INFECTION AND IMMUNITY, Jan. 1996, p. 182–188
0019-9567/96/$04.00+0

Vol. 64, No. 1

Protease Cleavage of Iron-Transferrin Augments Pyocyanin-Mediated Endothelial Cell Injury via Promotion of Hydroxyl Radical Formation

RACHEL A. MILLER,[1,2]* GEORGE T. RASMUSSEN,[1,2] CHARLES D. COX,[3] AND BRADLEY E. BRITIGAN[1,2]

Research Service and Department of Internal Medicine, VA Medical Center, Iowa City, Iowa 52246,[1] *and Departments of Internal Medicine*[2] *and Microbiology,*[3] *University of Iowa College of Medicine, Iowa City, Iowa 52242*

Received 20 April 1995/Returned for modification 15 May 1995/Accepted 26 October 1995

Although a number of bacterium- and host-derived factors have been suggested to contribute to the pathogenesis of *Pseudomonas aeruginosa*-associated tissue injury, the mechanism remains unclear. We have previously shown that protease modification of iron (Fe)-transferrin generates new iron chelates capable of catalyzing hydroxyl radical (˙OH) formation from superoxide and hydrogen peroxide. The latter two oxidants are generated during redox cycling of another *P. aeruginosa* secretory product, pyocyanin. The lung is a major site of *P. aeruginosa* infection, with damage to local endothelial cells contributing to the pathogenesis of such infections. Endothelial cells are highly susceptible to oxidant-mediated injury. Therefore, we examined whether pseudomonas elastase-cleaved Fe-transferrin and pyocyanin synergistically enhance pulmonary artery endothelial cell injury via ˙OH formation. By measuring ^{51}Cr release from cultured endothelial cell monolayers, pseudomonas elastase-cleaved Fe-transferrin significantly augmented cell injury resulting from cellular exposure to sublethal concentrations of pyocyanin. This enhancement in injury was not protease specific, as similar results were obtained with pyocyanin in combination with trypsin- or porcine pancreatic elastase-cleaved Fe-transferrin. The association of iron with the transferrin appeared to be necessary in this process. Supporting the involvement of ˙OH generation via the Haber-Weiss reaction in augmenting cell injury, catalase, dimethyl thiourea, superoxide dismutase, deferoxamine, and dimethyl sulfoxide significantly inhibited cell injury resulting from exposure to pyocyanin and protease-cleaved Fe-transferrin. Furthermore, spin trapping demonstrated the production of ˙OH in this cellular system. We conclude that ˙OH formation resulting from the interaction of protease-cleaved Fe-transferrin and endothelial cell redox cycling of pyocyanin may contribute to *P. aeruginosa*-associated tissue injury via endothelial cell injury.

Pseudomonas aeruginosa is a major cause of nosocomial pneumonia and septicemia (48). The organism also plays a role in the chronic progressive pulmonary dysfunction observed with cystic fibrosis (28, 56). Both bacterial and host factors likely contribute to the marked tissue damage observed with these infections. A variety of neutrophil- and bacterium-derived secretory products, particularly proteases, have been implicated in this process (25, 28, 37, 39, 46, 49, 59, 67). Oxidants, largely generated by local neutrophils, have also been suggested as important contributors to tissue injury at sites of inflammation under a variety of conditions (11, 21, 55, 61).

Products of *P. aeruginosa* may also lead to oxidant-mediated tissue injury. Pyocyanin, a blue pigment produced by many strains of *P. aeruginosa*, is known to have numerous deleterious effects on host defense mechanisms, most notably leukocytes and mucociliary transport (47, 54, 64, 65). It is also cytotoxic in vitro to a number of eukaryotic and prokaryotic cells (2, 31, 34). In each case, these deleterious effects likely occur via pyocyanin's ability to undergo cellular redox cycling in the presence of various reducing agents and oxygen to form superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) (34).

Superoxide and H_2O_2 are only moderately cytotoxic but can react in the presence of an iron catalyst to form the highly cytotoxic hydroxyl radical (˙OH) via the Haber-Weiss reaction (13). Hydroxyl radical is one of the most potent oxidizing agents described and has received considerable interest as a cause of oxidative cell injury at sites of inflammation (63). In vivo, free extracellular iron is essentially nonexistent (16, 41, 43). Transferrin serves as the primary iron-binding protein in serum and is also present at mucosal surfaces (43). When bound to this 76- to 80-kDa protein, iron cannot act as an ˙OH catalyst (3, 4, 15, 66). This property may allow transferrin to function as an antioxidant in vivo (33, 62).

A number of neutrophil- and bacterium-derived proteases cleave transferrin into smaller protein fragments (6, 8, 24, 26, 27, 42). We have previously demonstrated that the *P. aeruginosa*-derived protease, pseudomonas elastase (PAE), in addition to trypsin and porcine pancreatic elastase (PPE), cleaves diferric transferrin (Fe-transferrin) into smaller protein fragments able to catalyze ˙OH formation (9) and augment endothelial cell injury in the presence of polymorphonuclear leukocyte-derived oxidants (45). Evidence supporting the potential clinical relevance of these findings has been provided by the detection of transferrin cleavage products in bronchoalveolar lavage (BAL) specimens from *P. aeruginosa*-infected cystic fibrosis patients but not from healthy individuals (10).

P. aeruginosa-associated lung infection involves injury to microvascular endothelial cells (5, 7, 19, 29). These cells are also well known for their susceptibility to oxidant-mediated injury (36). We have previously shown that pyocyanin interacts synergistically with iron bound to another *P. aeruginosa* secretory product, the siderophore pyochelin, to damage pulmonary en-

* Corresponding author. Mailing address: Department of Medicine, The University of Iowa, 200 Hawkins Dr., SW34 GH, Iowa City, IA 52242. Phone: (319) 356-7228. Fax: (319) 356-4600.

dothelial cells through the production of ·OH (11). Therefore, we examined the possibility that the synergistic action of pyocyanin and protease-cleaved Fe-transferrin could result in oxidant-mediated pulmonary artery endothelial cell injury via ·OH formation. Here we report the results of these studies and discuss them as indicating a potential mechanism of microvascular injury at sites of *P. aeruginosa* infection.

