Interaction of the *Pseudomonas aeruginosa* Secretory Products Pyocyanin and Pyochelin Generates Hydroxyl Radical and Causes Synergistic Damage to Endothelial Cells

Implications for *Pseudomonas*-associated Tissue Injury

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Abstract

Pyocyanin, a secretory product of *Pseudomonas aeruginosa*, has the capacity to undergo redox cycling under aerobic conditions with resulting generation of superoxide and hydrogen peroxide. By using spin trapping techniques in conjunction with electron paramagnetic resonance spectrometry (EPR), superoxide was detected during the aerobic reduction of pyocyanin by NADH or porcine endothelial cells. No evidence of hydroxyl radical formation was detected. Chromium oxalate eliminated the EPR spectrum of the superoxide-derived spin adduct resulting from endothelial cell exposure to pyocyanin, suggesting superoxide formation close to the endothelial cell plasma membrane. We have previously reported that iron bound to the *P. aeruginosa* siderophore pyochelin (ferripyochelin) catalyzes the formation of hydroxyl free radical from superoxide and hydrogen peroxide via the Haber-Weiss reaction. In the present study, spin trap evidence of hydroxyl radical formation was detected when NADH and pyocyanin were allowed to react in the presence of ferripyochelin. Similarly, endothelial cell exposure to pyocyanin and ferripyochelin also resulted in hydroxyl radical production which appeared to occur in close proximity to the cell surface. As assessed by ^{51}Cr release, endothelial cells which were treated with pyocyanin or ferripyochelin alone demonstrated minimal injury. However, endothelial cell exposure to the combination of pyochelin and pyocyanin resulted in 55% specific ^{51}Cr release. Injury was not observed with the substitution of iron-free pyochelin and was diminished by the presence of catalase or dimethyl thiourea. These data suggest the possibility that the *P. aeruginosa* secretory products pyocyanin and pyochelin may act synergistically via the generation of hydroxyl radical to damage local tissues at sites of pseudomonas infection. (*J. Clin. Invest.* 1992. 90:2187–2196.) Key words: free radical • iron • pyocyanin • siderophore • spin trap • superoxide

Introduction

Nosocomial pneumonia remains a major cause of morbidity and mortality in the United States. In most clinical series *Pseudomonas aeruginosa* ranks among the top three Gram-negative organisms causing such infections[1]. *Pseudomonas* pneumonia is particularly severe with a mortality approaching 70% even with appropriate antibiotic therapy (1, 2). *P. aeruginosa* also plays a major role in the chronic progressive pulmonary dysfunction in patients with cystic fibrosis, which is manifested as progressive bronchiectasis and hemoptysis (3) and which currently accounts for > 90% of deaths in these patients (4). It is unclear why *Pseudomonas* lung infections carry such a high fatality rate. Most work has focused on the destructive nature of *Pseudomonas*-derived proteases and other toxins (2, 4). However, alternative mechanisms may be involved.

Many *P. aeruginosa* strains secrete pyocyanin, a phenazine derivative (5-methyl-1-hydroxyphenazine), which is toxic for eukaryotic and prokaryotic cells (5–7). Wilson et al. (8) reported that pyocyanin, at concentrations present in the sputum sol of cystic fibrosis patients, induced ciliary dysfunction in human nasal epithelial cells (9, 10). Like paraquat, menadione, and other xenobiotics, pyocyanin can redox cycle in the presence of various reducing agents and oxygen with resultant formation of superoxide ($^{•}O_2^{-}$) and hydrogen peroxide (H_2O_2) (5). Pyocyanin-induced free radical production appears to be responsible for much of its microbicidal activity (5), but its role in *P. aeruginosa*-associated tissue injury is less clear.

Superoxide and H_2O_2 are only moderately reactive oxidants. In the presence of an exogenous iron catalyst, $^{•}O_2^{-}$ and H_2O_2 react to form hydroxyl radical via the Haber-Weiss reaction (11). This extremely reactive and toxic oxidant has been incriminated as the actual mediator of several forms of cell injury associated with $^{•}O_2^{-}/H_2O_2$ exposure (12). In vivo, extracellular iron is tightly complexed to host iron-binding proteins such as transferrin and lactoferrin. These iron chelates cannot participate in the Haber-Weiss reaction (13–15), thereby protecting tissue from the potentially damaging effects of untoward hydroxyl radical formation (16). Although some intracellular iron chelates such as ferritin and haemosiderin have been reported to be capable of acting as hydroxyl radical catalysts (17, 18), the presence of intracellular antioxidants such as the superoxide dismutases and catalase limit the access of $^{•}O_2^{-}$ and H_2O_2 to them. This suggests that pathologic in vivo hydroxyl radical generation may sometimes depend on the presence of nonphysiologic iron chelates.

Like many microorganisms, *P. aeruginosa* secretes iron-binding compounds (siderophores) which allow the organism

to compete with host iron-binding proteins for iron (19, 20). Recently, we found that iron bound to the *P. aeruginosa* siderophore pyochelin was capable of catalyzing the formation of hydroxyl radical from a continuous flux of $^\bullet O_2^-/H_2O_2$ generated by stimulated neutrophils or a cell-free enzymatic system (21). These data suggested that ferripyochelin might contribute to *Pseudomonas*-mediated tissue injury via the generation of cytotoxic hydroxyl radical.

Given: the evidence that *Pseudomonas*-derived secretory products could lead to generation of oxygen-centered free radicals; the tendency of this microorganism to invade vascular structures (22); the substantial evidence for the susceptibility of pulmonary endothelial cells to oxidant injury; and a role for such endothelial cell injury in the pathogenesis of several types of lung injury (23), we examined the effect of exposure of pulmonary artery endothelial cell monolayers to pyocyanin and/or ferripyochelin. By using spin trapping techniques, the nature of free radical species generated under such conditions was determined as well as the capacity of such exposures to cause cell injury. The implications of these results to the pathogenesis of tissue injury at sites of *Pseudomonas* infection are discussed.

Methods

Purification of pyocyanin and pyochelin

Apopyochelin was purified from broth culture of *P. aeruginosa* strain PA01 (ATCC 15692; American Type Culture Collection, Rockville, MD) by dichloromethane extraction and thin layer chromatography as previously described (20, 24). Ferripyochelin was generated by the addition of $FeCl_3$ to a solution (100% ethanol) of apopyochelin at pH 5.0 to achieve 80% iron loading based on the known stoichiometry of iron/pyochelin of 1:2 (24). Fully loaded pyochelin was not employed to avoid any possibility of "free" iron in the experimental systems.

