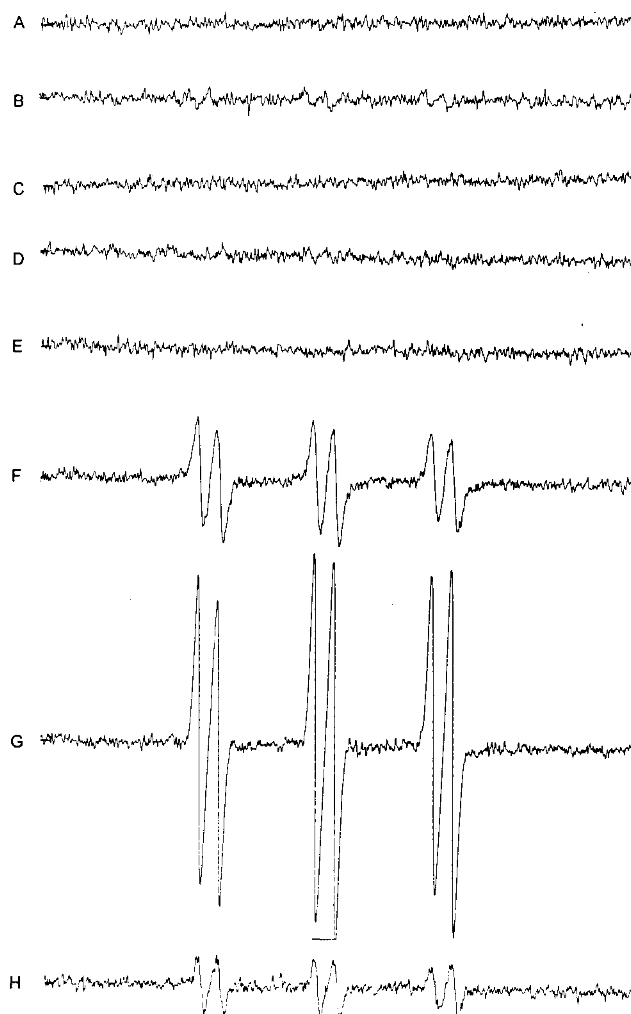

FIG. 6. EPR tracings representative of three to five separate experiments in which A549 cells were exposed in the presence of 10 mM 4-POBN and 170 mM ethanol for 20 to 30 min (tracing A) plus either ferripyochelin (10 μM Fe) (tracing B), 5 mM H_2O_2 (tracing C), PMA-stimulated neutrophils (10^5/ml) (tracing D), or pyocyanin (80 mM) (tracing E). Tracings F, G, and H were obtained when the A549 cells were exposed to ferripyochelin plus the same concentrations of H_2O_2 (tracing F), pyocyanin (tracing G), and stimulated neutrophils (tracing H).

DISCUSSION

P. aeruginosa causes necrotizing bacterial pneumonia associated with considerable morbidity and mortality in hospitalized patients (14). In addition, chronic infection with the same bacterial species is linked to the progressive pulmonary dysfunction which is currently responsible for the death of most patients with cystic fibrosis (13). In both the acute and chronic forms of *P. aeruginosa* lung infection noted above, airway epithelial cells are among the lung cells most extensively damaged (12, 14). The exact mechanism(s) whereby such cellular injury occurs remains unclear and may well be multifactorial (13, 14).

Formation of $HO^{\cdot}$ generated by the iron-catalyzed Haber-Weiss reaction has been implicated as an important mediator of oxidant-mediated damage to numerous cell types (7, 18, 37). Most iron in vivo is chelated in a form in which the metal is unable to participate in this reaction (18). We have recently demonstrated that iron bound to the *P. aeruginosa* siderophore pyochelin is an efficient catalyst of the Haber-Weiss reaction (9) and that this lipophilic iron chelate is capable of augmenting oxidant-mediated injury to pulmonary artery endothelial cells (5, 6). These results suggested that such a process could contribute to the endothelial cell damage which occurs at sites of *P. aeruginosa* infection (12, 14).