MATERIALS AND METHODS

Protease cleavage of apotransferrin and diferric transferrin. Apotransferrin and diferric transferrin (20 mg/ml, Sigma Chemical Co., St. Louis, Mo.) were suspended in Hanks' balanced salt solution without phenol red and with calcium chloride (1.67 mM) and magnesium sulfate (0.82 mM) (HBSS) (University of Iowa Cancer Facility, Iowa City, Iowa). They were incubated in the presence of 20 μg of PAE (64.7 U/mg of protein; Nagase Biochemicals, Tokyo, Japan) per ml, 200 μg of PPE (70 U/mg of solid, 120 U/mg of protein; Sigma) per ml, or 50 μg of trypsin (12,600 U/mg of solid, 13,000 U/mg of protein; Sigma) per ml for 48 to 72 h at 37°C. Previous work has shown this incubation period to be optimal for generating redox active transferrin cleavage products (9). Solutions composed of apo- or diferric transferrin or each protease alone were incubated in parallel as controls. Cleavage of transferrin by each protease was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with transferrin preparations solubilized in Laemmli solubilizing buffer (10) containing 1% 2-mercaptoethanol for 5 min at 100°C. A 4.4- to 8.7-μg amount of solubilized transferrin (with or without protease) was electrophoresed (150 V · h) into a 14% polyacrylamide minigel, following which the gel was stained with silver (Accurate Chemical & Scientific Corporation, Westbury, N.Y.).

Purification of pyocyanin. As previously described (20), pyocyanin was extracted from culture supernatant of *P. aeruginosa* PA01 (ATCC 15692; American Type Culture Collection, Rockville, Md.) by consecutive chloroform extractions followed by alternating extractions with acidified and neutralized water layers to allow separation of the red, acid, and blue forms of pyocyanin. The pyocyanin was then crystallized, washed with water, and dried under a vacuum. It was dissolved in water before being used in cell injury and spin-trapping assays.

Cell culture. Pulmonary artery endothelial cells were obtained from porcine pulmonary artery by techniques described by Britigan et al. (12). For cell injury assays (see below), porcine pulmonary artery endothelial cells at passages 5 to 9 were plated at a concentration of 3.75×10^4 cells per well in 48-well tissue culture plates (Costar, Cambridge, Mass.) in 0.5 ml of medium 199 (University of Iowa Cancer Facility) with 10% serum, basal medium amino acids, basal minimal essential vitamins, 2 mM L-glutamine, and 10 U of penicillin-streptomycin (Gibco Laboratories, Grand Island, N.Y.) per ml. The plates were then incubated at 37°C, 5% CO_2 for 4 to 5 days to obtain confluent monolayers. For spin-trapping experiments, confluent monolayers of porcine pulmonary artery endothelial cells were generated on Cytodex 3 microcarrier beads (Pharmacia, Piscataway, N.J.) as previously described (12, 17).

^{51}Chromium release assay. The ^{51}Cr release assay was performed as previously described (11), with a slight modification. Endothelial cell monolayers were placed in HBSS and then preincubated for 30 min (37°C) in the presence of protease-cleaved Fe (apo)-transferrin (4 mg/ml) or corresponding controls, following which pyocyanin was added. The experimental result was not altered if the 30-min preincubation step was omitted. The pyocyanin concentration was adjusted such that it caused no more than 10% of the maximum ^{51}Cr release, generally 80 to 160 μM. These varying pyocyanin concentrations were used as endothelial cell susceptibility to pyocyanin-mediated injury varies depending on the number of times the cells are passaged in culture and among individual animals from which the cells originated. Data are expressed as the mean specific ^{51}Cr release for triplicate samples, where specific ^{51}Cr release is defined as follows: ^{51}Cr release = (test well ^{51}Cr cpm − spontaneous-release ^{51}Cr cpm)/(maximum-release ^{51}Cr cpm − spontaneous-release ^{51}Cr cpm), where cpm is counts per minute.

Spontaneous ^{51}Cr release from the monolayers by cells incubated in HBSS over 4 h only was a mean of 23.0% (range, 14.8 to 29.9%) of the maximum release for all experiments performed. In some experiments, the ability of ·OH scavengers and antioxidant enzymes to prevent cell injury was determined by adding catalase (500 U/ml; Sigma), CuZn superoxide dismutase (SOD) (300 U/ml; Sigma), dimethyl sulfoxide (DMSO) (1% [vol/vol]; Fisher Scientific Co., Fairlawn, N.J.), or dimethyl thiourea (DMTU) (50 μM; Aldrich Chemical Co., Milwaukee, Wis.) along with the transferrin cleavage products to the endothelial cell monolayer and allowing them to remain throughout the subsequent exposure to pyocyanin. Similar experiments were performed with the iron chelator deferoxamine (0.1 or 0.5 mM; Sigma).

Spin trapping. Endothelial cell monolayers on Cytodex 3 beads were washed with HBSS three times and resuspended in HBSS with 25% (vol/vol) microcarrier beads (0.5×10^7 to 1.0×10^7 cells per ml) containing diethylenetriaminepentaacetic acid (DTPA) (0.1 mM; Sigma), α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN) (20 mM; Sigma), and 1% ethanol (Aaper Chemical Co., Shelbyville, Ky.), with or without the protease cleavage products or controls. Pyocyanin (40 μM) was added the reaction mixture, which was then transferred to a flat quartz electron paramagnetic resonance (EPR) cell. The EPR spectrum was determined with signal at an average temperature of 25°C by using an EPR spectrometer (model ESP 300; Bruker, Karlsruhe, Germany) at the following settings: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.941 G; time constant, 0.164 s; and gain, 5.0×10^5. In this system, ·OH formation is reflected by detection of the ·OH-derived spin adduct, 4-POBN–·CHOHCH$_3$ (A_N = 15.5 G; A_H = 2.6 G [A_N and A_H are the splitting constants for nitrogen and hydrogen, respectively]) (50). All buffers were routinely treated with chelating resin (Sigma) to reduce the presence of adventitious iron. The EPR spectra shown are representative of experiments performed a minimum of three times.

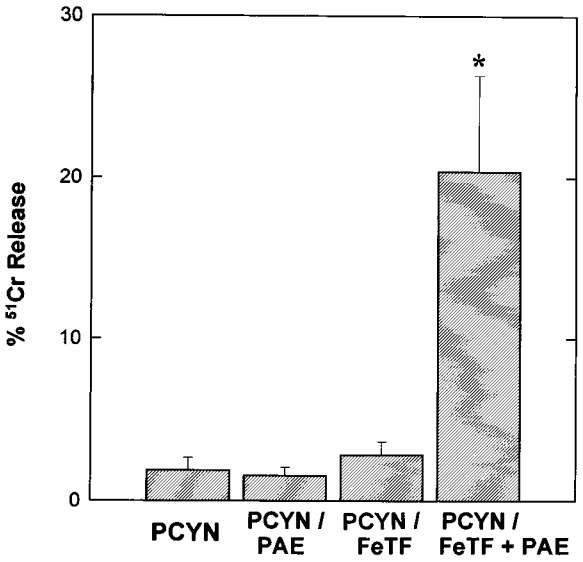

FIG. 1. Mean (n = 13, ± standard error of the mean [error bars]) specific ^{51}Cr release obtained with porcine pulmonary artery endothelial cell monolayers incubated in the presence of pyocyanin (PCYN) (80 to 160 μM) alone and with cells preincubated for 30 min in the presence of PCYN and PAE-cleaved iron-transferrin (FeTF + PAE), where * indicates a P value of < 0.001 versus the result for PCYN alone. Paired samples containing PCYN plus either uncleaved FeTF or PAE alone are shown for comparison.

