Induction of Neutrophil Apoptosis by the 
Pseudomonas aeruginosa Exotoxin Pyocyanin: A Potential Mechanism of Persistent Infection


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Induction of Neutrophil Apoptosis by the *Pseudomonas aeruginosa* Exotoxin Pyocyanin: A Potential Mechanism of Persistent Infection


*Pseudomonas aeruginosa* colonizes and infects human tissues, although the mechanisms by which the organism evades the normal, predominantly neutrophilic, host defenses are unclear. Phenazine products of *P. aeruginosa* can induce death in *Caenorhabditis elegans*. We hypothesized that phenazines induce death of human neutrophils, and thus impair neutrophil-mediated bacterial killing. We investigated the effects of two phenazines, pyocyanin and 1-hydroxyphenazine, upon apoptosis of neutrophils in vitro. Pyocyanin induced a concentration- and time-dependent acceleration of neutrophil apoptosis, with 50 μM pyocyanin causing a 10-fold induction of apoptosis at 5 h (*p* < 0.001), a concentration that has been documented in sputum from patients colonized with *P. aeruginosa*. 1-hydroxyphenazine was without effect. In contrast to its rapid induction of neutrophil apoptosis, pyocyanin did not induce significant apoptosis of monocyte-derived macrophages or airway epithelial cells at time points up to 24 h. Comparison of wild-type and phenazine-deleted strains of *P. aeruginosa* showed a highly significant reduction in neutrophil killing by the phenazine-deleted strain. In clinical isolates of *P. aeruginosa* pyocyanin production was associated with a proapoptotic effect upon neutrophils in culture. Pyocyanin-induced neutrophil apoptosis was not delayed either by treatment with LPS, a powerfully antiapoptotic bacterial product, or in neutrophils from cystic fibrosis patients. Pyocyanin-induced apoptosis was associated with rapid and sustained generation of reactive oxygen intermediates and subsequent reduction of intracellular cAMP. Treatment of neutrophils with either antioxidants or synthetic cAMP analogues significantly abrogated pyocyanin-induced apoptosis. We conclude that pyocyanin-induced neutrophil apoptosis may be a clinically important mechanism of persistence of *P. aeruginosa* in human tissue. *The Journal of Immunology*, 2002, 168: 1861–1868.

The neutrophil granulocyte is a major participant in the acute inflammatory response in tissues, being recruited from the circulation when local defenses are overwhelmed. The constitutively short life span of the neutrophil, less than 24 h in the circulation, is regulated by the onset of neutrophil apoptosis (programmed cell death) (1, 2). Apoptosis is important in the normal resolution phase of inflammation, since it leads to functional downregulation (3) and to recognition and clearance of the apoptotic neutrophils by macrophages (1). The process of apoptosis is susceptible to modulation by host cytokines, suggesting active control of cell death during the course of inflammation in vivo (4). There is also evidence that this physiological process can be subverted by pathogens (5). Inappropriate induction of neutrophil apoptosis during infection could deplete neutrophil numbers and function, impairing host defense and favoring bacterial persistence.

The human opportunistic pathogen *Pseudomonas aeruginosa* is a major cause of pulmonary damage and mortality in patients with cystic fibrosis and other forms of bronchiectasis (6, 7). Colonization of the respiratory tract with *P. aeruginosa* leads to an exuberant inflammatory response in the airways, with large numbers of activated neutrophils present. Despite the central role of neutrophils in preventing bacterial colonization, killing bacteria with their toxic products including proteases and reactive oxygen intermediaries (8), *P. aeruginosa* persists in the tissues, resulting in chronic colonization and infection. A number of *P. aeruginosa* virulence factors have been described, but the mechanisms by which the organism evades neutrophil defenses are unclear (7).