Pyocyanin was isolated from similar broth culture using a previously developed technique (25). Briefly, pyocyanin was extracted from culture supernatant of *P. aeruginosa* strain PA01 by consecutive chloroform extractions after which the chloroform layer was subjected to alternating extractions with acidified and neutralized water layers to allow separation of the red, acid form, and blue forms of pyocyanin. After five such conversions the pyocyanin was crystallized, washed with water, and dried under vacuum. Pyocyanin was solubilized in H_2O before use.

Endothelial cell culture

Microcarrier beads. Confluent monolayers of porcine pulmonary artery endothelial cells were cultured on microcarrier beads (Cytodex 3, Pharmacia, Inc., Piscataway, NJ) as previously described (26, 27). Multiple cell lines (passages 6–9) were employed for these studies. Cells were cultured for 2–3 d after confluence. Control experiments were performed simultaneously to eliminate any contribution of variation in cell line or passage number to the results. Before use, microcarrier beads were washed free of cell culture media (medium 199 containing 10% heat-inactivated fetal bovine serum, University of Iowa Cancer Center, Iowa City, IA) by transferring the beads to a 50-ml conical tube (Sarstedt, Princeton, NJ) and allowing them to sediment ($\sim$ 5 min). Culture media was then removed by aspiration and replaced with 10 ml of Hanks' balanced salt solution without phenol red (HBSS, University of Iowa Cancer Facility). Beads and HBSS were gently mixed and the beads allowed to resediment. The HBSS was then replaced with fresh HBSS and the process repeated three times. After the final wash the cells were resuspended in HBSS at 25% (vol/vol) microcarrier beads which yields a final cell concentration of 0.5–1.0 × 10^7 cells/ml.

Microtiter plates. Porcine pulmonary artery endothelial cells were plated at a concentration of 5 × 10^4 cells per well in 24-well tissue culture plates (Costar, Cambridge, MA) in 0.5 ml medium 199 with 10% serum, 2× basal medium amino acids, basal minimal essential vitamins, 2 mM L-glutamine, 10 U/ml penicillin/streptomycin (Gibco Laboratories, Grand Island, NY). The plates were then placed in culture at 37°C, 5% CO_2 for 4–5 d.

Spin trapping

Desired reaction mixtures (0.5 ml) were assembled in 12 × 75-mm glass tubes and transferred to a quartz electron paramagnetic resonance (EPR) flat cell which was in turn placed into the cavity of the EPR spectrometer (model E104A, Varian Associates, Inc., Palo Alto, CA; or model ESP300, Bruker, Karlsruhe, FRG). Spin trapping systems consisted of 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and the following: 100 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) with and without 170 mM ethanol (27); 10 mM α-phenyl-*N*-*t*-butyl-nitrone (PBN) plus 140 mM dimethyl sulfoxide (DMSO) (28); or 10 mM α-(4-pyridyl-1-oxide)-N-t-butyl-nitrone (4-POBN) plus 170 mM ethanol (29, 30). DMPO, DMSO, PBN, and 4-POBN were obtained from Sigma Chemical Co., St. Louis, MO and ethanol from Aaper Chemical Co., Shelbeysville, KY. Resulting EPR spectra were recorded sequentially at 25°C. Routinely, for the Varian instrument spectrometer microwave power was 20 mW, modulation frequency was 100 kHz with a modulation amplitude of 1.0 G, response time was 1 s, and the gain was 5 × 10^4. For experiments performed using the Bruker microwave power was 20 mW, modulation frequency was 100 kHz with a modulation amplitude of 0.892 G, time constant was 0.327 or 0.655 s, and the gain was 5.0 × 10^5. In experiments containing endothelial cells the reaction mixtures contained 25% (vol/vol) microcarrier beads in HBSS. In some cases experiments were performed under oxygen depleted conditions by bubbling the reaction mixture with N_2 before initiation of the reaction. All buffers were routinely treated with chelating resin (Sigma Chemical Co.) to reduce the presence of adventitious iron, generally $\sim$ 1 μM in most buffers. All EPR spectra shown are representative of experiments performed a minimum of three times.

^{51}Chromium Release

Microtiter plate-adherent pulmonary artery endothelial cells were loaded with ^{51}Cr, by incubation for 18 h (37°C, 5% CO_2) in medium 199 (no additives) containing 20 μCi/ml ^{51}Cr (sodium chromate, 2 mCi/ml; Amersham Corp., Arlington Heights, IL). Each well was then washed three times in (1 ml per well). After the final wash, 1 ml HBSS was added to each well and the plates were incubated for 30 min at 4°C. Wells were then washed again with PBS after which 0.5 ml medium 199 (no additives) was added to each well except for maximum release controls which contained medium 199 with 10% Triton X-100. Desired compounds were then added to each well (triplicate samples) and the cells allowed to incubate for 4 h (37°C, 5% CO_2) after which the supernatants were removed and radioactivity determined by gamma counter. For experiments in which ferripyochelin, apopyochelin, or other iron chelates were used, cells were allowed to preincubate for 30 min in the presence of these compounds (or media alone) before the addition of other agents. At ethanol concentrations ≤ 0.4% (vol/vol), at which all experiments were performed, there was no detectable increase in ^{51}Cr release above the spontaneous rate. Spontaneous release was measured as the amount of ^{51}Cr release over 4 h by cells incubated in medium 199 alone and was a mean of 21% of maximum release for all experiments performed.

Results of each experiment are expressed as the mean specific ^{51}Cr release of triplicate samples where specific ^{51}Cr release is defined as:

$$\frac{(\text{test well } ^{51}\text{Cr cpm} - \text{spontaneous release } ^{51}\text{Cr cpm})}{(\text{maximum release } ^{51}\text{Cr cpm} - \text{spontaneous release } ^{51}\text{Cr cpm})}.$$

Other reagents

Reduced β-nicotinamide adenine dinucleotide (NADH), catalase, human erythrocyte CuZn superoxide dismutase (SOD), and paraquat (methyl viologen) were obtained from Sigma. Dimethyl thiourea (DMTU) was purchased from Aldrich Chemical Co., Milwaukee, WI. Chromium oxalate which had been synthesized according to the method of Bailar and Jones (31) was kindly provided by Dr. Gerald Rosen, Dept. of Pharmacology, University of Maryland, Baltimore, MD.