Statistical analysis. Statistical comparisons among three or more groups were made with a repeated-measure analysis of variance by using the Bonferroni or Tukey post-hoc multiple-comparison method when significant differences were seen. A two-tailed paired t test was used for two-group comparisons. The P values reported throughout are derived from the results of the post-hoc multiple-comparison tests unless otherwise noted. Differences were considered significant at $P < 0.05$. Statistical calculations were performed by using the Graph Pad Instat program (version 2.00, copyright 1990 to 1993) from Graph Pad Software.

RESULTS

(i) Does the interaction of pyocyanin and PAE-cleaved Fe-transferrin synergistically augment endothelial cell injury? As endothelial cells are susceptible to oxidant-mediated injury, the ability of PAE-cleaved Fe-transferrin and pyocyanin to interact and augment this injury was examined. Consistent with our previous results (9), SDS-PAGE of PAE-exposed Fe-transferrin confirmed Fe-transferrin cleavage by showing the appearance of two or more bands with molecular weights lower than that of the Fe-transferrin standard. Endothelial cell injury was studied in the presence of sublethal concentrations of pyocyanin. As quantitated by the release of ^{51}Cr from prelabelled endothelial cells, 80 to 160 μM pyocyanin resulted in a release of ^{51}Cr which was 1.9% of the maximum (Fig. 1). The addition of the same concentration of pyocyanin to paired endothelial cell monolayers which had been preincubated for 30 min in the presence of PAE-cleaved Fe-transferrin resulted in a 20.4% specific ^{51}Cr release (Fig. 1). This was a statistically significant difference relative to the result seen with pyocyanin alone (n = 13; $P < 0.001$). Cells incubated with uncleaved

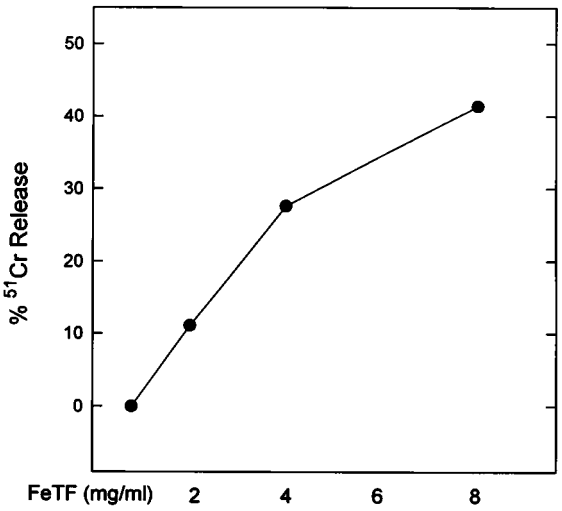

FIG. 2. Dose-response curve representative of four experiments demonstrating a concentration-dependent increase in ^{51}Cr release with PAE-cleaved iron-transferrin (FeTF + PAE) at a constant pyocyanin concentration (160 μM). The pyocyanin concentration used in the three studies whose results are not shown was either 80 or 160 μM.

Fe-transferrin or PAE alone followed by pyocyanin addition yielded ^{51}Cr release which was not statistically different from that obtained with pyocyanin alone (Fig. 1). Likewise, the addition of $FeCl_3$ to the pyocyanin-PAE test group did not elicit a significant increase in ^{51}Cr release.

Experiments performed with increasing concentrations of PAE-cleaved Fe-transferrin and Fe-transferrin and PAE controls in the presence of 80 or 160 μM pyocyanin revealed a concentration-dependent increase in ^{51}Cr release with the Fe-transferrin cleavage products (Fig. 2), in comparison with the minimal increases observed with Fe-transferrin and PAE controls. Although use of the higher concentration of the Fe-transferrin cleavage products yielded the most ^{51}Cr release, the variability among the triplicates samples also increased. In addition, this Fe-transferrin concentration is in excess of that expected under most in vivo conditions (2 to 4 mg/ml) (53). Therefore, the concentrations of Fe-transferrin and PAE used in all subsequent endothelial cell injury assays were 4 mg/ml and 3 μg/ml, respectively.

To further assess the mechanism whereby PAE-cleaved Fe-transferrin and pyocyanin augment oxidant-mediated endothelial cell injury, a series of experiments were done to determine which components of the system were necessary in mediating the increase in endothelial cell injury. Studies performed with pyocyanin and PAE-cleaved apotransferrin yielded no significant increase in endothelial cell injury above the level obtained with pyocyanin alone (Fig. 3). The role of iron in mediating injury was further established by the addition of deferoxamine, an iron chelator, to the reaction mixture. The presence of 100 or 500 μM deferoxamine produced 54.7 and 52.9% inhibitions of endothelial cell injury, respectively, resulting from endothelial cell exposure to pyocyanin and PAE-cleaved Fe-transferrin ($n = 5$, $P < 0.001$). The iron critical for injury appears to be associated with the cleaved Fe-transferrin rather than released from the protein, as the combination of pyocyanin and free iron in the form of $FeCl_3$ (100 μM) did not result in a significant increase in injury (Fig. 3). The combination of pyocyanin and Fe-transferrin treated with heat-inactivated (100°C for 10 min) PAE also failed to augment oxidant-mediated endothelial cell injury (Fig. 3), demonstrating that Fe-transferrin first interacts with an active enzyme to bring about endothelial cell injury. To determine whether pyocyanin was necessary for endothelial cell injury with the PAE-cleaved Fe-transferrin, studies assessing ^{51}Cr release with and without pyocyanin were performed. The preincubation of endothelial cells with PAE-cleaved Fe-transferrin caused significant injury only when pyocyanin was later added ($n = 11$; $P < 0.001$). These findings suggest that the synergistic interaction of PAE-cleaved Fe-transferrin and pyocyanin enhances endothelial cell injury via an iron-dependent, oxidant-mediated mechanism which requires Fe-transferrin cleavage.