The secreted products of *P. aeruginosa* include highly diffusible phenazine pigment exotoxins. The most abundant phenzone, pyocyanin, is present in significant quantities in the sputum of patients with cystic fibrosis and bronchiectasis whose lungs are colonized by this organism (9). Recent studies of the mechanisms of *P. aeruginosa* virulence have shown that the organism’s ability to generate phenazines is critical for killing both in *Caenorhabditis elegans* and in a model of murine septicemia (10). We therefore hypothesized that phenazines might induce apoptosis in neutrophils as a mechanism of *P. aeruginosa* evasion of the host inflammatory response. The studies presented show that the predominant phenazine pigment, pyocyanin, induces rapid and overwhelming apoptosis in neutrophil populations in vitro, which is associated with rapid reactive oxygen intermediate (ROI) generation and...
lowering of intracellular cAMP ([cAMP]). Inhibition of either of these downstream effects abrogates pyocyanin-induced apoptosis. Moreover, the neutrophil-killing abilities of different *P. aeruginosa* strains correlate closely with their production of pyocyanin, suggesting phenazine-induced neutrophil apoptosis may be a clinically relevant mechanism of *P. aeruginosa* persistence.

Materials and Methods

**Isolation of neutrophils**

Human peripheral blood neutrophils were obtained from healthy volunteers and also from four patients with cystic fibrosis (three males, age range 11–19 years, all studied when clinically well at annual review). Full ethical approval for these studies was obtained from the South Sheffield Research Ethics Committee, and all subjects gave fully informed consent. Neutrophils were isolated from citrated venous blood by dextran sedimentation and centrifugation through a discontinuous plasma-Percoll (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) gradient, as previously described (11). Purity was assessed by counting >500 cells on duplicate cytospin preparations and was always >95%, with contaminating cells being almost exclusively eosinophils.

**Cell culture**

Neutrophils were suspended at a concentration of 2.5 × 10^6/ml in RPMI 1640, with 10% FCS and with penicillin and streptomycin (100 U/L; all from Life Technologies, Glasgow, U.K.) (12). The age of neutrophils in culture was calculated, designating this stage as time 0. Neutrophils were incubated in 96-well Falcon Flexiwell plates (BD Pharmingen, Cowley, Oxford, U.K.) at 37°C in a 5% CO₂ atmosphere, for 5 h, unless otherwise stated. The 5-h time point was chosen because there is little (typically <5%) constitutive apoptosis in control (untreated) populations. For reagents reconstituted in DMSO (Merck, Dorset, U.K.) (final concentration <0.001% v/v), control cells were cultured in the presence of DMSO, to ensure there was no effect of DMSO at these concentrations upon neutrophil apoptosis (data not shown). Monoocyte-derived macrophages (MDM) were prepared from the mononuclear cell layer obtained by plasma-Percoll gradient centrifugation, followed by culture of adherent cells in Iscove’s DMEM with 10% autologous serum, as previously described (1). A549 pulmonary epithelial cells were cultured in RPMI 1640 and 10% FCS.

**Preparation and analysis of pyocyanin**

Pyocyanin and 1-hydroxyphenazine (1-HP) were prepared by photolysis of phenazine methosulfate and phenazine (Sigma-Aldrich, St. Louis, MO), respectively, and purified, as previously described (13-15). [1H₃]Pyocyanin was prepared by treatment of [1H₃]-1-HP with [1H₃]dimethyl sulfate at 120°C for 10 min (16). Pyocyanin was characterized by its UV spectrum by positive ion electrospray mass spectrometry (14). In the case of 1-HP, samples were also analyzed by gas chromatography-electron impact mass spectrometry. After purification, pyocyanin and 1-HP were reconstituted in sterile H₂O to a concentration of 10⁻⁴ M and stored in the dark at -20°C. All subsequent dilutions were made in RPMI 1640 and added to neutrophils at the concentrations specified in the text.

For analysis of pyocyanin content of culture supernatants, supernatants were spiked with [1H₃]pyocyanin as a positive control and loaded onto Oasis columns (Waters, Watford, U.K.). The columns were washed with water, followed by 5% methanol, and pyocyanin was eluted in methanol. HPLC was conducted on a Waters isocratic system using an Aquashim C18 column (10 cm × 2 mm; Phenomenex, Macclesfield, U.K.). Elution was undertaken at 0.2 ml/min with a 15-min linear gradient from 0% to 1% aqueous formic acid to acetonitrile:0.1% formic acid (75:25 v/v), followed by 5 min in 75% acetonitrile:formic acid (all solvents were of analytical grade and were obtained from BDH, Poole, Dorset, U.K.). Under these conditions, pyocyanin eluted at 9.5 min. The HPLC flow was directed into the electrospray source of the Quadrto II, which was operated in the positive ion MS:MS mode. Detection and quantitation of pyocyanin were by MRM (211/168, 221/175).