The work reported herein extends these same observations to airway epithelial cells, cells which appear to be more extensively damaged than endothelial cells during acute and chronic *P. aeruginosa* lung infections (12, 14). With exposure to either H_2O_2, neutrophil-derived H_2O_2 and $O_2^{-\cdot}$, or the redox active *P. aeruginosa* secretory product pyocyanin, the resulting damage (^{51}Cr release) to monolayers of immortalized A549 lung epithelial cells was markedly enhanced by the presence of ferripyochelin. Pyocyanin has previously been shown to induce the formation of $O_2^{-\cdot}$ in a variety of cells (6, 19, 20), and we confirmed using DMPO spin trapping techniques that this free radical species was also generated upon exposure of epithelial cells to this agent. Using an alternative method, Gardner has also obtained evidence for pyocyanin-induced $O_2^{-\cdot}$ production by lung epithelial cells in vitro (15). These results are consistent with our previous data for porcine pulmonary artery endothelial cells (5, 6) except that the amount of H_2O_2 (but not pyocyanin) required was far greater than that needed to produce comparable endothelial cell damage. Other investigators have noted that the resistance of epithelial cells to oxidant-mediated cell injury is greater than that of endothelial cells (30, 31), the reason for which remains unclear.

The ability of pyochelin to augment oxidant-mediated damage to A549 cells required that it have iron chelated to it, suggesting that the mechanism responsible involves the formation of an iron-dependent oxidant such as $HO^{\cdot}$. Consistent with this hypothesis, spin trapping techniques confirmed $HO^{\cdot}$ production under these conditions. Furthermore, a variety of

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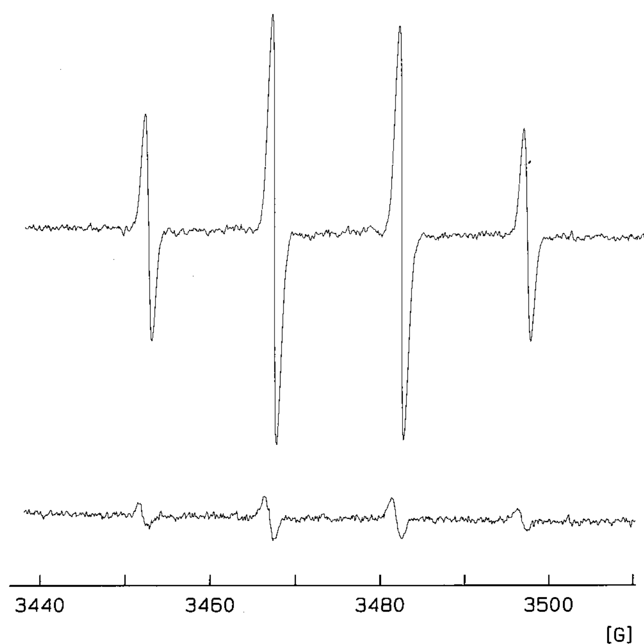

FIG. 7. EPR tracings representative of three separate experiments in which A549 cells were exposed to pyocyanin (80 μM) alone (top tracing) and in the presence of SOD (30 U/ml) (bottom tracing).

oxidant scavengers which would be expected to interrupt either the formation of or damage mediated by HO˙ decreased the ability of ferripyochelin to augment A549 cell damage by each of the three oxidant systems studied. Surprisingly, the ability of various antioxidants to provide protection from ferripyochelin-dependent injury varied with the source of O_2^- and/or H_2O_2-pyocyanin, neutrophils, or H_2O_2. Furthermore, the pattern was different in some cases from that which we had previously observed with endothelial cells (5, 6). The reason for these variations is not clear. It is likely that these antioxidant agents differ in their access to potential sites of HO˙ formation. If this is the case, it suggests that the critical site of A549 injury may vary somewhat with each system. Alternatively, in some cases, damage could be mediated by iron-dependent oxidants other than HO˙ (e.g., ferryl species) whose reactivity which each antioxidant may be different from that of HO˙ (21, 27, 33, 38).