(ii) Is the ability of cleaved Fe-transferrin and pyocyanin to synergistically augment endothelial cell injury protease specific? Proteases other than PAE are responsible for much of the increased proteolytic activity detectable at sites of *P. aeruginosa* infection (1, 22, 23, 58–60). Several of these proteases can also cleave Fe-transferrin (6, 8, 9, 24, 26, 27, 42), yielding redox active iron (9). Therefore, we assessed if oxidant-mediated endothelial cell injury was augmented in the presence of these iron chelates. Cleavage of Fe-transferrin by proteases other than PAE (trypsin or PPE) also augmented pyocyanin-mediated endothelial cell injury. PPE, an enzyme similar in function to human neutrophil elastase (HNE) (57), was used as a model of HNE as the cost of HNE precluded its use in these studies given the quantity necessary to generate the Fe-transferrin cleavage products required. Cleavage of Fe-transferrin by PPE and trypsin was illustrated by SDS-PAGE, demonstrating the presence of two prominent and several other, lower-molecular-weight bands which were not seen with the Fe-transferrin standard (data not shown). These banding patterns were consistent with those seen previously (9). Although trypsin, PPE, and PAE induced different patterns of transferrin cleavage, the relative magnitudes of cleavage were similar. As shown in Fig. 4, the inclusion of trypsin- or PPE-cleaved Fe-transferrin significantly increased endothelial cell injury (^{51}Cr release) resulting from exposure to pyocyanin. The proteases alone failed to

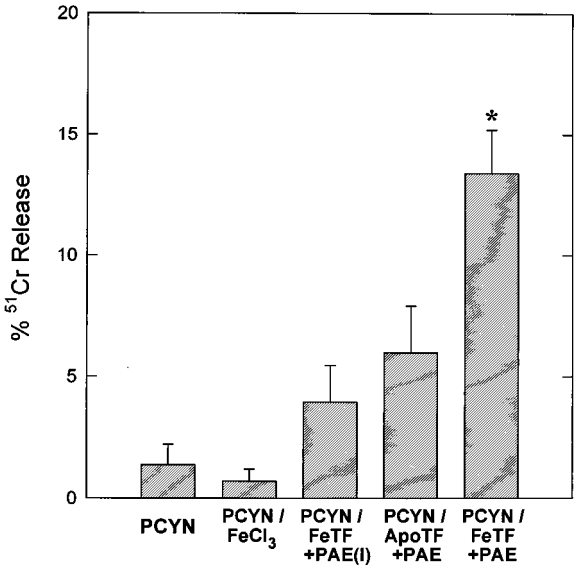

FIG. 3. Mean ($n = 5$, ± standard error of the mean [error bars]) specific ^{51}Cr release obtained with porcine pulmonary artery endothelial cell monolayers incubated in the presence of pyocyanin (PCYN) (80 to 160 μM) alone and with cells preincubated for 30 min in the presence of PCYN plus PAE-cleaved iron-transferrin (FeTF + PAE), where * indicates a P value of <0.01 versus the result for PCYN alone; PAE-cleaved apotransferrin (ApoTF + PAE); FeTF previously treated with heat-inactivated PAE [FeTF + PAE(I)]; and iron chloride ($FeCl_3$).

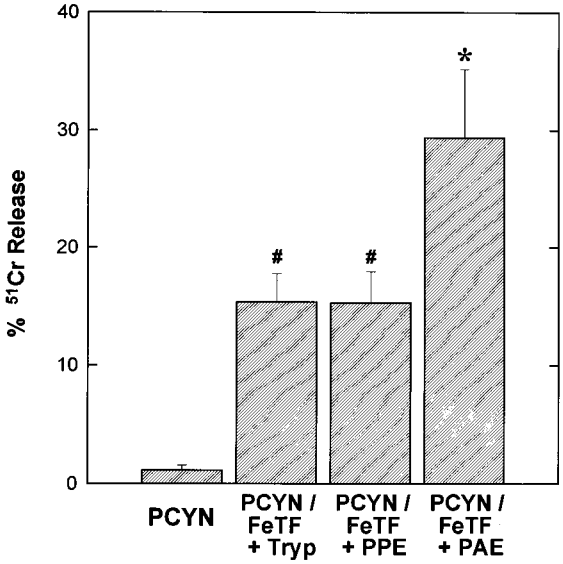

FIG. 4. Mean (n = 18, ± standard error of the mean [error bars]) specific ^{51}Cr release obtained with porcine pulmonary artery endothelial cell monolayers incubated in the presence of pyocyanin (PCYN) (80 to 160 μM) alone and with cells preincubated for 30 min in the presence of PCYN plus iron-transferrin (FeTF) previously cleaved with PAE, PPE, or trypsin (Tryp), where * and # indicate P values of <0.001 and <0.05, respectively, versus results for PCYN alone.

augment cell injury. Trypsin-cleaved Fe-transferrin and PPE-cleaved Fe-transferrin were generally less potent than PAE-cleaved Fe-transferrin in increasing endothelial cell injury resulting from pyocyanin exposure (Fig. 4). However, this difference did not reach statistical significance.

As seen with the PAE system (Fig. 3), trypsin- and PPE-cleaved apotransferrin did not significantly increase pyocyanin-induced ^{51}Cr release, establishing the necessity of the presence of iron in association with the cleaved transferrin in mediating endothelial cell injury. Furthermore, Fe-transferrin treated with heat-inactivated trypsin or PPE (100°C for 10 min) also showed no augmentation in endothelial cell injury, demonstrating the necessity of protease cleavage of Fe-transferrin in mediating cell injury.

(iii) Is the endothelial cell injury mediated by ·OH? We have previously demonstrated that ·OH is formed when protease-cleaved Fe-transferrin is added to human neutrophil-derived $\cdot O_2^-$ and H_2O_2 (9). Hypothesizing that the iron-dependent augmentation in pyocyanin-associated endothelial cell injury is mediated via ·OH formation, we performed studies assessing the role of ·OH scavengers and antioxidant enzymes in inhibiting cell injury. The addition of catalase or DMTU to test wells containing PAE-cleaved Fe-transferrin and pyocyanin resulted in a 40% decrease in ^{51}Cr release (Fig. 5). The addition of SOD and DMSO produced 27 and 21% decreases in ^{51}Cr release, respectively (Fig. 5). The simultaneous addition of SOD, catalase, and DMTU resulted in a 99% decrease in ^{51}Cr release. The percent decreases in ^{51}Cr release observed with each individual inhibitor and the combination were statistically significant by a two-tailed paired t test, consistent with involvement of reactive oxygen intermediates such as ·OH in contributing to endothelial cell injury resulting from the synergistic action of pyocyanin and PAE-cleaved Fe-transferrin.

Further evidence supporting the role of ·OH in mediating endothelial cell injury was obtained via spin-trapping studies using the highly sensitive and specific 4-POBN–ethanol ·OH spin-trapping detection system. Exposure of porcine pulmonary artery endothelial cells cultured on microcarrier beads to PAE-cleaved Fe-transferrin and pyocyanin resulted in significantly more ·OH formation than seen with endothelial cells exposed to pyocyanin alone or in association with noncleaved Fe-transferrin (Fig. 6). ·OH was also detected when endothelial cells were exposed to pyocyanin and trypsin- or PPE-cleaved Fe-transferrin (Fig. 7). The magnitude of ·OH generated, however, was less than that seen with PAE-cleaved Fe-transferrin (Fig. 7). This correlated with the extent to which pyocyanin-mediated cell injury was increased by the three protease cleavage products (Fig. 4). The substitution of protease-cleaved apotransferrin for cleaved Fe-transferrin in the presence of pyocyanin and endothelial cells generated no detectable spin adduct (data not shown), illustrating the necessity for iron in the system and providing further evidence for involvement of the Haber-Weiss reaction.