**Assessment of cell viability and apoptosis**

At the time points indicated, apoptosis was quantified by assessment of nuclear morphology on Giemsa-stained cytosphin preparations, counting >300 cells per slide on duplicate cytospins with the observer blinded to cell treatment (17). This method has been shown to correlate closely with other measurements of neutrophil apoptosis, including annexin V binding (17) and shedding of CD16 (18). In three individual experiments in which apoptosis had been modulated by pyocyanin treatment, we confirmed that morphological appearances of apoptosis correlated with surface binding of annexin V. Briefly, untreated and pyocyanin-treated neutrophils were retrieved from culture, washed with PBS, and stained for phosphatidylserine (PS) exposure by adding 5 μl each of annexin V and Via-Probe (BD Pharmingen) (19). A549 cells and MDM were removed from culture dishes using Cell Dissociation Fluid (Sigma-Aldrich) and stained for PS exposure, as described above. Where apoptosis was calculated by morphometry alone, necrosis was assessed at all time points by exclusion of the vital dye, trypan blue, and was <2%, unless otherwise indicated.

**Modulation of pyocyanin-induced apoptosis**

Neutrophils were incubated in the presence or absence of pyocyanin with candidate modulators of pyocyanin-induced apoptosis. These included the bacterial product, LPS, derived from *P. aeruginosa*, serotype 10, the cAMP analogue, dibutyryl cAMP (dbcAMP), and the antioxidants N-acetylcysteine (NAC) and diphenyleneiodonium chloride (DPI) (all from Sigma-Aldrich). LPS and dbcAMP were added simultaneously with pyocyanin, while neutrophils were preincubated for 1 h with NAC, DPI, or medium alone before addition of pyocyanin.

**Preparation of *P. aeruginosa* culture supernatants**

Seven clinical pathological isolates of *P. aeruginosa* were maintained on nutrient agar (Oxoid, Basingstoke, U.K.). Five isolates were obtained from sputum of infected patients; one was a urine isolate, and one a blood isolate. By observation of pigment color on plates, four isolates (all from sputum) were found to be pigment producing and three nonproducing. Each isolate was subcultured from individual colonies and, following 18 h at 37°C, the agar plates were left at room temperature by a light source for at least 1 day to optimize pigment production. To remove the pigment from the agar, the agar plates were washed by adding 6 ml of tissue culture medium (RPMI 1640 + 10% FCS), then left at room temperature for 2 h. The media were removed and centrifuged at 3000 rpm for 5 min, after which the supernatant was decanted and spun again. This was repeated until no visible pellet remained. The supernatant was then filtered using a 0.2-μm filter to remove any traces of bacteria. The filtrates were aliquoted and stored at −70°C until required. Before use in neutrophil apoptosis assays, 100 μl of each filtrate was used to inoculate Luria-Bertani agar plates, which were incubated for 48 h at 37°C. This confirmed there was no bacterial contamination of the supernatants. As a control, sterile agar plates were also washed, as described above, and used in neutrophil apoptosis assays.

The same methods were used for preparation of supernatants from wild-type (PA14) and phenazine-deficient (ΔphnAB) strains of *P. aeruginosa* provided by F. M. Ausubel (Harvard Medical School, Cambridge, MA) (10). The strains were grown overnight on Luria-Bertani agar supplemented with 100 μg/ml rifampicin and supernatants made as described above.

**Measurement of LPS concentrations in *P. aeruginosa* culture supernatants**

LPS concentrations in culture supernatants were measured using the Limusus amebocyte lysate-based method from Kinetic-QCL. System according to the manufacturer’s instructions (BioWhittaker, Wokingham, U.K.).

**Measurement of ROI**

The generation of intracellular ROI was measured using the cell-permeable molecule 2,7'-dichloro-dihydroflourescein diacetate (DCF; Sigma-Aldrich), which becomes fluorescent upon reaction with hydrogen peroxide, as previously described (20). Neutrophils were preincubated with DCF for 30 min before treatment with appropriate stimulus. Cells were collected, washed in PBS at room temperature, and immediately analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA).

