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Pseudomonas Pyocyanin Increases Interleukin-8 Expression by Human Airway Epithelial Cells

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***Pseudomonas aeruginosa*, an opportunistic human pathogen, causes acute pneumonia in patients with hospital-acquired infections and is commonly associated with chronic lung disease in individuals with cystic fibrosis (CF). Evidence suggests that the pathophysiological effects of *P. aeruginosa* are mediated in part by virulence factors secreted by the bacterium. Among these factors is pyocyanin, a redox active compound that increases intracellular oxidant stress. We find that pyocyanin increases release of interleukin-8 (IL-8) by both normal and CF airway epithelial cell lines and by primary airway epithelial cells. Moreover, pyocyanin synergizes with the inflammatory cytokines tumor necrosis factor alpha and IL-1α. RNase protection assays indicate that increased IL-8 release is accompanied by increased levels of IL-8 mRNA. The antioxidant *n*-acetyl cysteine, general inhibitors of protein tyrosine kinases, and specific inhibitors of mitogen-activated protein kinases diminish pyocyanin-dependent increases in IL-8 release. Conversely, inhibitors of protein kinases C (PKC) and PKA have no effect. In contrast to its effects on IL-8 expression, pyocyanin inhibits cytokine-dependent expression of the monocyte/macrophage/T-cell chemokine RANTES. Increased release of IL-8, a potent neutrophil chemoattractant, in response to pyocyanin could contribute to the marked infiltration of neutrophils and subsequent neutrophil-mediated tissue damage that are observed in *Pseudomonas*-associated lung disease.**

The gram-negative bacterium *P. aeruginosa* is the most frequently reported pathogen associated with nosocomial pneumonias (14). Moreover, it is commonly associated with the chronic, progressive lung disease that is the leading cause of morbidity and mortality in individuals with cystic fibrosis (CF) (9, 27). *P. aeruginosa* secretes numerous virulence factors that may contribute to the pathophysiological effects observed in *Pseudomonas*-infected airways (4). However, the molecular mechanisms by which these factors exert their effects are poorly understood. Among these factors is the redox active phenazine derivative pyocyanin (17).

Pyocyanin readily enters cells, leading to increased intracellular formation of reactive oxygen species (13). Reactive oxygen species have been shown to affect a variety of physiological functions. Our laboratory is interested in studying the effects of pyocyanin on human airway epithelial cells, particularly those effects that might contribute to the development of lung disease.

Pseudomonas infections are characterized by a marked influx of polymorphonuclear cells (PMNs) (neutrophils) (10). These cells, when activated in the presence of the bacterium, release oxidant species and proteases that may contribute to the tissue injury that is observed in *Pseudomonas*-infected airways (12, 19). Little is known about the stimuli that are responsible for influx and activation of PMNs in response to this bacterium. However, interleukin-8 (IL-8) is the major PMN chemoattractant responsible for PMN influx and activation in a variety of disease states and thus likely plays an important role in *P. aeruginosa* infections as well. Previous studies by other investigators identify a *Pseudomonas* secretory factor with the properties of pyocyanin that increases IL-8 release by airway epithelial cells both *in vitro* (18) and *in vivo* (15). Based on these studies, we examined the effect of pyocyanin on IL-8 release by human airway epithelial cell lines and by primary epithelial cells cultured from bronchial brushings. We report herein that pyocyanin increases IL-8 release by these cells and synergizes with inflammatory cytokines. This increase in IL-8 release is accompanied by increases in the steady-state levels of IL-8 mRNA. Conversely, pyocyanin inhibits tumor necrosis factor alpha (TNF-α)-dependent increases in expression of the chemokine RANTES. Our studies suggest that pyocyanin increases IL-8 release through signal transduction pathways that include oxidants, protein tyrosine kinases (PTKs), and MAP kinases (MAPKs).

MATERIALS AND METHODS

Materials. Human placental collagen, *n*-acetyl cysteine (NAC), and menadione were purchased from Sigma Chemical Co. (St. Louis, Mo.). Bovine collagen (type I) and human fibronectin were purchased from Life Technologies (Grand Island, N.Y.) and Collaborative Biomedical Products (Bedford, Mass.), respectively. TNF-α, IL-1α, and IL-8 were purchased from R&D Systems (Minneapolis, Minn.). Staurosporine, bisindolylmaleimide, Calphostin C, genistein, tyrphostin 23, herbimycin A, PD98059, PD169316, and KT5720 were obtained from Calbiochem-Novabiochem (La Jolla, Calif.). The oxidant-sensitive fluorescent probe carboxy-dihydrodichlorofluorescein diacetate bis(acetoxymethyl) ester (C-2938) was purchased from Molecular Probes (Eugene, Oreg.).

Pyocyanin preparation. Pyocyanin was isolated from broth culture of *P. aeruginosa* PAO1 by Charles Cox as previously described (5). *Pseudomonas* proteins were removed during purification by chloroform extraction of the pyocyanin. In addition, final stock pyocyanin preparations (1 to 4 mM) had no detectable levels of *Pseudomonas* lipopolysaccharide as determined by the *Limulus* amoebocyte lysate assay (E-TOXATE Assay; Sigma) or of autoinducer as measured by high-performance liquid chromatography separation and bioassay (11a). We estimate that <0.1 U of endotoxin/ml and <0.01 μM autoinducer were present under our experimental conditions.