DISCUSSION

The intense inflammatory response characterizing *P. aeruginosa* lung infections in patients with pneumonia and cystic fibrosis often leads to marked tissue damage. This likely contributes to the high rates of morbidity and mortality associated with these infections (28, 43, 48, 56). A variety of neutrophil and *P. aeruginosa* secretory products have been implicated in mediating this process (18, 25, 28, 39, 46, 49, 59, 67). Among these agents, the *P. aeruginosa* secretory product pyocyanin has been shown to be cytotoxic for a number of cell types because of its ability to induce cellular formation of $\cdot O_2^-$ and H_2O_2 (34). We have shown that PAE and other proteases can cleave Fe-transferrin into smaller iron chelates which in the presence of phorbol myristate acetate-stimulated neutrophils or other sources of $\cdot O_2^-$ and H_2O_2 are able to catalyze ·OH formation (9) and augment endothelial cell injury (45). Previous data from our laboratory have also demonstrated ·OH formation and endothelial cell injury as a result of the synergistic inter-

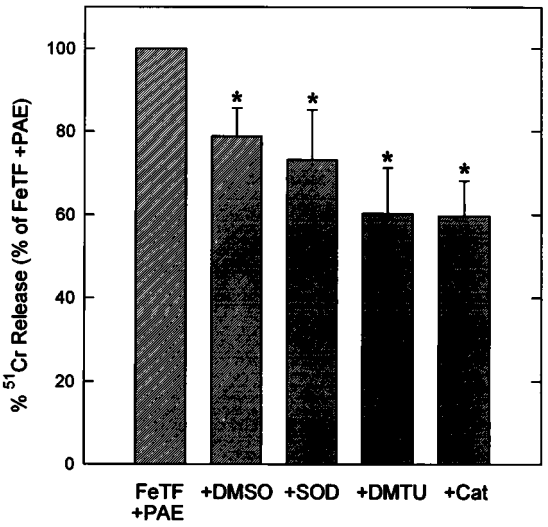

FIG. 5. Mean (n = 12, ± standard error of the mean [error bars]) specific ^{51}Cr release obtained with porcine pulmonary artery endothelial cell monolayers incubated in the presence of pyocyanin (80 to 160 μM) and PAE-cleaved iron-transferrin (FeTF + PAE), with or without antioxidant enzymes and ·OH scavengers. The data represent the mean percent ^{51}Cr release from endothelial cells treated with pyocyanin and FeTF + PAE (expressed as 100%) in the presence of DMSO, SOD, DMTU, or catalase (Cat), where * indicates a P value of <0.01 versus the result for FeTF + PAE by a two-tailed paired t test.

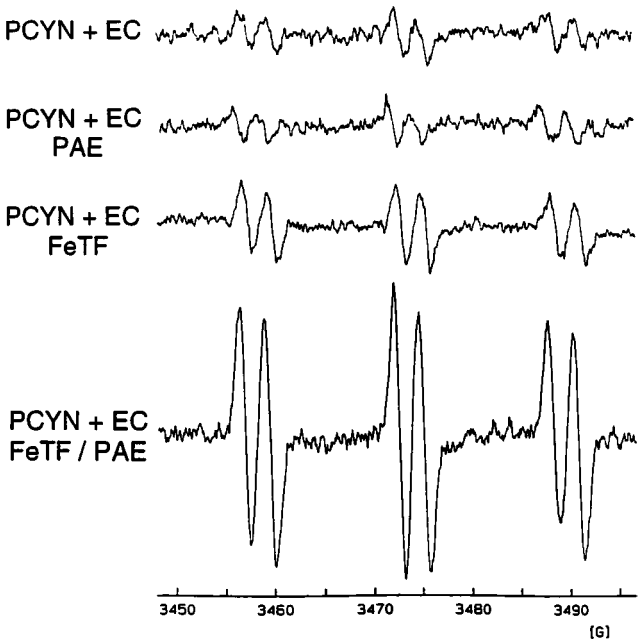

FIG. 6. EPR spectra representative of three separate experiments demonstrating the ·OH-derived spin adduct, 4-POBN–$CHOHCH_3$, resulting from the combination of DTPA, 4-POBN, ethanol, pyocyanin (PCYN) (40 μM), and porcine pulmonary artery endothelial cells on microcarrier beads (EC) (25% [vol/vol]) and the same reaction mixture supplemented with iron-transferrin (FeTF) (50 μM Fe, 25 μM TF), PAE, or PAE-cleaved FeTF (FeTF/PAE).

action of pyocyanin and iron bound to the *P. aeruginosa* siderophore, pyochelin (11). On the basis of these findings, the ability of PAE-cleaved Fe-transferrin and pyocyanin to augment pyocyanin-mediated endothelial cell injury via ·OH formation was examined.

Using a ^{51}Cr release assay, we demonstrated that Fe-transferrin previously cleaved by PAE as well as two other proteases (PPE and trypsin) significantly augmented pyocyanin-mediated porcine pulmonary artery endothelial cell injury. Additional experiments established the need for pyocyanin and Fe-transferrin which had been previously exposed to an active protease in order for cell injury to occur.

The above findings are consistent with the hypothesis that the iron is acting as a catalyst for the generation of ·OH via the Haber-Weiss reaction, resulting in an increase in oxidant-mediated endothelial cell injury. Consistent with these findings, when pyocyanin and endothelial cells were combined with PAE-, PPE-, or trypsin-cleaved Fe-transferrin, significantly more ·OH was spin trapped than with pyocyanin and endothelial cells alone or in combination with uncleaved Fe-transferrin. The quantity of ·OH generated with trypsin- and PPE-cleaved Fe-transferrin was generally less than that observed with PAE-cleaved Fe-transferrin; however, this difference did not reach statistical significance. Also supporting the role of ·OH in cell injury, catalase, DMTU, SOD, DMSO, and deferoxamine each inhibited the magnitude of cell injury resulting from exposure to pyocyanin and PAE-cleaved Fe-transferrin. SOD and DMSO were not as effective as catalase and DMTU in preventing cell injury. This may be due to the inability of these compounds to reach the site where $\cdot O_2^-$ and H_2O_2 interact with the iron chelate. Alternatively, with DMSO, methyl radical or formaldehyde from the reaction of ·OH with the methyl groups of DMSO may also be cytotoxic.