**Measurement of neutrophil [cAMP]**

[cAMP], levels were measured using a Biotrak enzyme immunoassay according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Neutrophils were stimulated with salbutamol (2 μM; Glaxo Wellcome, Stevenage, U.K.) as a positive control.

**Statistical analysis**

Results are expressed as mean ± SE of the mean of the number (n) of independent experiments, with each experiment using cells from separate donors and performed in duplicate. Data were analyzed as appropriate by paired t tests or ANOVA with Bonferroni posttest, using the Prism 3.0 program (GraphPad, San Diego, CA). Results were considered to be statistically significant where p < 0.05.
Results
The phenazine pigment, pyocyanin, induces neutrophil apoptosis in a time- and concentration-dependent manner

We first investigated whether two phenazine pigments, pyocyanin and its degradation product 1-HP, could induce neutrophil apoptosis. Time course experiments were performed in the presence or absence of a concentration (50 μM) of each compound previously shown to have biological effects in cell culture (21). After 3 h, an acceleration of apoptosis was observed in cells cultured with pyocyanin, which was statistically significant at 6 h in which 35.4 ± 7.5% (mean ± SE) of pyocyanin-treated cells were apoptotic compared with 7.1 ± 2.4% of untreated controls (p = 0.001, n = 4) (Fig. 1A). Both control and pyocyanin-treated cell populations remained trypan blue negative for all early time points studied (6 h and before). At later time points (>12 h), there was significant secondary necrosis of pyocyanin-treated neutrophils, as assessed by trypan blue staining (data not shown).

Neutrophils were then cultured with increasing concentrations of pyocyanin or 1-HP for 5 h (Fig. 1B). A 5-h time point was chosen because there is little apoptosis in control populations and because, in pyocyanin-treated cells, there are high levels of secondary necrosis at later time points. Pyocyanin treatment resulted in a dramatic, concentration-dependent acceleration of neutrophil death. Assessed by light microscopy, apoptosis was 3.3 ± 0.6% in control populations compared with 17.5 ± 0.6% in pyocyanin-treated cells (p < 0.001, n = 5) (Fig. 1A). Both control and pyocyanin-treated cell populations remained trypan blue negative for all early time points studied (6 h and before). At later time points (>12 h), there was significant secondary necrosis of pyocyanin-treated neutrophils, as assessed by trypan blue staining (data not shown).

Neutrophils were cultured with or without 50 μM pyocyanin or 1-HP and sampled after 1, 2, 3, and 6 h in culture. Apoptosis was assessed by cytospin morphology, as described in Materials and Methods. Percentage of apoptosis (mean ± SE of four independent experiments) in control ( ), 1-HP ( ), and pyocyanin-treated ( ) neutrophils is plotted against time (hours). At t = 0, apoptosis was <1%. *** Statistically significant difference between control and pyocyanin-treated populations after 6 h in culture (p < 0.001).

Data shown are mean ± SE percent apoptosis. There were no significant differences between the two methods in either the untreated (p = 0.27) or treated cells (p = 0.58).

We determined whether the phenomenon of pyocyanin-induced apoptosis was of specific relevance to neutrophils by studying the effect of pyocyanin upon two other cell types, MDM and a pulmonary epithelial cell line (A549). In three independent experiments, pyocyanin did not induce significant apoptosis of MDM or A549 cells at time points up to 24 h, in contrast to the observed proapoptotic effect upon neutrophils (Fig. 2).

Pyocyanin induces neutrophil apoptosis in the presence of LPS

Bacterial LPS have been found to have potent and long-lasting antiapoptotic effects upon neutrophils (4, 24) and are produced in binding is comparable to other studies (22, 23), with a slight excess of annexin V positivity, as it is an earlier marker of apoptosis (18). 1-HP was without effect upon neutrophil apoptosis at concentrations up to 150 μM.