Statistical analysis of data

A paired Student's t test was used for all statistical analyses. Results were considered significant at $P < 0.05$.

Results

Spin tapping of free radicals generated by the interaction of pyocyanin and NADH. Before studying the nature of free radical species resulting from the interaction of pyocyanin and/or

pyochelin with cells, we evaluated the chemistry of these two compounds under more controlled conditions. Consistent with earlier reports (32-34), addition of NADH to a solution of pyocyanin under anaerobic conditions resulted in the generation of the pyocyanin free radical (not shown). Other investigators have reported that in the presence of molecular oxygen (O_2), this pyocyanin radical will undergo oxidation to reform pyocyanin with the resultant generation of $^{\bullet}O_2^{-}$ (34-36), as well as H_2O_2 via the spontaneous dismutation of $^{\bullet}O_2^{-}$. Superoxide and H_2O_2 can react with one another, albeit at a very slow rate (37), to generate the three electron reduction products of O_2, hydroxyl radical. Therefore the possibility that additional free radical products other than $^{\bullet}O_2^{-}$ are produced as a consequence of the aerobic redox cycling of pyocyanin was examined using spin trapping. Pyocyanin (40 μM) was added to a solution of NADH (40 μM), DTPA (0.1 mM) and the spin trap DMPO (100 mM) in H_2O after which the EPR spectrum of this solution was continually monitored at 25°C. The resulting EPR spectrum (Fig. 1 a) was dominated by a nitroxide species whose hyperfine splitting constants ($A_N = 14.3$, $A_H = 11.8$ G) are consistent with the superoxide-derived spin adduct of DMPO, DMPO/$^{\bullet}$OOH. An additional nitroxide species ($A_N = A_H = 14.9$ G) which is that of the hydroxyl radical

Pyocyanin + NADH
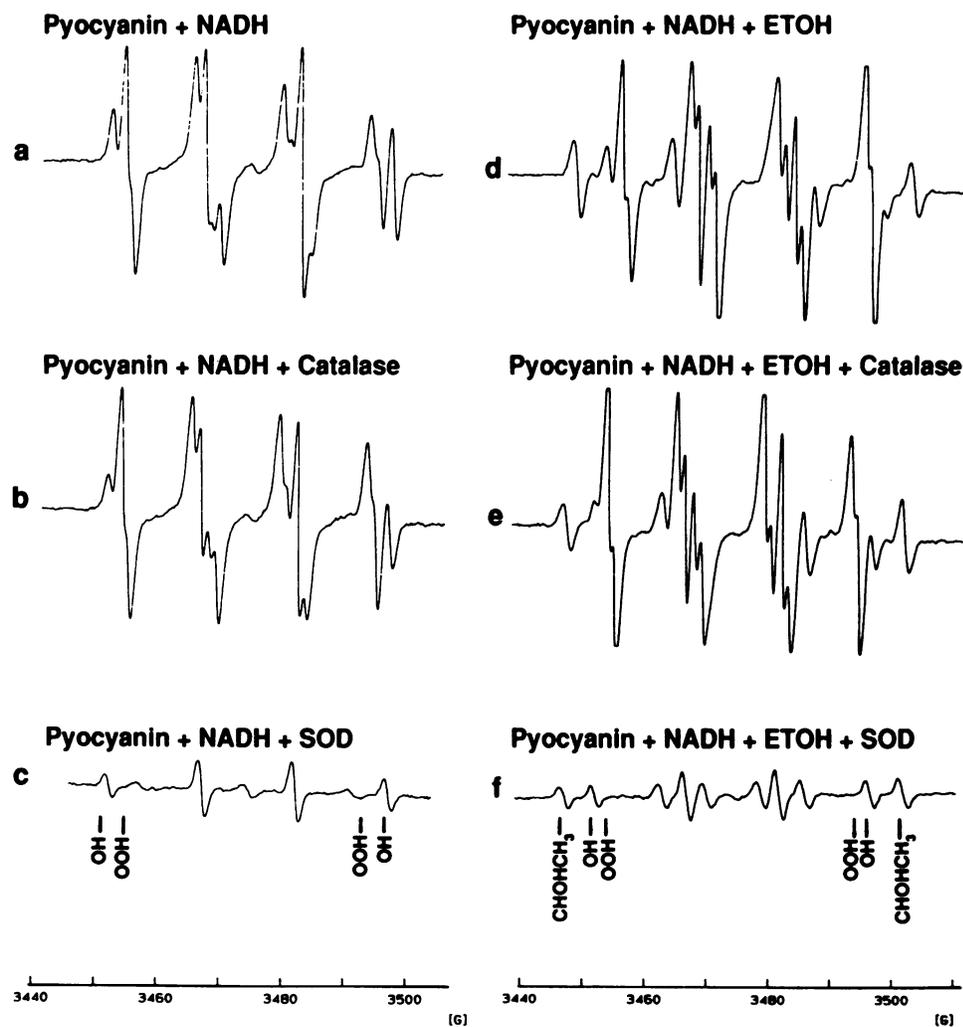
a

Pyocyanin + NADH + Catalase

b

Pyocyanin + NADH + SOD

c

OH OOH   OOH OH

3440   3460   3480   3500
[G]

Pyocyanin + NADH + ETOH

d

Pyocyanin + NADH + ETOH + Catalase

e

Pyocyanin + NADH + ETOH + SOD

f

CHOHCH₃ OH OOH   OOH OH CHOHCH₃

3440   3460   3480   3500
[G]

Figure 1. EPR spectra a-c were obtained after the mixture of pyocyanin (40 μM) and NADH (40 μM) in the presence of 100 mM DMPO and 0.1 mM DTPA (tracing a) plus 500 U/ml catalase (tracing b) or plus 30 U/ml SOD (tracing c). EPR spectra d-f were obtained under identical conditions to those corresponding to tracings a-c, respectively except that the reaction mixtures also contained 170 mM ethanol. Spectra a-c are those of the DMPO/$^{\bullet}$OOH and DMPO/$^{\bullet}$OH spin adducts which were inhibitable by the inclusion of SOD but not catalase consistent with the spin trapping of $^{\bullet}O_2^{-}$. Spectra d-f show no effect of ethanol on the results relative to spectra a-c except for the appearance of a small amount of DMPO/$^{\bullet}$CHOHCH₃ spin adduct which was inhibitable by SOD (tracing f) but not catalase (tracing e). These results are consistent with only the spin trapping of $^{\bullet}O_2^{-}$ without any definitive evidence of spin trapping hydroxyl radical. The location of high and low field peaks corresponding to DMPO/$^{\bullet}$OOH, DMPO/$^{\bullet}$OH, and DMPO/$^{\bullet}$CHOHCH₃ are designated OOH, OH, and CHOHCH₃, respectively.

spin adduct of DMPO, DMPO/ˈOH (38) was also detected (Fig. 1 a). Previous work has shown that detection of DMPO/ˈOH is not specific for the spin trapping of hydroxyl radical because it can also arise as a decomposition product of superoxide-induced DMPO/ˈOOH (39).