Cell culture. The human alveolar type II cell line A549 (ATCC 185-CCL; passages 78 to 120; American Type Culture Collection) and the human lung cell line Calu-3 (ATCC 55-HTB; passages 20 to 40) were cultured in Dulbecco modified Eagle medium–Ham's F-12 (1:1) supplemented with 10% fetal bovine

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serum, 2 mM glutamine, and 500 U (each) of penicillin and streptomycin per ml. The human bronchial epithelial cell line 16-HBEo$^-$ was cultured in collagen-coated tissue cultureware in the same medium (passages 8 to 30). Two CF cell lines homozygous for the ΔF508 mutation, ECFTEo$^-$ (passages 56 to 76) and IB3 (passages 86 to 110), as well as the IB3 rescue cell line C38 (passages 27 to 50), were cultured in bovine collagen-human fibronectin-bovine serum albumin (BSA)–coated tissue cultureware in LHC-8 medium (Biofluids Inc., Rockville, Md.) supplemented with 5% fetal bovine serum, glutamine, and penicillin-streptomycin as previously described (28). Primary human bronchial epithelial cells, generously provided by Michael Peterson, were isolated by bronchial brushing and seeded at a density of 5×10^4 cells per well in 12-well tissue culture plates with serum-free Basal Epithelial Growth Medium (Clonetics Corporation, San Diego, Calif.). Cells were cultured for 72 h with a medium change after 48 h. Pyocyanin was then added in fresh serum-free medium for the indicated time.

Enzyme-linked immunosorbent assay (ELISA). Cells were cultured in 48-well tissue culture plates until they were 80 to 90% confluent. To reduce basal IL-8 release, 16-HBEo$^-$ and Calu-3 cells were cultured for 24 h in serum-free medium before agonists were added and subsequent incubations were done in serum-free medium. Treatments of all other cell lines were done in complete medium. At the end of the treatment period, the medium was recovered and detached cells were removed by centrifuging at $15{,}000 \times g$ for 5 min. The medium was stored frozen at −20°C until assay. Total cell protein was measured using the micro-bicinchoninic acid assay (micro BCA; Pierce, Rockford, Ill.).

IL-8 levels were determined by ELISA. Briefly, 98-well Immulon plates (Dynatech Laboratories Inc., Alexandria, Va.) were coated overnight with monoclonal antibody against human IL-8 (4 μg/ml in 100 mM bicarbonate buffer [pH 9.6]; (MAB208; R&D Systems). Between each step plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Plates were blocked for 1 h with PBS containing 1% BSA, 5% sucrose, and 0.05% sodium azide. Samples were diluted in the appropriate medium and added to the wells with biotinylated goat anti-human IL-8 polyclonal antibody (40 ng/ml in Tris-buffered saline–0.05% Tween 20–0.1% BSA; BAF208; R&D Systems). Antibody binding was visualized with horseradish peroxidase-conjugated streptavidin (1:1,000 in Tris-buffered saline–0.05% Tween 20–0.1% BSA; Pierce) and a TMB Peroxidase EIA Substrate kit (Bio-Rad Laboratories, Hercules, Calif.). Development of the color was stopped by addition of 0.5 M H_2SO_4, and the absorbance at 450 nm was measured. Values were determined relative to a standard curve (15 to 1,000 pg/ml of IL-8). Some of the measurements were done with an IL-8 Cytoscreen Immunoassay Kit (Biosource International, Camarillo, Calif.).

Fluorescence assay for oxidant formation. Cells in 12-well culture dishes were washed twice with warm HEPES-buffered saline (135 mM NaCl, 5 mM KOH, 10 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose) containing 0.05% BSA (HBS-G-BSA) and incubated at 37°C for 30 min in HBS-G-BSA containing 5 μM fluorescent probe. At the end of this time, the indicated concentration of menadione was added and the cells were incubated for 1 h at 37°C. Cultures were subsequently washed twice with ice-cold PBS and incubated for 10 min with PBS containing 0.2% Triton X-100. The cell extract was removed from the cells, and the relative fluorescence intensity of the extract (excitation wavelength, 485 nm; emission wavelength, 535 nm) was determined with a Gilford Fluoro IV spectrofluorometer (CIBA-Corning Diagnostics Corp., Park Ridge, Ill.).

RNase protection assay (RPA). Total RNA was isolated by using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio). Biotinylated probe was prepared from linearized plasmid by using the T7 polymerase MaxiScript (Ambion, Austin, Tex.). For these studies, a customized mixture of plasmids was used to generate probe sequences to detect mRNA for several human proteins (in order of decreasing probe size): RANTES, IL-10, IL-1β, IL-1 receptor antagonist, IL-8, L32 (ribosomal protein), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Pharmingen, San Diego, Calif.). The RPA was performed according to the directions of the Ambion HybSpeed RPA kit using 40 μg of total RNA per sample. Following RNase digestion, samples were run on a 5% polyacrylamide–8% urea gel and transferred to Ambion BrightStar Plus nylon membrane. Membranes were incubated with alkaline phosphatase-conjugated streptavidin, and binding was visualized with an Ambion CDP-Star kit and by autoradiography (Kodak X-Omat AR film).

Statistical analysis. Statistical analysis was done on raw data by using the analysis of variance test. Values were considered to be significantly different if P was <0.05. For results from the analysis, see the figure legends and Table 1.

RESULTS

Pyocyanin increases IL-8 release. Previous studies have identified a *Pseudomonas* secretory factor that stimulates IL-8 production by 16-HBEo$^-$ cells (18) and by surface epithelial cells in superfused dog trachea in vivo (15). Based on the physical properties of this factor, we hypothesized that it was the bacterial secretory product pyocyanin. To test this hypothesis, we exposed human airway epithelial cells to purified pyocyanin and measured the release of IL-8 into the medium.

Figure 1 shows that pyocyanin increased IL-8 release by several human airway epithelial cell lines in a concentration-dependent manner (Fig. 1a and b). Pyocyanin also increased IL-8 release by primary cultures of human bronchial epithelial cells (Fig. 1b). Increases in IL-8 above control levels were observed as early as 4 to 8 h after pyocyanin addition (Fig. 1c), and these levels continued to increase relative to controls between 24 and 48 h (data not shown). Longer times were not tested.

Measurable increases in IL-8 release were observed with pyocyanin concentrations as low as 5 μM. Maximal increases, up to 10-fold higher than control levels, were observed at pyocyanin concentrations varying from 25 to 100 μM. Concentrations as high as 75 to 100 μM are observed in sputum from patients with *Pseudomonas* infections (26), suggesting that the concentrations used in our studies are likely to be physiologically relevant.