Table 1. Apoptosis of neutrophil populations assessed by annexin V positivity and morphology in the presence or absence of pyocyanin

<table>
<thead>
<tr>
<th>Mean Annexin V Positivity (%)</th>
<th>Mean % Apoptosis as Assessed by Morphology</th>
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<tbody>
<tr>
<td>Control</td>
<td>Pyocyanin</td>
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<td>Control</td>
<td>Pyocyanin</td>
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<tr>
<td>7.7 ± 1.3</td>
<td>26.5 ± 3.8</td>
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<tr>
<td>3.3 ± 0.6</td>
<td>17.5 ± 3.4</td>
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Neutrophils were cultured with pyocyanin (12.5 μM) or medium alone for 5 h. Data shown are mean ± SE percent apoptosis. There were no significant differences between the two methods in either the untreated (p = 0.27) or treated cells (p = 0.58).

FIGURE 1. Pyocyanin induces time- and concentration-dependent apoptosis of neutrophils. A, Neutrophils were cultured with or without 50 μM pyocyanin or 1-HP and sampled after 1, 2, 3, and 6 h in culture. Apoptosis was assessed by cytospin morphology, as described in Materials and Methods. Percentage of apoptosis (mean ± SE of four independent experiments) in control ( ), 1-HP ( ), and pyocyanin-treated ( ) neutrophils is plotted against time (hours). At t = 0, apoptosis was <1%. *** Statistically significant difference between control and pyocyanin-treated populations after 6 h in culture (p < 0.001). B, Neutrophils were cultured in the presence of various concentrations of 1-HP ( ) or pyocyanin ( ) for 5 h. Percentage of apoptosis (mean ± SE of five independent experiments) is plotted against concentration of phenazine (micromolar). Significant differences from untreated cells are indicated by **, p < 0.01, and ***, p < 0.001.

FIGURE 2. Differential induction of neutrophil apoptosis by pyocyanin compared with other cell types. In three independent experiments, A549 pulmonary epithelial cells and MDM were treated for 24 h with pyocyanin (50 μM) and compared with neutrophils treated with pyocyanin (50 μM) for 5 h. A549 cells and MDM were retrieved from culture, and apoptosis was determined by annexin V staining. Neutrophil apoptosis was assessed by light microscopy. Data are expressed as mean ± SE of the fold increase in apoptosis in pyocyanin-treated compared with untreated cells. In each case, untreated cells are represented by open bars, and pyocyanin-treated cells by hatched bars, with a significant difference indicated by ***, p < 0.001.
significant quantities by *P. aeruginosa* (25, 26). Antiapoptotic factors have been shown to inhibit apoptosis when added along with a proapoptotic stimulus, with Fas-mediated neutrophil apoptosis being inhibited by coadministration of a range of different antiapoptotic stimuli (12, 27). We therefore sought to investigate whether *P. aeruginosa* LPS could inhibit the proapoptotic effect of pyocyanin in coculture experiments (Fig. 3). After 5 h in culture, LPS (100 ng/ml) significantly retarded constitutive apoptosis (3.8 ± 0.7%) as compared with control (5.9 ± 0.6%, p < 0.05), in keeping with published data (4, 24). LPS did not inhibit pyocyanin-induced apoptosis. In three experiments, cells cultured with pyocyanin alone (100 μM) showed no significant difference in apoptosis compared with cells cultured with pyocyanin and LPS at either concentration. Apoptosis was 55.9 ± 5% for pyocyanin-treated populations compared with 60.5 ± 5.3% for cells treated with both pyocyanin and LPS.

Peripheral blood neutrophils were also purified from four patients with cystic fibrosis during clinical remission of their respiratory disease. Circulating neutrophils from these patients are known to be primed (28), and circulating inflammatory mediators have the potential to delay neutrophil apoptosis (4, 29). In four parallel experiments, pyocyanin (50 μM) induced apoptosis of the patients’ neutrophils to levels equivalent to those seen with neutrophils from normal donors: at 5 h apoptosis was 24.9 ± 8.6% in cystic fibrosis patient neutrophils compared with 30.1 ± 4.9% in parallel samples from normal volunteers (p = 0.26).