To determine whether DMPO/ˈOH generation resulted from the spin trapping of $^•O_2^-$ or hydroxyl radical, experiments were repeated in the presence of SOD (30 U/ml), catalase (500 U/ml), or ethanol (170 mM). If DMPO/ˈOH resulted from hydroxyl radical generation, all three of these compounds should inhibit its formation. In addition, in the case of ethanol which acts via its ability to scavenge hydroxyl radical, a new spin adduct derived from the spin trapping of the α-hydroxyethyl radical product (ˈCHOHCH₃) of that reaction should be formed and should be inhibitable by catalase. In contrast, if DMPO/ˈOH formation reflected spin trapping of $^•O_2^-$, only SOD would be expected to have a major effect. As shown in Fig. 1 c, the presence of SOD (30 U/ml) in the reaction mixture totally eliminated DMPO/ˈOH production. In contrast, catalase (500 U/ml) had no effect (Fig. 1 b). Although inclusion of ethanol did result in the appearance of small DMPO/ˈCHOHCH₃ peaks (A_N = 16.0, A_H = 22.8 G, Fig. 1 d), these were only inhibitable by SOD (Fig. 1 f) but not catalase (Fig. 1 e), indicating that they likely resulted from spin trapping of hydroxyl radical which was also derived from the decomposition of DMPO/ˈOOH as has been reported (39).

Recently, the sensitivity of DMPO spin trapping systems for hydroxyl radical has been brought into question because of the capacity of $^•O_2^-$ to destroy the paramagnetic nature of hydroxyl radical-derived spin adducts of DMPO (40–42). Therefore, to confirm that the reaction of pyocyanin and NADH did not form hydroxyl radical, additional spin trapping experiments were performed in which the reaction was allowed to take place in the presence of either PBN and DMSO (28) or 4-POBN and ethanol (29). Neither of these two systems is capable of spin trapping $^•O_2^-$ in a stable form. However, in the presence of hydroxyl radical both systems result in the formation of a spin adduct, PBN/ˈOCH₃ and 4-POBN/ˈCHOHCH₃, respectively, which is not modified by $^•O_2^-$ (28, 29). Recent data suggests that the 4-POBN/ethanol system is 10 times more sensitive for hydroxyl radical than that of DMPO (29, 30). Nevertheless, when pyocyanin and NADH were mixed in the presence of 4-POBN/ethanol (Fig. 2 a) or PBN/DMSO (not shown) no EPR signal above background was detectable confirming the lack of hydroxyl radical formation by this reaction.

Ferripyochelin will catalyze formation of hydroxyl radical from the interaction of pyocyanin and NADH. We previously showed that iron bound to the *Pseudomonas* siderophore pyochelin can catalyze hydroxyl radical generation from $^•O_2^-$/ H_2O_2 produced by the reaction of xanthine oxidase and hypoxanthine or by PMA-stimulated neutrophils (21). Given the considerable likelihood that pyocyanin and ferripyochelin would coexist in vivo, evidence for formation of hydroxyl radical was sought during the reaction of pyocyanin and NADH in the presence of ferripyochelin using the 4-POBN/ethanol spin trapping system. The hydroxyl radical-derived spin adduct, 4-POBN/ˈCHOHCH₃ (A_N = 15.8, A_H = 2.4 G) was detectable with the combination of pyocyanin and ferripyochelin (Fig. 2 b) but not with either alone (Fig. 2, a and c). 4-POBN/

Pyocyanin + NADH

Pyocyanin + NADH + Ferripyochelin
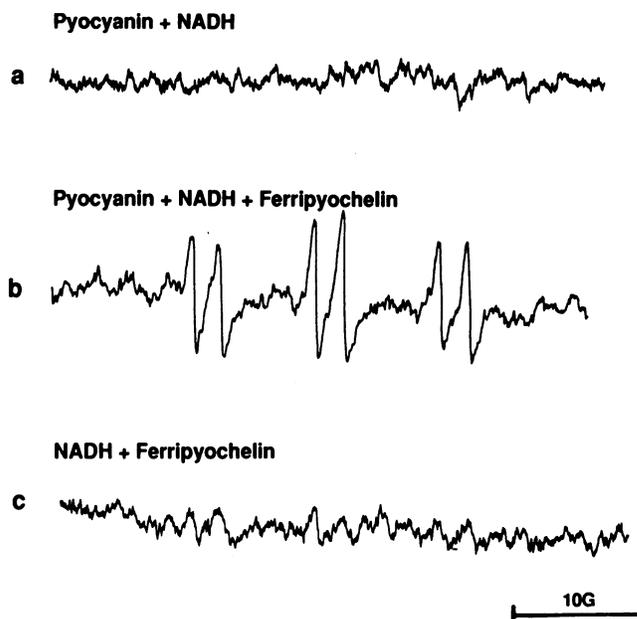
NADH + Ferripyochelin

|———— 10G ————|

Figure 2. EPR spectra obtained by using the 4-POBN/ethanol spin-trapping system and resulting from the reaction of pyocyanin (40 μM) and NADH (40 μM) in the presence (tracing b) and absence (tracing a) of 10 μM ferripyochelin. Also shown is the result obtained with NADH and ferripyochelin in the absence of pyocyanin (tracing c). The species detected with the mixture of NADH, pyocyanin, and ferripyochelin is that of 4-POBN/ˈCHOHCH₃ indicating hydroxyl radical production.

ˈCHOHCH₃ was inhibited by catalase and SOD (not shown) consistent with hydroxyl radical formation via the Haber-Weiss (superoxide-driven Fenton) reaction (43).