For each cell line, higher concentrations of pyocyanin (≥100 μM) caused a decrease in IL-8 release relative to maximal levels. Two observations suggest that this was not due to nonspecific cytotoxicity by pyocyanin. First, pyocyanin at concentrations less than 160 μM did not increase ^{51}Cr release (tested up to 6 h) relative to control cells (data not shown) (2). Additionally, in previous studies by our laboratory, we reported that pyocyanin (≤200 μM) has no effect on calcium signaling in epithelial cells when measured 24 h after pyocyanin addition (7). We speculate that the decreased IL-8 release at higher pyocyanin concentrations was due to inhibitory effects on total protein biosynthesis (data not shown).

Pyocyanin synergizes with inflammatory cytokines. Release of IL-8 is regulated by a variety of inflammatory stimuli, including cytokines such as TNF-α and IL-1 (1). Moreover, multiple stimuli can combine to increase IL-8 release additively or synergistically. To determine whether pyocyanin affects the release of IL-8 in response to inflammatory cytokines, we measured IL-8 in the medium from cells treated with pyocyanin alone, cytokines alone, or both together (Table 1). We found that pyocyanin synergized with each cytokine in both normal and CF cell lines. Because the concentrations of cytokines used in these studies were found to be maximal (data not shown), increased release in the presence of pyocyanin suggests that pyocyanin exerts its effect at least in part by mechanisms distinct from those of cytokines. 16-HBEo$^-$ cells did not consistently respond to cytokines (data not shown), so results with these cells are not included.

Pyocyanin increases steady-state levels of IL-8 mRNA. IL-8 expression is regulated at the level of transcription in most cells, and factors that increase IL-8 expression increase steady-state levels of IL-8 mRNA (1). To determine whether pyocyanin increases IL-8 mRNA levels, we treated cells for 16 h with or without 5 μM pyocyanin, with TNF-α (10 ng/ml), IL-1α (10 ng/ml), or with each cytokine in combination with pyocyanin. We then measured steady-state levels of IL-8 mRNA using an RPA. For the RPA, a mixture of probes to detect multiple mRNAs was used (see Materials and Methods). Figure 2 shows representative results from one such experiment. This figure illustrates several points. First, band intensities for the housekeeping genes, L32 and GAPDH, indicate that similar amounts of sample were loaded in each lane. Second, pyocyanin (lane 2) increased IL-8 mRNA relative to control levels (lane 1). Third, TNF-α (lane 3) and IL-1α (lane 5) each increased IL-8 mRNA levels relative to controls, and a further increase was consistently observed when cells were treated with both pyocyanin and cytokine together (lanes 4 and 6). Increased IL-8 mRNA levels in response to pyocyanin were observed as early as 8 h after addition and appeared to be