**Effect of *P. aeruginosa* culture supernatants on neutrophil apoptosis**

To confirm that phenazine production by *P. aeruginosa* significantly affects neutrophil apoptosis, we compared supernatants from a wild-type strain of *P. aeruginosa* (PA14) and a phenazine-deficient strain (ΔphnAB). ΔphnAB is genetically identical to PA14 except for deletion of the *phnA* and *phnB* genes encoding the anthranilate-synthase complex that catalyzes phenazine production by *P. aeruginosa* (10). There was a clear difference in the effects of strain supernatants upon neutrophil apoptosis, with wild-type supernatants having a significantly greater proapoptotic effect (p < 0.05, Fig. 4). Supernatants from the deleted strain did have a modest proapoptotic effect; this strain produces ~10% of the pyocyanin of the wild-type organism in the absence of the catalytic complex (10). We also examined seven clinical isolates of *P. aeruginosa*; four were pigment producing, and three nonproducing. Supernatant from each isolate was tested in three independent experiments for effect upon neutrophil apoptosis, and supernatant pyocyanin concentration was determined by HPLC-mass spectrometry (data not shown). LPS concentrations were also measured in the supernatants, and all had LPS concentrations in excess of 1 μg/ml, a concentration that significantly inhibits neutrophil apoptosis, even at early time points (4, 24). As expected, therefore, supernatants of the three pyocyanin-negative strains inhibited neutrophil apoptosis. Two of the four cultures from pigment-producing strains showed a proapoptotic effect, with a pyocyanin concentration that parallelled their induction of apoptosis. Two pyocyanin-producing cultures showed no proapoptotic effect, but the pyocyanin concentrations of these supernatants (1 and 2.3 μM) were below the levels producing a significant proapoptotic effect in the concentration-response curve (Fig. 1B).

**Pyocyanin induces generation of ROI by neutrophils**

Pyocyanin exerts its toxic effects upon mammalian cells and other bacteria (30), and in *C. elegans* (10), via its ability to undergo redox cycling, generating superoxide anions and other ROI. ROI have been shown to accelerate neutrophil apoptosis (23, 31). Production of hydrogen peroxide was measured by flow cytometry, using DCF (20). Pyocyanin treatment of neutrophils was found to induce rapid ROI generation, detectable within 5 min of pyocyanin addition (Fig. 5A) and continuing up to 5 h (Fig. 5C). ROI production was comparable with that observed with FMLP, although, in contrast to pyocyanin, ROI production had returned to background levels 60 min after FMLP treatment (Fig. 5B). Pyocyanin caused concentration-dependent ROI generation (Fig. 5D), at concentrations of pyocyanin that also accelerate apoptosis (Fig. 1B).

Two different antioxidant molecules were used with the aim of inhibiting pyocyanin-induced neutrophil apoptosis: DPI, an inhibitor both of mitochondrial ROS generation and of NADPH oxidase (32), and NAC, which protects cells from a reduction in glutathione levels following oxidative stress (33). Neutrophils were preincubated for 1 h with medium alone, DPI (10 μM), and/or NAC (500 μg/ml) before addition of pyocyanin (34). These antioxidant concentrations were predicted to be effective free radical scavengers from previous studies of neutrophil apoptosis (23, 35). Neither DPI (10 μM) nor NAC (500 μg/ml) altered the rate of constitutive neutrophil apoptosis at 5 h (data not shown). Both NAC and DPI significantly inhibited pyocyanin-induced apoptosis.

**FIGURE 3.** Pyocyanin induces neutrophil apoptosis in the presence of LPS. Neutrophils were cultured with and without pyocyanin (100 μM) in the absence (■) or presence (□) of LPS (100 ng/ml), a concentration known to retard constitutive neutrophil apoptosis. Percentage of apoptosis (mean ± SE of duplicate measurements from three experiments) is shown. After 5 h in culture, LPS inhibited constitutive apoptosis, but was without effect upon pyocyanin-induced cell death. *, Statistically significant difference (p < 0.05) between means of control and LPS-treated populations.