Detection of free radicals formed after the exposure of endothelial cells to pyocyanin. The above and earlier data (34–36) suggested that redox cycling of pyocyanin could serve as a continuous source of $^•O_2^-$ (and therefore H_2O_2) in biological systems. Endothelial cells are susceptible to H_2O_2-mediated injury (44–49). Hydroxyl radical resulting from the interaction of exogenous H_2O_2 and endothelial cell associated-iron has been suggested to be responsible for this cell injury (49). Consistent with these data, we have recently spin-trapped hydroxyl radical after exposure of endothelial cells to H_2O_2 (27). In that endothelial cells may well be exposed to pyocyanin during the course of localized or bacteremic infections with *P. aeruginosa*, the nature of free radical species generated by the exposure of endothelial cell monolayers to pyocyanin was examined. This was done using a previously developed spin trapping technique which utilizes microcarrier bead-adherent porcine pulmonary artery endothelial cells (27). When such a "suspension of adherent endothelial cells" was exposed to 40 μM pyocyanin in the presence of DMPO, an EPR spectrum consistent with DMPO/ˈOH was detected (Fig. 3 a). Generation of this spin adduct was inhibited by SOD (Fig. 3 b) but not catalase (Fig. 3 c) or ethanol (Fig. 3 d). These results are consistent with the spin trapping of $^•O_2^-$ as had been observed with the reaction of pyocyanin and NADH (Fig. 1 a). No evidence of hydroxyl radical was apparent in these experiments or when they were
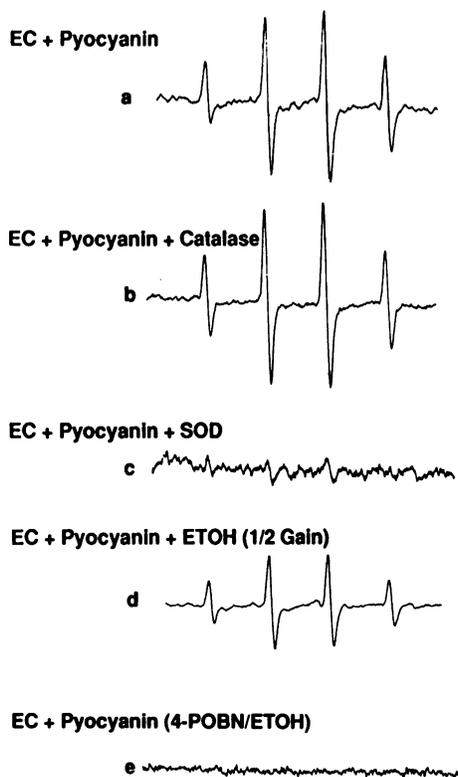
EC + Pyocyanin

a

EC + Pyocyanin + Catalase

b

EC + Pyocyanin + SOD

c

EC + Pyocyanin + ETOH (1/2 Gain)

d

EC + Pyocyanin (4-POBN/ETOH)

e

Figure 3. EPR spectra obtained after the addition of 40 μM pyocyanin to microcarrier bead adherent porcine pulmonary artery endothelial cells (25% vol/vol) in the presence of DMPO (tracing a) as well as 500 U/ml catalase (tracing b), or 30 U/ml SOD (tracing c), or 170 mM ethanol (tracing d). Note that tracing d was obtained at half the gain of the other three scans. Tracing a reveals a single nitroxide species whose splitting constants correspond to DMPO/$^\bullet$OH. As shown in tracings b–d its detection was inhibitable by SOD but not catalase or ethanol consistent with spin trapping of $^\bullet O_2^-$. Also shown is the result obtained under conditions identical to those of tracing a except that the combination of 10 mM 4-POBN and 170 mM ethanol was substituted for DMPO. The resulting EPR spectrum reveals no species above background further confirming the absence of detectable hydroxyl radical production.

repeated using the more sensitive 4-POBN/ethanol spin trapping system (Fig. 3 e).

In our earlier study (27), hydroxyl radical generated by endothelial cell exposure to H_2O_2 appeared to occur at an intracellular site as indicated by the inability of the "EPR line-broadening agent" chromium oxalate, which is excluded from intracellular sites, to eliminate the EPR spectrum of the resulting spin adduct. Therefore, the presumed catalyst for hydroxyl radical generation was likely an intracellular iron chelate. In the present set of experiments, chromium oxalate almost totally eliminated the DMPO/$^\bullet$OH spectrum resulting from the spin trapping of $^\bullet O_2^-$ generated by endothelial cells exposed to pyocyanin (Fig. 4), indicating it arose extracellularly or in close proximity to the cell membrane. As previously noted with paraquat (27), the site of $^\bullet O_2^-$/H_2O_2 generation induced by pyocyanin is therefore quite distant from potential intracellular catalytic iron chelates.

Generation of hydroxyl radical during the exposure of endothelial cells to pyocyanin and ferripyochelin. The *Pseudomonas* siderophore pyochelin is hydrophobic (20) suggesting that it will readily associate with cellular membranes. Given the above data that exposure of endothelial cells to pyocyanin results in the continuous formation of $^\bullet O_2^-/H_2O_2$ near the cell surface the possibility that treatment of endothelial cells with pyocyanin and ferripyochelin would result in the generation of hydroxyl radical was examined. Microcarrier bead-adherent porcine pulmonary artery endothelial cells were incubated with pyocyanin (40 μM) in the presence of ferripyochelin (10 μM) and hydroxyl radical production assessed by spin trapping with 4-POBN/ethanol. As shown in Fig. 5, the 4-POBN/$^\bullet$CHOHCH$_3$ spin adduct was readily detectable after such treatment. Formation of this spin adduct was inhibited by catalase and to a lesser extent SOD (Fig. 5), as would be expected if it arose from spin trapping of hydroxyl radical generated through the Haber-Weiss reaction. Substitution of apopyochelin also eliminated spin adduct generation (Fig. 5), demonstrating the necessity for iron in the system and providing further evidence for involvement of the Haber-Weiss reaction. Addition of chromium oxalate to a suspension of endothelial cells treated with pyocyanin and ferripyochelin almost total eliminated the 4-POBN/$^\bullet$CHOHCH$_3$ spectrum (Fig. 6) consistent with spin trapping of hydroxyl radical generated extracellularly or in close proximity to the cell membrane. Unfortunately, DMPO spin trapping could not be employed to confirm our
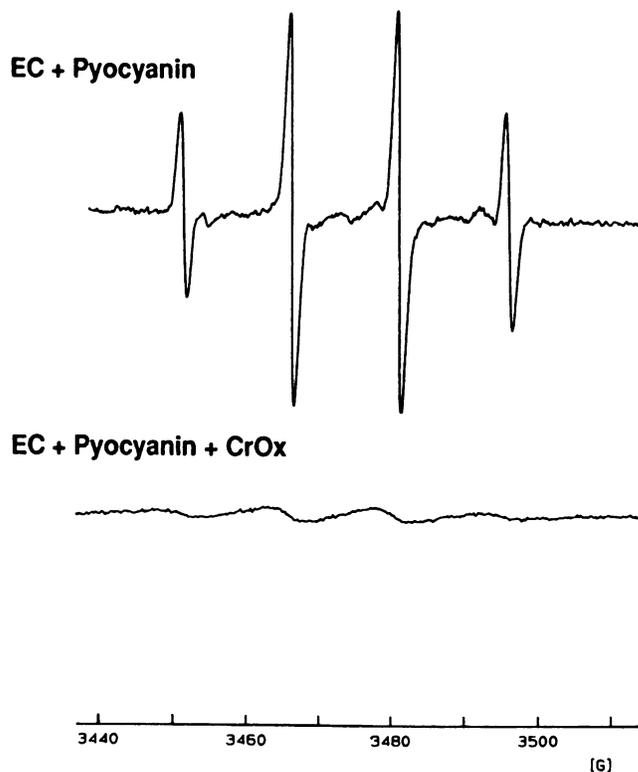
EC + Pyocyanin