The above data support the hypothesis that endothelial cell injury resulting from exposure to pyocyanin is enhanced by Fe-transferrin previously cleaved by PAE and other proteases via the ability of this iron to convert pyocyanin-derived $\cdot O_2^-$ and H_2O_2 to the more cytotoxic ·OH. We are not aware of articles in which the concentrations of pyocyanin or PAE at tissue sites of acute *P. aeruginosa* pneumonia have been reported. The concentrations of proteases and Fe-transferrin used in this study approximated those reported to be found in vivo by some investigators in the airways of cystic fibrosis patients. Wide variations in these concentrations have been reported, however, with PAE activity in airway fluids from *P. aeruginosa*-infected cystic fibrosis patients being found to vary from nearly 200 μg/ml to 0 (14, 30, 32, 38, 59, 60). PPE was used as an HNE analog in the present study as it has been shown to have similar enzymatic properties and is more economical (57). In vivo concentrations of HNE in sputum samples from cystic fibrosis patients have been in the range of 55 μg/ml (60) or, in some cases, higher (44). Although trypsin is released from damaged cells (35, 51), no data regarding in vivo trypsin concentrations at sites of pulmonary inflammation and injury are available. Thus, we can only presume that the concentration used in this study was that of physiologic importance. Unfortunately, none of these studies address the enzyme concentrations present near a cell surface to which *P. aeruginosa* is attached, where levels of PAE could be very high. In serum, concentrations of transferrin range from 2 to 4 mg/ml (53), and transferrin concentrations in BAL specimens from

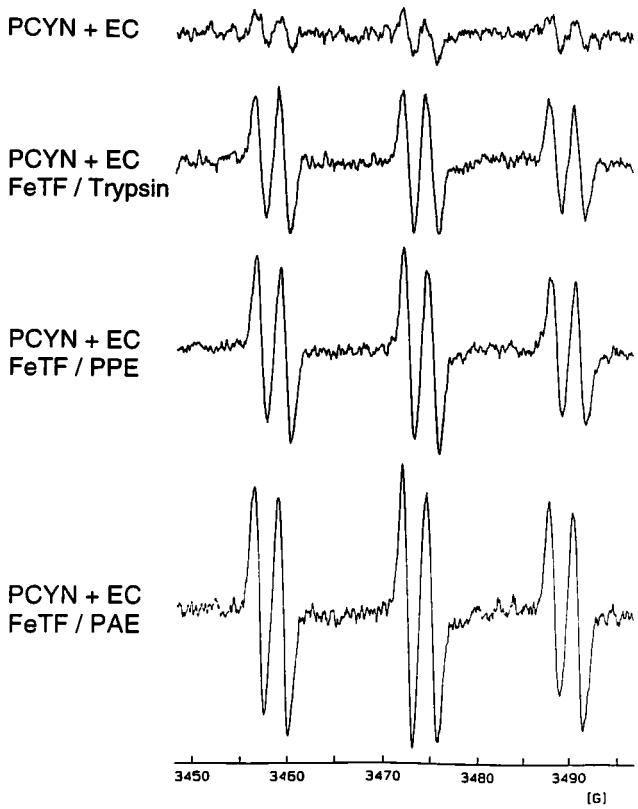

FIG. 7. EPR spectra representative of three separate experiments demonstrating the ·OH-derived spin adduct, 4-POBN–$CHOHCH_3$, resulting from the combination of DTPA, 4-POBN, ethanol, pyocyanin (PCYN) (40 μM), and porcine pulmonary artery endothelial cells on microcarrier beads (EC) (25% [vol/vol]) and the same reaction mixture supplemented with iron-transferrin (FeTF) (50 μM Fe, 25 μM TF) or FeTF previously cleaved with trypsin, PPE, or PAE.

cystic fibrosis patients are measured to be approximately 0.03 mg/ml (30). Considering the reported 100-fold dilution BAL specimens are felt to represent (52), the transferrin concentration used in this study approximates that found in the lung in vivo, as well as in serum. The concentration of pyocyanin used in the cell injury assays described in this report was somewhat higher than that detected in sputum sol obtained from *P. aeruginosa*-colonized individuals with cystic fibrosis or bronchiectasis (65). The in vivo pyocyanin concentration in the small airways and alveoli of these individuals is not known but is likely much higher than that detected in sputum sol.

We have recently reported additional evidence suggesting the biologic relevance of the data reported in this communication (10). Protease-cleaved transferrin was detected in nearly all BAL samples from *P. aeruginosa*-infected cystic fibrosis patients and 40% of BAL samples from individuals with *P. aeruginosa* pulmonary infections in the setting of idiopathic bronchiectasis. No such products were detected in samples from eight healthy controls. The ability of the transferrin cleavage products detected in these BAL samples to catalyze ·OH formation was not assessed. However, our in vitro data strongly suggest that these chelates are capable of acting as ·OH catalysts and could potentially contribute to tissue injury resulting from exposure of airway cells to pyocyanin. Further evaluation of the impact of these *P. aeruginosa* products on airway epithelial cells which are also damaged in such infections is currently under way.

In summary, we have obtained in vitro evidence that pyocyanin and protease-cleaved Fe-transferrin interact in a synergistic manner to enhance oxidant-mediated endothelial cell injury via formation of ·OH. The findings with PPE-cleaved Fe-transferrin, in particular, suggest that the recruitment of neutrophils to sites of *P. aeruginosa* infection may potentiate this injury through an increase in neutrophil elastase which would further enhance the formation of these catalytic Fe-transferrin products. The results of the in vitro studies reported in this communication provide justification for future studies of the role of Fe-transferrin cleavage in *P. aeruginosa* tissue injury.

ACKNOWLEDGMENTS

We thank Garry Buettner of the University of Iowa EPR facility for his assistance with the spin-trapping studies, Mary Lou Booth and Greg Aylsworth of the laboratory of D. Michael Shasby for their cell culture work, and Sherry Flanagan for her secretarial assistance.

This work was supported in part by awards from the VA Research Service; NIH grants HL-44275, AI-28412, and AI-34954; and a grant-in-aid from the American Heart Association. R.A.M. is supported as an Interdisciplinary Cardiovascular Research Fellow (NIH grant HL-07121).

This work was performed in part during the tenure of B.E.B. as an Established Investigator of the American Heart Association.