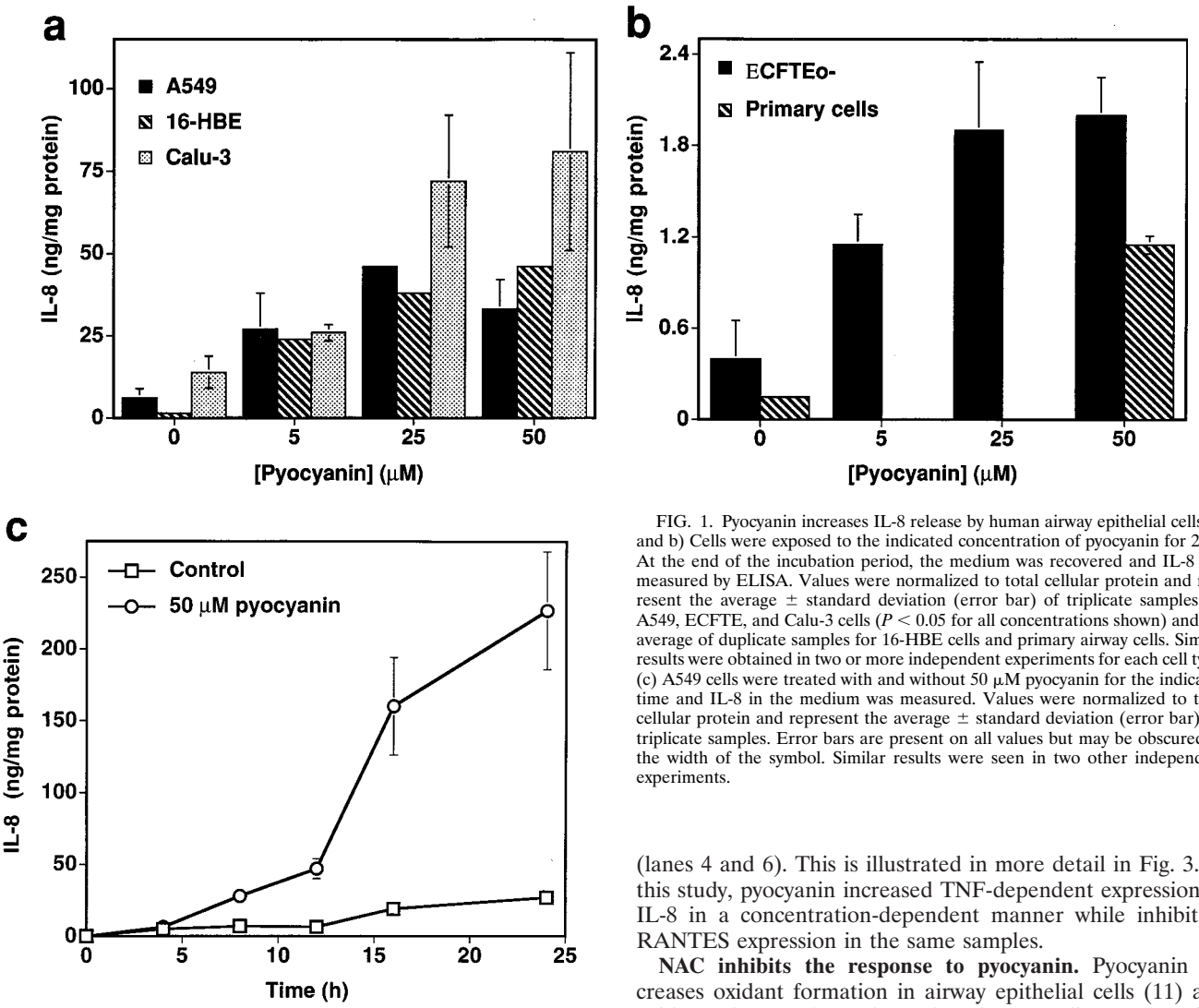

FIG. 1. Pyocyanin increases IL-8 release by human airway epithelial cells. (a and b) Cells were exposed to the indicated concentration of pyocyanin for 24 h. At the end of the incubation period, the medium was recovered and IL-8 was measured by ELISA. Values were normalized to total cellular protein and represent the average ± standard deviation (error bar) of triplicate samples for A549, ECFTE, and Calu-3 cells ($P < 0.05$ for all concentrations shown) and the average of duplicate samples for 16-HBE cells and primary airway cells. Similar results were obtained in two or more independent experiments for each cell type. (c) A549 cells were treated with and without 50 μM pyocyanin for the indicated time and IL-8 in the medium was measured. Values were normalized to total cellular protein and represent the average ± standard deviation (error bar) for triplicate samples. Error bars are present on all values but may be obscured by the width of the symbol. Similar results were seen in two other independent experiments.

maximal by 12 h (data not shown). These results are consistent with the time course for IL-8 release.

Interestingly, the effects of pyocyanin appeared to be specific for IL-8. In the case of the chemokine RANTES, while both TNF-α (lane 3) and IL-1α (lane 5) upregulated RANTES expression, pyocyanin alone did not have an effect (lane 2). Moreover, pyocyanin inhibited the response to each cytokine (lanes 4 and 6). This is illustrated in more detail in Fig. 3. In this study, pyocyanin increased TNF-dependent expression of IL-8 in a concentration-dependent manner while inhibiting RANTES expression in the same samples.

NAC inhibits the response to pyocyanin. Pyocyanin increases oxidant formation in airway epithelial cells (11) and oxidants that are formed are thought to mediate pyocyanin's effects. To determine whether oxidant formation contributes to the pyocyanin-dependent increase in IL-8 release, we tested the effect of adding the thiol antioxidant NAC. We have previously shown, using an oxidant-sensitive fluorescent probe, that NAC scavenges oxidants that are formed in response to pyocyanin (7).

For these experiments, we pretreated cells with increasing concentrations of NAC for 2 h. Because NAC acidifies the

TABLE 1. Effect of pyocyanin on cytokine-dependent increases in IL-8 release[a]

Cell type	IL-8 (ng/mg protein)				
	Pyo	TNF	TNF-Pyo	IL-1	IL-1–pyo
A549	55 ± 16	3,100 ± 138	6,400 ± 870	9,200 ± 310	11,700 ± 1,400
Calu-3	572 ± 106	790 ± 130	1,900 ± 340	3,600 ± 930	5,900 ± 870
ECFTE	23 ± 4	280 ± 71	860 ± 160	670 ± 270	1,470 ± 210
C38	8.1 ± 1.3	190 ± 40	330 ± 39	250 ± 28	1,270 ± 80
IB3	13 ± 0.5	1,930 ± 541	2,600 ± 300[b]	1,500 ± 170	2,890 ± 96

[a] Cells were exposed for 24 h to pyocyanin (Pyo) alone (5 μM) or to the indicated cytokine ± 5 μM pyocyanin. At the end of the incubation period, the medium was recovered and IL-8 was measured by ELISA. Values represent the average ± standard deviation of triplicate samples. Unless otherwise indicated, values for cytokines with pyocyanin are statistically different ($P < 0.05$) from values for cytokines alone. Similar results were obtained for each cell type in two or more independent experiments.

[b] $P < 0.1$

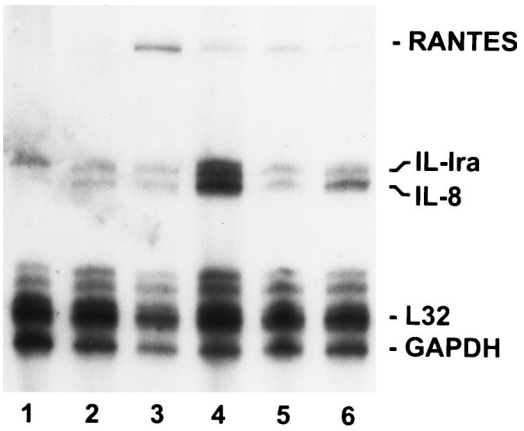

FIG. 2. Effect of pyocyanin on steady-state levels of IL-8 mRNA. A549 cells were treated for 24 h without agonist (lane 1) or with 5 μM pyocyanin (lane 2), 10 ng of TNF-α per ml (lane 3), TNF-α (10 ng/ml) plus pyocyanin (5 μM) (lane 4), 10 ng of IL-1α per ml (lane 5), or IL-1α (10 ng/ml) plus pyocyanin (5 μM) (lane 6). Total RNA was then isolated and levels of mRNA were determined for the indicated human proteins as described in Materials and Methods. Internal controls were L32 (ribosomal protein) and GAPDH. Note that the bands directly above L32 were variably present and likely reflect incomplete digestion by the RNase. Bands representing IL-10 and IL-1β, which would migrate between RANTES and IL-1 receptor antagonist (IL-1ra), were not detected in our studies. Similar results were seen in two other independent experiments.