EC + Pyocyanin + CrOx

3440   3460   3480   3500

[G]

Figure 4. EPR spectra resulting from the exposure of endothelial cells to 40 μM pyocyanin in the presence of 100 mM DMPO and 0.1 mM DTPA in the absence (*top scan*) and presence (*bottom scan*) of the EPR line-broadening agent chromium oxalate (10 mM).
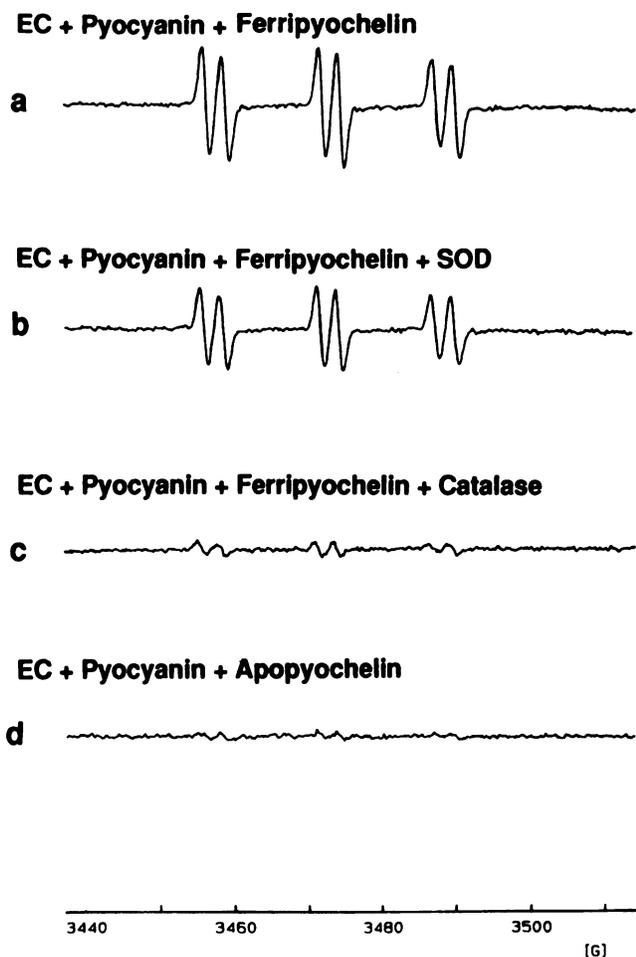
EC + Pyocyanin + Ferripyochelin

a

EC + Pyocyanin + Ferripyochelin + SOD

b

EC + Pyocyanin + Ferripyochelin + Catalase

c

EC + Pyocyanin + Apopyochelin

d

3440   3460   3480   3500

[G]

Figure 5. EPR spectra obtained using the 4-POBN/ethanol spin-trapping system in which microcarrier bead-adherent endothelial cells were exposed to 40 μM pyocyanin and 10 μM ferripyochelin in the presence of 0.1 mM DTPA alone (tracing a) plus 10 U/ml SOD (tracing b) or plus 500 U/ml catalase (tracing c). Results reveal that endothelial cell exposure to the combination of ferripyochelin and pyocyanin results in the generation of the 4-POBN/$^{\bullet}$CHOHCH$_3$ spin adduct, which is inhibited slightly by the presence of SOD and to a greater extent by catalase. Tracing d was obtained under the same conditions as tracing a except that apopyochelin was substituted for ferripyochelin. Tracing d therefore demonstrates that the generation of hydroxyl radical is dependent on the presence of iron.

results. Consistent with our earlier experience (21), in the buffer systems necessary to maintain endothelial cell viability, the simultaneous presence of ferripyochelin and DMPO resulted in the generation of confounding nitroxide species.

Endothelial cell damage by pyocyanin and ferripyochelin. Given the susceptibility of endothelial cells to oxidant-mediated injury, the effect of pyocyanin and ferripyochelin exposure on endothelial cell viability was assessed. As quantitated by the release of ^{51}Cr from prelabeled adherent endothelial cells, 40 μM pyocyanin resulted in only slight, although statistically significant, cell injury (Table I). Increasing the concentration of pyocyanin 10-fold failed to increase the magnitude of endothelial cell injury (data not shown). Similarly, when endothelial cells were exposed to ferripyochelin alone, a small but significant increase in ^{51}Cr release was detectable (6.8±1.8%
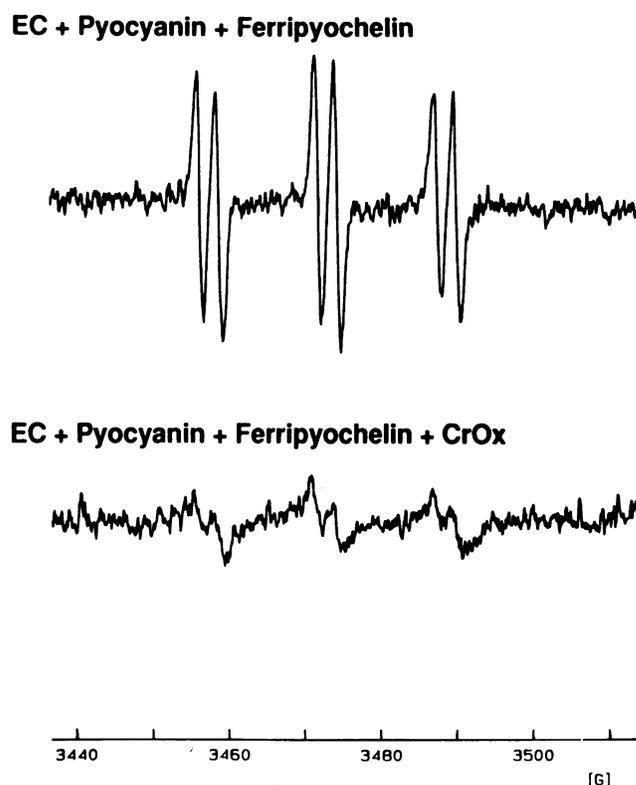
EC + Pyocyanin + Ferripyochelin