REFERENCES

1. **Amitani, R., R. Wilson, A. Rutman, R. Read, C. Ward, D. Burnett, R. A. Stockley, and P. J. Cole.** 1991. Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. Am. J. Respir. Cell Mol. Biol. **4:**26–32.
2. **Armstrong, A. V., D. E. S. Stewart-Tull, and J. S. Roberts.** 1971. Characterization of the Pseudomonas aeruginosa factor that inhibits mouse-liver mitochondrial respiration. J. Med. Microbiol. **4:**249–262.
3. **Aruoma, O. I., and B. Halliwell.** 1987. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl-radical generation? Biochem. J. **241:**273–278.
4. **Baldwin, D. A., E. R. Jenny, and P. Aisen.** 1984. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J. Biol. Chem. **259:**13391–13394.
5. **Bedrossian, C. W. M., S. D. Greenberg, D. B. Singer, J. J. Hansen, and H. S. Rosenberg.** 1976. The lung in cystic fibrosis. A quantitative study including prevalence of pathologic findings among different age groups. Hum. Pathol. **7:**195–204.
6. **Bluard-Deconinck, J., J. Williams, R. W. Evans, J. van Snick, P. A. Osinski, and P. L. Masson.** 1978. Iron-binding fragments from the N-terminal and C-terminal regions of human lactoferrin. Biochem. J. **171:**321–327.
7. **Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja.** 1983. Infections caused by *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5:**279–313.
8. **Brines, R. D., and J. H. Brock.** 1983. The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum: unusual resistance of human apolactoferrin to proteolytic digestion. Biochim. Biophys. Acta **759:**229–235.
9. **Britigan, B. E., and B. L. Edeker.** 1991. *Pseudomonas* and neutrophil products modify transferrin and lactoferrin to create conditions that favor hydroxyl radical formation. J. Clin. Invest. **88:**1092–1102.
10. **Britigan, B. E., M. B. Hayek, B. N. Doebbeling, and R. B. Fick, Jr.** 1993. Transferrin and lactoferrin undergo proteolytic cleavage in the *Pseudomonas aeruginosa*-infected lungs of patients with cystic fibrosis. Infect. Immun. **61:** 5049–5055.
11. **Britigan, B. E., G. T. Rasmussen, and C. D. Cox.** 1994. *Pseudomonas* siderophore pyochelin enhances neutrophil-mediated endothelial cell injury. Am. J. Physiol. **266:**L192–L198.
12. **Britigan, B. E., T. L. Roeder, and D. M. Shasby.** 1992. Insight into the nature and site of oxygen-centered free radical generation by endothelial cell monolayers using a novel spin trapping technique. Blood **79:**699–707.
13. **Britigan, B. E., G. M. Rosen, Y. Chai, and M. S. Cohen.** 1986. Do human neutrophils make hydroxyl radical? Detection of free radicals generated by human neutrophils activated with a soluble or particulate stimulus using electron paramagnetic resonance spectrometry. J. Biol. Chem. **261:**4426–4431.
14. **Bruce, M. C., L. Poncz, J. D. Klinger, R. C. Stern, J. F. Tomashefski, Jr., and D. G. Dearborn.** 1985. Biochemical and pathologic evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. Am. Rev. Respir. Dis. **132:**529–535.
15. **Buettner, G. R.** 1987. The reaction of superoxide, formate radical, and hydrated electron with transferrin and its model compound, Fe(III)-ethylenediamine-N,N′-bis [2-(2-hydroxyphenyl) acetic acid] as studied by pulse radiolysis. J. Biol. Chem. **262:**11995–11998.
16. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. **80:**1–35.
17. **Busch, C., P. A. Cancilla, L. E. DeBault, J. C. Goldsmith, and W. G. Owen.** 1982. Use of endothelium cultured on microcarriers as a model for the microcirculation. Lab. Invest. **47:**498–504.
18. **Cabelli, D. E., D. Allen, B. H. J. Bielski, and J. Holeman.** 1988. The interaction between Cu(I) superoxide dismutase and hydrogen peroxide. J. Biol. Chem. **264:**9967–9971.
19. **Cash, H. A., D. E. Woods, B. McCullough, W. G. Johanson, and J. A. Bass.** 1979. A rat model of chronic respiratory infection with Pseudomonas aeruginosa. Am. Rev. Respir. Dis. **119:**453–459.
20. **Cox, C. D.** 1986. Role of pyocyanin in the acquisition of iron from transferrin. Infect. Immun. **52:**263–270.
21. **Cross, C. E., B. Halliwell, E. T. Borish, W. A. Pryor, R. L. Saul, J. M. McCord, and D. Harman.** 1987. Oxygen radicals and human disease. Ann. Intern. Med. **107:**526–545.
22. **Doring, G., W. Goldstein, A. Roll, P. O. Schiotz, N. Hoiby, and K. Botzenhart.** 1985. Role of *Pseudomonas aeruginosa* exoenzymes in lung infections of patients with cystic fibrosis. Infect. Immun. **49:**557–562.
23. **Doring, G., H.-J. Obernesser, K. Botzenhart, B. Flehmig, N. Hoiby, and A. Hofmann.** 1983. Proteases of Pseudomonas aeruginosa in patients with cystic fibrosis. J. Infect. Dis. **147:**744–750.
24. **Doring, G., M. Pfestorf, K. Botzenhart, and M. A. Abdallah.** 1988. Impact of proteases on iron uptake of *Pseudomonas aeruginosa* pyoverdin from transferrin and lactoferrin. Infect. Immun. **56:**291–293.
25. **Dunn, M. M., M. Dunne, and D. W. Kamp.** 1990. Polymorphonuclear leukocyte- and Pseudomonas aeruginosa-induced damage to a human pulmonary epithelial cell line. J. Infect. Dis. **162:**172–177.
26. **Esparza, I., and J. H. Brock.** 1980. The effect of trypsin digestion on the structure and iron-donating properties of transferrins from several species. Biochim. Biophys. Acta **622:**297–307.
27. **Evans, R. W., and J. Williams.** 1978. Studies of the binding of different iron donors to human serum transferrin and isolation of iron-binding fragments from the N- and C-terminal regions of the protein. Biochem. J. **173:**543–552.
28. **Fick, R. B., Jr.** 1989. Pathogenesis of the Pseudomonas lung lesion in cystic fibrosis. Chest **96:**158–164.
29. **Fick, R. B., Jr., and J. S. Hata.** 1989. Pathogenetic mechanisms in lung disease caused by Pseudomonas aeruginosa. Chest **95:**206S–213S.
30. **Fick, R. B., Jr., G. P. Naegel, S. U. Squier, R. E. Wood, J. B. L. Gee, and H. Y. Reynolds.** 1984. Proteins of the cystic fibrosis respiratory tract: fragmented immunoglobulin G opsonic antibody causing defective opsonophagocytosis. J. Clin. Invest. **74:**236–248.
31. **Friedheim, E. A. H.** 1934. XXV. The effect of pyocyanine on the respiration of some normal tissues and tumours. Biochem. J. **28:**173–179.