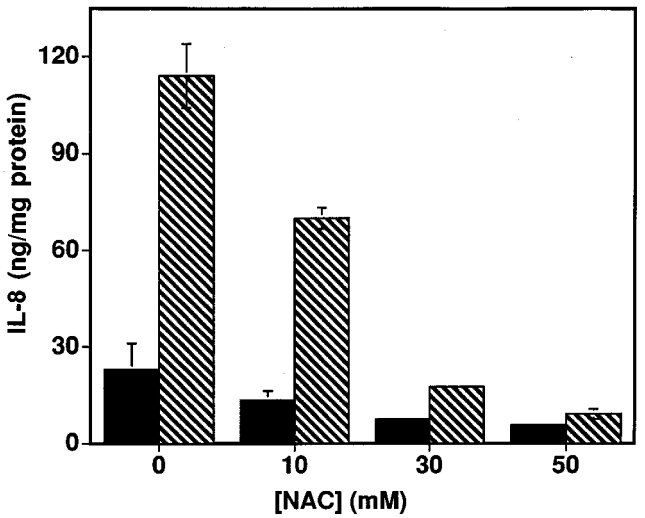

FIG. 4. Effect of NAC on pyocyanin-dependent IL-8 release. A549 cells were treated for 2 h with the indicated concentration of NAC and then stimulated for 24 h with (hatched bars) and without (solid bars) 50 μM pyocyanin. At the end of the incubation, IL-8 in the medium was measured by ELISA. Values were normalized to total cell protein and represent the average ± standard deviation (error bar) of triplicate samples ($P < 0.05$ for all concentrations of NAC in pyocyanin-treated cells). Similar results were seen in two other independent experiments.

medium, all NAC-containing solutions were titrated to pH 7.3 to 7.5 before use. We then exposed the cells to 50 μM pyocyanin for 24 h and measured the release of IL-8 into the medium. Figure 4 shows that NAC significantly reduced both basal and pyocyanin-dependent IL-8 release by A549 cells. Similar results were obtained with 16-HBEo$^-$ cells (data not shown). Moreover, NAC reduced cytokine-dependent increases in IL-8 release in both the presence and absence of pyocyanin. These data suggest that oxidants contribute to both cytokine-dependent and pyocyanin-dependent increases in IL-8 release. Similarly, at concentrations above 10 mM, NAC inhibited pyocyanin-dependent increases in IL-8 mRNA (data not shown), suggesting that oxidants exert their effects, at least in part, at the level of transcription.

Although oxidant formation appears to play a role in pyocyanin-dependent release of IL-8, not all redox active compounds have this effect. Figure 5 demonstrates that the redox active quinone, menadione, increased oxidant formation measured using an oxidant-sensitive fluorescent probe (Fig. 5a) but inhibited IL-8 release (Fig. 5b) over the same concentration range. The mechanism by which menadione inhibits IL-8 release is currently unknown but likely does not reflect cytotoxicity: concentrations of >100 μM menadione were required to observe increases in ^{51}Cr release relative to control cells (data not shown). These results suggest a difference between pyocyanin- and menadione-dependent oxidant stress. This may be a result of a difference in the type, location, or amount of oxidants that are formed, or it may reflect an inhibitory effect specific for menadione.

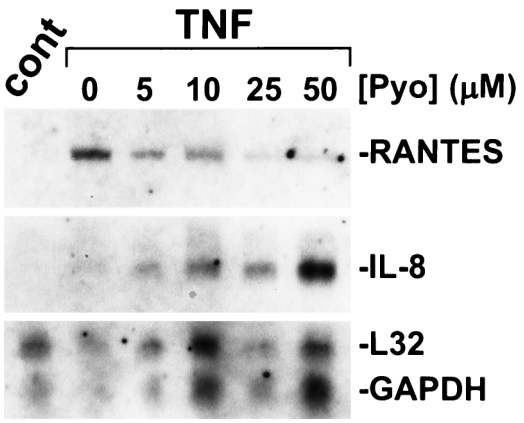

FIG. 3. Effect of pyocyanin on TNF-dependent increases in IL-8 and RANTES mRNA. Cells were treated with and without TNF-α (10 ng/ml) plus the indicated concentration of pyocyanin. Total RNA was then isolated, and levels of mRNA were determined for the indicated human proteins. Similar results were seen in a separate independent experiment. cont, control.

PKC and cyclic AMP-dependent PKA inhibitors do not inhibit the response to pyocyanin. Activation of protein kinases is involved in regulating expression of numerous genes, including genes coding for inflammatory factors such as IL-8. Moreover, activation of protein kinase C (PKC) appears to be involved in increased release of IL-8 by protease-treated human bronchial epithelial cells (23). Although not demonstrated directly with pyocyanin, oxidant stress can activate PKC (24). Thus, we tested the hypothesis that pyocyanin increases IL-8 release by activating PKC. To do this, we used several PKC inhibitors: staurosporine (50% inhibitory concentration [IC_{50}], 10 nM), bisindolylmaleimide (IC_{50}, 14 nM), and Calphostin C (IC_{50}, 6 μM). Additionally, we tested the effect of the cyclic AMP-dependent PKA inhibitor KT5720 (IC_{50}, 59 nM). Cells were incubated for 1 h with 100 nM staurosporine, 300 nM bisindolylmaleimide or KT5720, or 60 μM Calphostin C before the start of the experiment, and inhibitor was present throughout the subsequent incubations.

Figure 6 shows that neither PKA (Fig. 6a) nor PKC (Fig. 6b) inhibitors prevented the response to pyocyanin. In fact, we often observed that PKC inhibitors increased both constitutive and pyocyanin-dependent IL-8 release. These data suggest that PKC may negatively affect IL-8 release by these cells.