EC + Pyocyanin + Ferripyochelin + CrOx

3440   3460   3480   3500

[G]

Figure 6. EPR spectra resulting from the exposure of endothelial cells to 40 μM pyocyanin and 10 μM ferripyochelin in the presence of 10 mM 4-POBN, 170 mM ethanol, and 0.1 mM DTPA in the absence (*top scan*) and presence (*bottom scan*) of the EPR line-broadening agent chromium oxalate (10 mM).

mean±SEM specific ^{51}Cr release, $n = 12, P \leq 0.05$). However, addition of pyocyanin (40 μM) to an endothelial cell monolayer which had been preincubated in the presence of 10 μM ferripyochelin for 30 min resulted in a much greater magnitude of cell injury than observed with either agent alone: 55% specific ^{51}Cr release (Table I). No increase in cell injury above that induced by pyocyanin alone was detected when apopyochelin

Table I. Specific ^{51}Cr Release

	Alone	FePCH	ApoPCH	FeNTA	FeEDTA
Pyocyanin	6.5±2.0*	54.7±5.2‡	13.5±3.4	4.1±2.3	3.5±2.4
Paraquat	8.5±4.8	11.2±3.7	ND	ND	ND

Shown is the mean±SEM of specific ^{51}Cr release as calculated per Methods, obtained after 4-h incubation of porcine pulmonary artery endothelial cell monolayers in the presence of 40 μM pyocyanin alone and under conditions in which the cells were preincubated for 30 min in the presence of 10 μM ferripyochelin, apopyochelin, ferric nitrilotriacetic acid (FeNTA), or ferric ethylenediaminetetraacetic acid (FeEDTA) before the addition of pyocyanin. In each of the latter cases the system also contained 0.4% (vol/vol) ethanol. Also shown are results obtained when 10 mM paraquat was substituted for pyocyanin. Each experiment was performed a minimum of three times (range 3–11). ND, not done. * Statistically significant, $P \leq 0.05$. ‡ Statistically significant, $P \leq 10^{-6}$.

was used in place of ferripyochelin (Table I), indicating that ferripyochelin augmentation of pyocyanin-mediated injury was dependent on the presence of iron and not related to the ethanol vehicle in which the pyochelin was prepared. ^{51}Cr release above background was not detected at ferripyochelin concentrations below 10 μM, even when the pyocyanin concentration was increased to 400 μM (data not shown). The effect of increasing the concentration of ferripyochelin above 10 μM could not be evaluated because such experiments required ethanol concentrations (the pyochelin vehicle) which produced variable cytotoxicity by themselves.

Catalase and the $H_2O_2/{}^{\bullet}OH$ scavenger DMTU protected endothelial cells from pyocyanin/ferripyochelin-mediated injury. The presence of 500 U/ml catalase or 50 mM DMTU decreased pyocyanin/ferripyochelin-induced ^{51}Cr release by 63.2±9.2% and 57.9±11.4% (mean±SEM, n = 5), respectively. DMSO (140 mM) and SOD (300 U/ml), however, had little effect on pyocyanin/ferripyochelin-induced injury. ^{51}Cr release resulting from pyocyanin/ferripyochelin exposure decreased only 10.5±4.2% and 5.9±3.1% (mean±SEM, n = 5) in the presence of DMSO and SOD, respectively.

In order to ascertain whether ferripyochelin was unique among iron chelates in its ability to increase injury in pyocyanin-exposed endothelial cells, experiments were repeated in which two other iron-complexes known to be active as hydroxyl radical catalysts replaced ferripyochelin. Substitution of equimolar concentrations (10 μM) of ferric nitrilotriacetic acid or ferric EDTA for ferripyochelin did not increase cell injury above that observed with pyocyanin-treated endothelial cell monolayers even when ethanol was included in the system (Table I). Similarly, to assess the uniqueness of the pyocyanin role in the above process, the redox active xenobiotic paraquat (methyl viologen), which induces $^{\bullet}O_2^-$ formation in porcine pulmonary artery endothelial cells (27), was substituted for pyocyanin in the above experiments. At a 10 mM concentration of paraquat, which, as previous spin-trapping data (27) indicates, induces magnitudes of $^{\bullet}O_2^-$ production in porcine endothelial cells similar to that of 40 μM pyocyanin, no significant specific ^{51}Cr release was observed in the absence or presence of 10 μM ferripyochelin (Table I). Thus, within the limitations of the experimental system employed only the combination of pyocyanin and ferripyochelin appeared to induced a large amount of endothelial cell injury.

Discussion

A common sequela of septicemia as well as localized infection with *P. aeruginosa* is microvascular injury and thrombosis (22). The susceptibility of endothelial cells to oxidant-mediated injury is well recognized and has been implicated in the pathogenesis of a number of disease states (23). The *P. aeruginosa* secretory product pyocyanin can undergo redox cycling with resultant formation of $^{\bullet}O_2^-/H_2O_2$ (34–36). In addition, we have recently demonstrated that iron bound to the *P. aeruginosa* siderophore pyochelin is capable of catalyzing the formation of hydroxyl radical from $^{\bullet}O_2^-/H_2O_2$ (21). Therefore, experiments were performed to assess the potential role of pyocyanin and/or ferripyochelin-mediated oxidant production in *Pseudomonas*-associated endothelial cell injury.