32. **Goldstein, W., and G. Doring.** 1986. Lysosomal enzymes from polymorphonuclear leukocytes and proteinase inhibitors in patients with cystic fibrosis. Am. Rev. Respir. Dis. **134:**49–56.
33. **Gutteridge, J. M. C., S. K. Paterson, A. W. Segal, and B. Halliwell.** 1981. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem. J. **199:**259–261.
34. **Hassan, H. M., and I. Fridovich.** 1980. Mechanism of the antibiotic action of pyocyanine. J. Bacteriol. **141:**156–163.
35. **Hatanaka, M., and K. Tsuboi.** 1991. Initiation of wound healing by proteinases released from damaged cells. Int. J. Tissue React. **13**(5)**:**249–255.
36. **Heffner, J. E., and J. E. Repine.** 1989. Pulmonary strategies of antioxidant defense. Am. Rev. Respir. Dis. **140:**531–554.
37. **Holder, I. A.** 1983. Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa: effect of treatment with protease inhibitors. Rev. Infect. Dis. **5:**S914–S921.
38. **Jackson, A. H., S. L. Hill, S. C. Afford, and R. A. Stockley.** 1984. Sputum sol-phase proteases and elastase activity in patients with cystic fibrosis. Eur. J. Respir. Dis. **65:**114–124.
39. **Kreger, A. S.** 1983. Pathogenesis of Pseudomonas aeruginosa ocular disease. Rev. Infect. Dis. **5:**S931–S935.
40. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
41. **Legrand, D., J. Mazurier, J. Montreuil, and G. Spik.** 1988. Structure and spatial conformation of the iron-binding sites of transferrins. Biochimie **70:** 1185–1195.
42. **Line, W. F., D. A. Sly, and A. Bezkorovainy.** 1976. Limited cleavage of human lactoferrin with pepsin. Int. J. Biochem. **9:**203–208.
43. **Masson, P. L., J. F. Heremans, and C. H. Dive.** 1966. Studies on lactoferrin, an iron-binding protein common to many external secretions. Clin. Chim. Acta **14:**735–739.
44. **Meyer, K. C., J. R. Lewandoski, J. J. Zimmerman, D. Nunley, W. J. Calhoun, and G. A. Dopico.** 1991. Human neutrophil elastase and elastase/alpha$_1$-antiprotease complex in cystic fibrosis: comparison with interstitial lung disease and evaluation of the effect of intravenously administered antibiotic therapy. Am. Rev. Respir. Dis. **144:**580–585.
45. **Miller, R. A., and B. E. Britigan.** 1995. Protease cleaved iron-transferrin augments oxidant-mediated endothelial cell injury via hydroxyl radical formation. J. Clin. Invest. **95:**2491–2500.
46. **Mohammed, J. R., B. S. Mohammed, L. J. Pawluk, D. M. Bucci, N. R. Baker, and W. B. Davis.** 1988. Purification and cytotoxic potential of myeloperoxidase in cystic fibrosis sputum. J. Lab. Clin. Med. **112:**711–720.
47. **Munro, N. C., A. Barker, A. Rutman, G. Taylor, D. Watson, W. J. McDonald-Gibson, R. Towart, W. A. Taylor, R. Wilson, and P. J. Cole.** 1989. Effect of pyocyanin and 1-hydroxyphenazine on in vivo tracheal mucus velocity. J. Appl. Physiol. **67:**316–323.
48. **Pennington, J. P.** 1990. 280. Nosocomial respiratory infection, p. 2199–2205. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases, 2nd ed. Churchill Livingstone, New York.
49. **Pier, G. B.** 1985. Pulmonary disease associated with Pseudomonas aeruginosa in cystic fibrosis. Current status of the host bacterium interaction. J. Infect. Dis. **151:**515–580.
50. **Ramos, C. L., S. Pou, B. E. Britigan, M. S. Cohen, and G. M. Rosen.** 1992. Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. J. Biol. Chem. **267:**8307–8312.
51. **Reichart, E., P. Boerkmann, and F. Plenat.** 1992. Parenteral administration of trypsin triggers lung emphysema. Eur. Respir. J. **5:**810–814.
52. **Reynolds, H. Y.** 1987. Bronchoalveolar lavage. Am. Rev. Respir. Dis. **135:** 250–263.
53. **Russell, R. M.** 1992. 200. Nutritional assessment, p. 1154. *In* J. B. Wyngaarden, L. H. Smith, Jr., and J. C. Bennett (ed.), Cecil textbook of medicine, 19th ed. W. B. Saunders Co., Philadelphia.
54. **Shellito, J., S. Nelson, and R. U. Sorensen.** 1992. Effect of pyocyanine, a pigment of *Pseudomonas aeruginosa*, on production of reactive nitrogen intermediates by murine alveolar macrophages. Infect. Immun. **60:**3913–3915.
55. **Sibille, Y., and H. Y. Reynolds.** 1990. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am. Rev. Respir. Dis. **141:**471–501.
56. **Stern, R. C., R. E. Wood, T. F. Boat, L. W. Matthews, A. S. Tucker, and C. F. Doershuk.** 1978. Treatment and prognosis of massive hemoptysis in cystic fibrosis. Am. Rev. Respir. Dis. **117:**825–828.
57. **Stone, P. J., C. Franzblau, and H. M. Kagan.** 1982. Proteolysis of insoluble elastin. Methods Enzymol. **82:**588–592.
58. **Suter, S.** 1989. The imbalance between granulocyte neutral proteases and antiproteases in bronchial secretions from patients with cystic fibrosis. Antibiot. Chemother. **42:**158–168.
59. **Suter, S., O. B. Schaad, L. Roux, U. E. Nydegger, and F. A. Waldvogel.** 1984. Granulocyte neutral proteases and Pseudomonas elastase as possible causes of airway damage in patients with cystic fibrosis. J. Infect. Dis. **149:**523–531.
60. **Suter, S., O. B. Schaad, H. Tegner, K. Ohlsson, D. Desgrandchamps, and F. A. Waldvogel.** 1986. Levels of free granulocyte elastase in bronchial secretions from patients with cystic fibrosis. J. Infect. Dis. **153:**902–909.
61. **Ward, P. A.** 1991. Mechanisms of endothelial cell injury. J. Lab. Clin. Med. **118:**421–426.
62. **Ward, P. A., G. O. Till, R. Kunkel, and C. Beauchamp.** 1983. Evidence for the role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J. Clin. Invest. **72:**789–801.
63. **Weiss, S. J.** 1986. Oxygen, ischemia and inflammation. Acta Physiol. Scand. **548**(Suppl.)**:**9–37.
64. **Wilson, R., T. Pitt, G. Taylor, D. Watson, J. MacDermot, D. Sykes, D. Roberts, and P. Cole.** 1987. Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. J. Clin. Invest. **79:**221–229.
65. **Wilson, R., D. A. Sykes, D. Watson, A. Rutman, G. W. Taylor, and P. J. Cole.** 1988. Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. Infect. Immun. **56:**2515–2517.
66. **Winterbourn, C. C.** 1983. Lactoferrin-catalyzed hydroxyl radical production: additional requirements for a chelating agent. Biochem. J. **210:**15–19.
67. **Wretlind, B., and O. R. Pavlovskis.** 1983. Pseudomonas elastase and its role in pseudomonas infections. Rev. Infect. Dis. **5:**S998–S1004.

Editor: J. R. McGhee