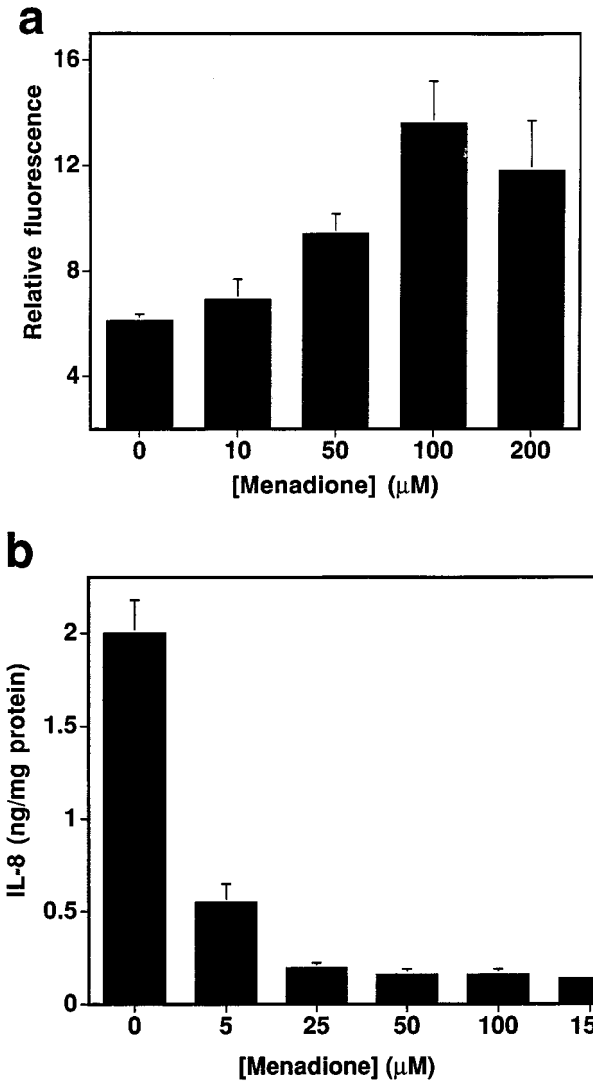

FIG. 5. Effect of the redox-active compound menadione on oxidant formation and IL-8 release. (a) A549 cells were preincubated with 5 μM of an oxidant-sensitive fluorescent probe for 30 min, and then exposed to the indicated concentration of menadione for 1 h. The relative fluorescence of cell-associated probe was determined as described in Materials and Methods. Values represent the average ± standard deviation (error bar) of triplicate samples ($P < 0.05$ for concentrations ≥50 μM). Similar results were seen in a separate independent experiment. (b) Cells were exposed to the indicated concentration of menadione for 24 h, and then IL-8 in the medium was measured by ELISA. Values were normalized to total cellular protein and represent the average + standard deviation (error bar) of triplicate samples ($P < 0.05$ for all concentrations). Similar results were seen in two other independent experiments.

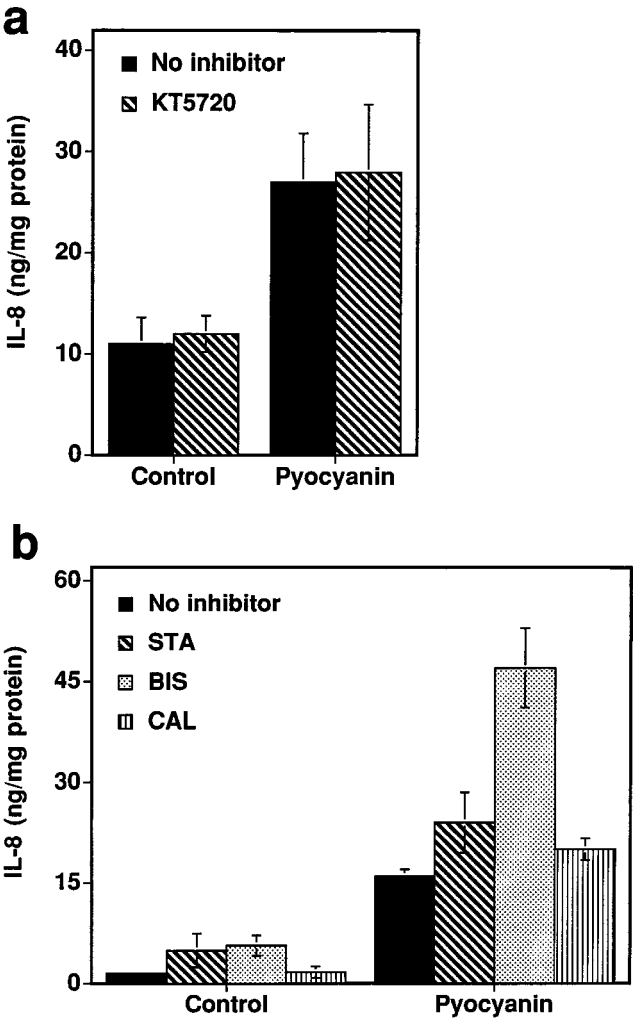

FIG. 6. Effect of PKA and PKC inhibitors on pyocyanin-dependent IL-8 release. A549 cells were pretreated with and without the PKA inhibitor KT5720 (300 nM) (a) or with and without the PKC inhibitors staurosporine (STA) (100 nM), bisindolylmaleimide (BIS) (300 nM), and Calphostin C (CAL) (60 μM) (b) for 1 h. Cells were then stimulated with and without 50 μM pyocyanin for 24 h in the continued presence of inhibitor. IL-8 in the medium was subsequently measured by ELISA. Values were normalized to total cell protein and represent the average ± standard deviation (error bar) of triplicate samples. Similar results were seen for each inhibitor in two other independent experiments.