Consistent with previous work with pyocyanin (5, 25, 34,

50, 51) and other redox active compounds (27, 52, 53), $^{\bullet}O_2^-$ but not hydroxyl radical was spin trapped during the reaction of pyocyanin with NADH or after the exposure of pulmonary artery endothelial cell monolayers to pyocyanin. In contrast, a small amount of hydroxyl radical has been reportedly spin trapped with addition of pyocyanin to *E. coli*, particularly a catalase-deficient strain (50). These data suggest that the reduction of pyocyanin by NADH or cellular enzyme systems does not generate hydroxyl radical within the limits of sensitivity of spin trapping. The potential for pyocyanin reduction to result in the formation of hydroxyl radical is likely dependent on the simultaneous presence of a transition metal catalyst and the relative antioxidant defenses of the cell. The location of free radical production is likely an important factor in determining the relative toxicity of such oxidants in biologic systems. Our data suggest that, like paraquat (27), most of the $^{\bullet}O_2^-$ spin trapped during endothelial cell exposure to pyocyanin is generated close to the cell surface.

Iron complexes capable of catalyzing the Haber-Weiss reaction are quite limited in vivo (16). Sites of *P. aeruginosa* infection would likely contain both pyocyanin and ferripyochelin (8, 54, 55). Consistent with our earlier work (21), the presence of ferripyochelin during the production of $^{\bullet}O_2^-/H_2O_2$ by pyocyanin and NADH resulted in hydroxyl radical generation. Of greater biologic relevance, addition of pyocyanin to endothelial cell monolayers preincubated with ferripyochelin also resulted in hydroxyl radical formation. The effects of SOD, catalase and chromium oxalate suggested this was the result of the Haber-Weiss reaction occurring in close proximity to the cell surface.

As assessed by the release of ^{51}Cr from prelabeled cells, pyocyanin and ferripyochelin alone caused minimal endothelial cell damage. Our detection of some pyocyanin-mediated injury differs from a previous report (51) which found no such damage as assessed by lactose dehydrogenase release. However, concerns have recently been expressed that pyocyanin may interfere with quantitation of lactose dehydrogenase activity (56). In addition, both lactose dehydrogenase and ^{51}Cr release are rather insensitive means of assessing endothelial cell injury. Pyocyanin has been reported to have deleterious effects on both respiratory epithelial cells (8–10, 57, 58) and cells involved in host defense (59–66). Of additional note, exposure to H_2O_2 or the induction of oxidant production via xenobiotics other than pyocyanin (e.g., menadione) have been shown to have effects on endothelial and/or epithelial cells which could augment the potential for inflammatory tissue injury (67–77). The possibility that pyocyanin-induced $^{\bullet}O_2^-/H_2O_2$ could lead to similar changes is likely worth investigating.

The combination of the same concentrations of pyocyanin and ferripyochelin which by themselves caused only minimal cytotoxicity resulted in nearly 55% cell lysis. The process is iron-dependent implying involvement of iron-catalyzed hydroxyl radical formation. Furthermore, both catalase and DMTU, a low molecular weight membrane permeable compound which prevents hydroxyl radical formation by scavenging H_2O_2 (78), were protective. However, SOD and DMSO offered no protection. This is consistent with the minimal decrease in hydroxyl radical production by pyocyanin/ferripyochelin treated endothelial cells in the presence of SOD (Fig. 5). SOD may not adequately reach the site of $^{\bullet}O_2^-$ interaction with ferripyochelin, presumably the cell membrane, to adequately

prevent redox cycling of the catalytic iron. Similarly, DMSO may not be able to achieve concentrations within the cell membrane sufficient to scavenge hydroxyl radical before it interacts with oxidizable membrane components. As additional evidence that this process likely involves hydroxyl radical-mediated toxicity, we have recently found that ferripyochelin markedly enhances the ability of products of the reaction of xanthine and xanthine oxidase ($^{\bullet}O_2^-/H_2O_2$) to damage pulmonary artery endothelial cells (manuscript in preparation).

Substitution of other catalytic iron chelates for ferripyochelin or another redox active xenobiotic (paraquat) for pyocyanin did not cause the magnitude of endothelial cell injury observed with the pyocyanin/ferripyochelin combination. These data suggest a unique interaction between these two compounds which apparently results in the generation of a highly toxic free radical species at a site which is capable of causing considerable cell injury.

The concentration of pyocyanin and pyochelin to which endothelial cells may be exposed in vivo is unknown. However, the concentration of pyocyanin employed in our study is similar to that detected in sputum sol of *P. aeruginosa*-infected patients (8). Quantitation of pyochelin present in vivo at sites of *P. aeruginosa* infection has not been reported. However, the concentration of pyochelin utilized in these studies was below that which routinely accumulates in in vitro broth culture of the organism (20). Given the lipophilic nature of pyochelin the concentration of the compound achievable at an endothelial cell membrane may be higher than this, particularly under conditions in which the organism is adherent to the cell surface. We have also recently found that another pseudomonas secretory product, pseudomonas elastase, is capable of cleaving ferritransferrin to yield novel iron chelates which are also able to catalyze the production of hydroxyl radical from $^{\bullet}O_2^-/H_2O_2$ (79). Such iron chelates, whose generation is enhanced by the simultaneous presence of human neutrophil elastase (79), could also potentially interact with pyocyanin-derived $^{\bullet}O_2^-/H_2O_2$ in a fashion analogous to ferripyochelin to enhance the potential for the formation of hydroxyl radical at sites of *P. aeruginosa* infection.

In summary, we have obtained in vitro evidence that two well-characterized secretory products of *P. aeruginosa*, pyocyanin and pyochelin, which have previously been suggested as virulence factors in vivo (2, 22), can act in a synergistic fashion to damage pulmonary artery endothelial cells. The mechanism appears to involve the endothelial cell-mediated redox cycling of pyocyanin leading to $^{\bullet}O_2^-/H_2O_2$ production which is then converted to the more cytotoxic hydroxyl radical via the catalytic activity of ferripyochelin. Clearly the tissue destruction so characteristic of *P. aeruginosa* infections does not likely result exclusively from oxidant-mediated injury. A host of other *P. aeruginosa* virulence factors have been described (4). Nevertheless, the results of studies carried out thus far suggest that the role of oxygen-centered free radicals in the pathogenesis of *P. aeruginosa* and other bacterial infections is an area worthy of additional study.

Acknowledgments

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