These data indicate that ferripyochelin can augment in vitro oxidant-mediated injury of human airway epithelial cells. However, it should be recognized that this evaluation has been

TABLE 1. Effect of antioxidants on ferripyochelin-dependent epithelial cell injury

Antioxidant	^{51}Cr release (%)[a]		
	H_2O_2	PMA-stimulated neutrophils	Pyocyanin
SOD	94 ± 15 (4)	22 ± 10 (3)*	42 ± 10 (7)**
Catalase	32 ± 8 (4)*	100 ± 7 (3)	11 ± 7 (4)**
DMTU	82 ± 9 (4)	27 ± 11 (3)*	32 ± 10 (5)**
DMSO	89 ± 14 (4)	ND	94 ± 5 (4)

[a] Results are the means ± standard errors of the means for A549 cells exposed to 10 μM ferripyochelin plus either (i) H_2O_2 (2.5 mM), (ii) PMA-stimulated neutrophils (10^7/ml), or (iii) pyocyanin (80 μM) in the presence of either SOD (30 U/ml), catalase (5,000 U/ml), DMTU (10 mM), or DMSO (140 mM), expressed as a percentage of the ^{51}Cr release observed in their absence. Numbers in parentheses are the number of separate experiments performed. *, P of <0.05; **, P of <0.01 by analysis of variance; ND, not done.

performed with a single human epithelial cell line. Limited studies performed to date with the HBE cell line have also demonstrated that concentrations of ferripyochelin similar to those employed in the present study augment oxidant-mediated cell injury. However, interexperimental variability of HBE cell susceptibility to the exogenous oxidant sources of interest (H_2O_2, pyocyanin, and neutrophils) have limited our ability to directly compare results with HBE and A549 cells. If eventually available in sufficient numbers, primary cultures of the human airway epithelium should be examined.

As previously discussed (6, 9), the concentrations of the agents used in these studies are likely present in vivo in the setting of cystic fibrosis. The concentrations of pyocyanin employed are similar to those detected in sputum sols of cystic fibrosis patients (36). Pyocyanin levels have not, to our knowledge, been quantitated in patients with acute *P. aeruginosa* pneumonia. Similarly, we are not aware of reports in which pyochelin has been quantitated at in vivo sites of either acute or chronic *P. aeruginosa* infection. However, data do suggest that local conditions should lead to pyochelin production in vivo (17, 28), and the concentration of pyochelin employed in our studies was equal to or less than that which accumulates during in vitro culture of the organism (11).

Although our data are consistent with a possible role of ferripyochelin in the epithelial cell injury occurring in acute and chronic *P. aeruginosa* lung infection, other conditions which could potentially enhance or inhibit the effect of ferripyochelin that we have observed in our in vitro system exist within the airway. The airway contains a variety of antioxidant molecules (1, 8, 16, 23) which could potentially limit the cytotoxicity of ferripyochelin-induced HO˙ formation. Two iron-binding proteins also exist in respiratory secretions, lactoferrin and, to a lesser extent, transferrin, which would compete with pyochelin for iron and would bind it in a form which does not catalyze HO˙ production. We have recently presented data that *P. aeruginosa* and neutrophil-derived proteins cleave both transferrin and lactoferrin in vitro (3) and in vivo (4), resulting in the formation of catalytic iron chelates capable of catalyzing HO˙ formation (3, 24, 26) and augmenting oxidant-mediated endothelial cell injury (24, 26). Thus, the normal protective mechanisms against redox active iron in the airway may be disturbed in *P. aeruginosa* infection. It is likely that these complex interactions will require the development of an animal system for study. In order to do so, technical issues, such as delivery and quantitation of agents and the parameter of injury for study, will need to be resolved. Nevertheless, given clinical (34) and recent in vitro (29, 32, 35) data indicating that host defense against this organism does not require neutrophil oxidant formation, investigations into the possible therapeutic benefit of antioxidant agents which would potentially decrease airway susceptibility to HO˙ and other oxidants may be warranted.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the Office of Research and Development, Medical Research Service, Department of Veteran Affairs, and the U.S. Public Health Service (HL44275 and AI34954). The work was performed during the tenure of B.E.B. as an Established Investigator of the American Heart Association.

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