PTK inhibitors inhibit the response to pyocyanin. Protein tyrosine kinases are implicated in pathways that increase expression of IL-8. Moreover, oxidant stress can activate PTKs (3). To determine whether activation of PTKs mediates the pyocyanin-dependent increase in IL-8 release, we tested the effect of several PTK inhibitors; genistein (IC_{50}, 100 μM), tyrphostin 23 (IC_{50}, 50 μM), and herbimycin A (IC_{50}, 100 ng/ml). For these experiments, cells were pretreated for 1 h with genistein or for 24 h with tyrphostin or herbimycin A, and inhibitors were present throughout. As shown in Fig. 7, all three PTK inhibitors diminished the pyocyanin-dependent increase in IL-8 release. Because these inhibitors act by different mechanisms, these data strongly suggest that pyocyanin activates pathways that include PTKs and that activation of these pathways increases IL-8 release. Consistent with this hypothesis is our observation that staurosporine concentrations that inhibit PTKs (300 nM) (6) also inhibited the pyocyanin-dependent increase in IL-8 release (data not shown).

We also tested the effect of these inhibitors on pyocyanin-dependent increases in IL-8 mRNA. Representative results from these studies are shown in Fig. 8. We found that both genistein (300 μM) and tyrphostin inhibited the increase in response to pyocyanin, suggesting that PTK activation contributes to pyocyanin-dependent increases in IL-8 transcription and/or mRNA stability. Interestingly, in parallel, genistein was a more potent inhibitor of IL-8 release than of mRNA expression. Similarly, herbimycin A inhibited IL-8 release at concentrations of 1 μg/ml but had no observable effect on mRNA levels even at higher concentrations (3 μg/ml)(data not shown). The latter observations suggest either that genistein and herbimycin A each have nonspecific effects on IL-8 bio-

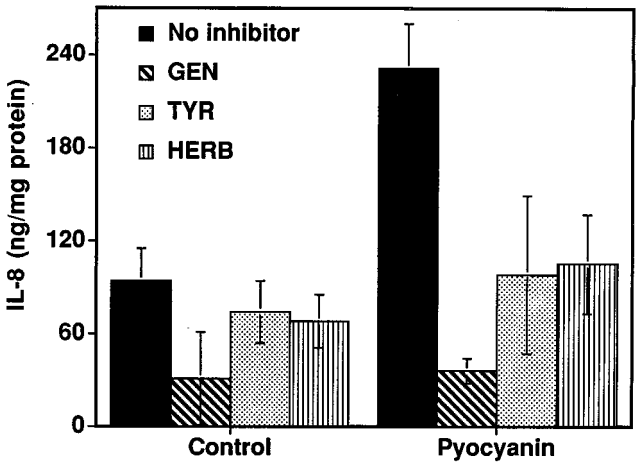

FIG. 7. Effect of PTK inhibitors on pyocyanin-dependent IL-8 release. A549 cells were treated with and without genistein (GEN) (300 μM) for 1 h or with tyrphostin 23 (TYR) (100 μM) or herbimycin A (HERB) (1 μg/ml) for 24 h prior to addition of pyocyanin. Cells were then stimulated with and without 50 μM pyocyanin for 24 h in the continued presence of inhibitor. IL-8 in the medium was measured by ELISA. Values were normalized to total cell protein and represent the average ± standard deviation (error bar) of triplicate samples ($P < 0.05$ for all inhibitors in pyocyanin-treated cells). Similar results were seen in two other independent experiments.

synthesis and/or release or that PTKs also modulate pyocyanin-dependent IL-8 expression at the posttranscriptional level.

MAPK inhibitors inhibit pyocyanin-dependent IL-8 release. There are three families of MAPKs, extracellular-signal regulated kinases 1 and 2 (ERK 1/2), c-Jun terminal kinase (JNK), and p38 (22). Studies indicate that TNF-α activates at least two transcription factors that regulate IL-8 expression, namely NF-κB and AP-1, by activating MAPK signal transduction pathways (22, 25). Moreover, oxidants have been implicated as early intermediates in MAPK signaling pathways (16). To determine whether pyocyanin increases IL-8 release by activating MAPKs, we pretreated cells with specific inhibitors of MEK (PD98059; IC_{50}, 2 μM), the kinase that activates ERK 1/2, and of p38 (PD169316; IC_{50}, 89 nM) for 1 h and then with pyocyanin for 24 h in the continued presence of inhibitor. Finally, we measured IL-8 release into the medium by ELISA (Fig. 9). We found that both inhibitors reduced the response to pyocyanin, suggesting a role for these kinases in pyocyanin's effects. Partial inhibition was observed with lower concentrations (5 μM PD98059 and 200 nM PD169316) of each inhibitor (data not shown).

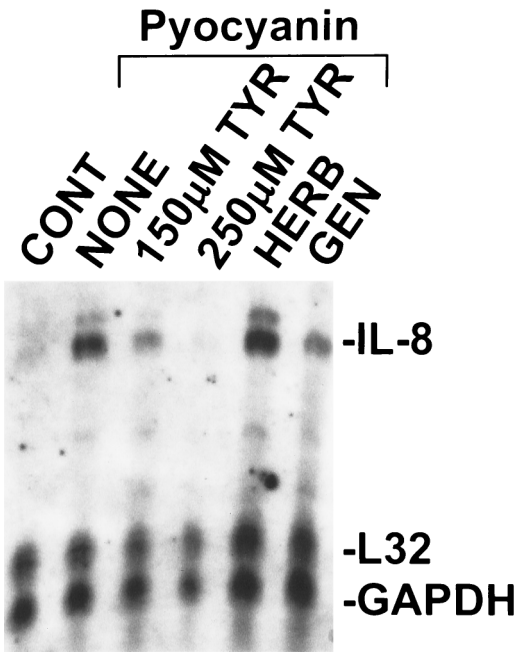

FIG. 8. Effect of PTK inhibitors on pyocyanin-dependent increases in IL-8 mRNA. The indicated cultures of A549 cells were treated with genistein (GEN) (300 μM) for 1 h or with tyrphostin 23 (TYR) (150 and 250 μM) or herbimycin A (HERB) (1 μg/ml) for 24 h prior to addition of pyocyanin. Cells were then stimulated with and without 50 μM pyocyanin for 24 h in the continued presence of inhibitor. Total RNA was isolated, and levels of mRNA were determined for the indicated human proteins. Similar results were seen in two other independent experiments.

DISCUSSION

Previous studies have described a low-molecular-weight, heat-stable *P. aeruginosa* secretory factor(s) that increases IL-8 release by human airway epithelial cells in vitro, as well as by surface epithelial cells from superfused dog trachea in vivo. Other studies have suggested that this activity may be due at least in part to autoinducer (8). Because these physical properties are also characteristic of *Pseudomonas* pyocyanin, we hypothesized that pyocyanin increases IL-8 release by airway cells.

Our studies are the first to demonstrate directly that pyocyanin increases steady-state levels of IL-8 mRNA and release of IL-8 from human airway epithelial cells. Pyocyanin-dependent increases in IL-8 release were observed with several cell lines and with primary cells under a variety of growth conditions, suggesting that this is likely to be a characteristic response by human airway epithelial cells. Moreover, as is observed with other stimuli (1), increased expression of IL-8 in response to pyocyanin was rapid (<8 h).

Pyocyanin is one of several *P. aeruginosa* factors, including pilin, flagellin, autoinducer (8), and nitrite reductase (20), that stimulate IL-8 release. Based on the purification protocol and subsequent analysis, it seems highly unlikely that the responses observed in our studies are due to contamination by any of these bacterial factors. Because an isogenic mutant deficient in pyocyanin production is not yet available, we are currently unable to test directly the relative contribution of pyocyanin to IL-8 release in vitro or in vivo in the context of the whole bacterium. However, there are several characteristics of the pyocyanin effect that suggest it plays an important role in the inflammatory response to the bacterium.

First, bacterial surface proteins exert effects only at the site of attachment. In contrast, pyocyanin is a readily diffusible secretory factor that could affect IL-8 release by cells at some distance from the site of colonization. Second, the concentration of autoinducer necessary to stimulate IL-8 release (~30 μM) (8) is severalfold higher than that normally achieved in stationary-phase bacterial cultures (~5 μM) (21) and thus is only likely to be present at or very near the site of colonization. Conversely, pyocyanin exerts a measurable effect at concentrations as much as 20-fold lower than those detected in sputum from patients with *Pseudomonas* infections (26). Third, pyocyanin synergizes with the inflammatory cytokines TNF-α and IL-1α. Increased levels of these cytokines are commonly found in bacterial lung infections. Finally, our results from RPA studies suggest that pyocyanin reduces release of the monocyte/macrophage/T-cell chemokine RANTES under the same conditions under which it increases IL-8 release. The delayed

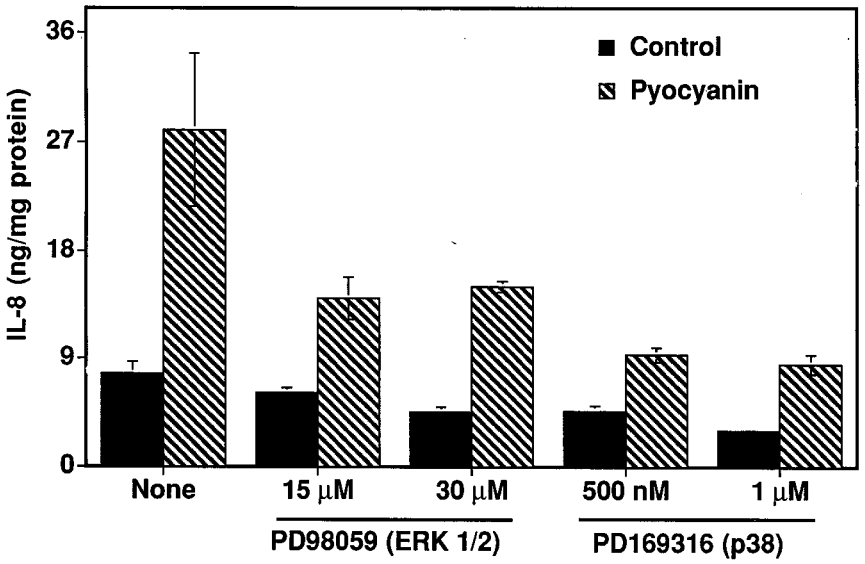

FIG. 9. Effect of MAPK inhibitors on pyocyanin-dependent IL-8 release. A549 cells were treated with the indicated concentration of inhibitor for 1 h and then with and without 50 μM pyocyanin for 24 h in the continued presence of inhibitor. At the end of the incubation period, IL-8 was measured in the medium by ELISA. Values were normalized to total cell protein and represent the average ± standard deviation (error bar) of triplicate samples ($P < 0.05$ for each inhibitor in pyocyanin-treated cells). Similar results were seen in two other independent experiments.

influx of T cells and monocytes/macrophages as a result of chemokine release is part of the resolving phase of inflammation. By preventing this phase, pyocyanin could prolong the inflammatory response. All of these characteristics suggest that pyocyanin can contribute to a more chronic and diffuse inflammatory response.

IL-8 expression is regulated by numerous inflammatory and stress-related factors. The signaling pathways by which these factors regulate expression are still poorly understood. Cytokine-stimulated pathways for IL-8 expression include oxidants, PTKs, and MAPKs. While our studies suggest that similar pathways are activated by pyocyanin, the observation that pyocyanin synergizes with cytokines at maximal concentrations suggests that pyocyanin activates additional pathways as well. Further studies will be necessary to understand fully the mechanisms by which pyocyanin regulates IL-8 expression and release.

Our results suggest that pyocyanin, alone or in combination with other factors, can cause significant increases in IL-8 release in *Pseudomonas*-infected airways. Increased IL-8 release in turn could contribute to the marked infiltration of neutrophils observed in *P. aeruginosa*-associated lung disease. Thus, pyocyanin could contribute to neutrophil-mediated airway damage by stimulating release of IL-8. Understanding the mechanisms by which *P. aeruginosa* exerts its pathophysiological effects is essential if we are to design effective therapies that target this microorganism.

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