**FIGURE 4.** Effects of wild-type and phenazine-deleted strains of *P. aeruginosa* upon neutrophil apoptosis. Filtered supernatants were prepared from a wild-type strain of *P. aeruginosa*, PA14 (■), and a phenazine-deficient strain, ΔphnAB (□), as described in Materials and Methods. Supernatants were cultured with neutrophils for 5 h, and apoptosis was assessed. Data from three independent experiments are expressed as mean ± SE of the fold increase in apoptosis in cells cultured with bacterial supernatant as compared with supernatant from sterile plate. *, Significant difference in proapoptotic effect between the two culture supernatants (p < 0.05).
Role of ROI in pyocyanin-induced neutrophil apoptosis. Neutrophils were pretreated for 1 h with medium alone (■), DPI (10 μM; □), NAC (500 μg/ml; ◊), or both antioxidants (□) before addition of pyocyanin (50 μM) or medium. Data from three experiments are expressed as mean ± SE of percentage of cells that were apoptotic. Apoptosis was measured by annexin V staining of peripheral blood neutrophils from healthy volunteers (45.2 ± 9.1% in cells treated with pyocyanin compared with 46.2 ± 9.9% in cells treated with medium alone; *p < 0.05). Neutrophils treated with pyocyanin alone (100 μM), or with pyocyanin and dbcAMP (10 or 100 μM) for 5 h, dbcAMP (100 μM) was able to protect against pyocyanin-induced apoptosis: in pyocyanin-treated populations, apoptosis at 5 h was 40.8 ± 14.1%, compared with 18.6 ± 8.2% in populations cultured with pyocyanin and 100 μM dbcAMP (p < 0.05; Fig. 6A). Intracellular concentrations of cAMP were measured in neutrophils, seeking evidence of a fall in [cAMP], preceding the onset of apoptosis in pyocyanin-treated cells. Neutrophils were incubated with representative doses of pyocyanin for 3 h, resulting in a concentration-dependent reduction in [cAMP], (Fig. 6B). Finally, time course experiments showed that the fall in neutrophil [cAMP], following treatment with 100 μM pyocyanin was not significant at 30 min, 1 h, or 2 h after pyocyanin treatment, but had fallen by 3 h. The reduction in [cAMP], thus occurs later than ROI generation following pyocyanin treatment of neutrophils. Cells treated with 2 μM salbutamol were used as a positive control in these experiments and showed a significant rise in [cAMP], within 30 min, together with an inhibitory effect upon pyocyanin-induced apoptosis, presumably due to the elevation of [cAMP], (data not shown).

Discussion

In these studies, we showed that pyocyanin, the major phenazine exotoxin produced by P. aeruginosa, rapidly induced high levels of apoptosis in peripheral blood neutrophils from healthy volunteers. 1-HP, a pyocyanin degradation product, was without effect upon neutrophil apoptosis. The proapoptotic effect of pyocyanin is far greater than, for example, that achieved by ligation of the death receptor, Fas (12), and neutrophils showed far greater sensitivity to pyocyanin than did macrophages or epithelial cells. Induction of neutrophil apoptosis by pyocyanin occurred in the presence of concentrations of LPS known to inhibit constitutive neutrophil apoptosis (4, 24) and did not differ in neutrophils from cystic fibrosis patients compared with healthy controls. That the cell death observed was apoptotic was evidenced by characteristic light microscopic features of apoptosis (1) and by binding of annexin V, indicative of PS exposure on the cell surface (18). Where effects upon apoptosis were compared for supernatants from wild-type P. aeruginosa and a strain deficient in phenazine production, there...
was a highly significant reduction in proapoptotic effect with the phenazine-deficient strain. These results suggest first that the net effect of phenazine exotoxins as group is proapoptotic to human neutrophils and, second, that the proapoptotic effect of *P. aeruginosa* supernatants is largely attributable to phenazine production.

Pyocyanin was found to induce neutrophil apoptosis at concentrations that have been reported in sputum samples of patients with cystic fibrosis or bronchiectasis. Studies by Wilson et al. (9) demonstrated the presence of detectable pyocyanin in 9 of 13 patients known to be colonized with *P. aeruginosa*, at concentrations ranging from 1 to >100 μM. Sputum from four of the patients had pyocyanin concentrations >10 μM, at which induction of neutrophil apoptosis would be predicted from our studies. The patients studied were colonized by *P. aeruginosa*, and it is likely that pyocyanin concentrations could increase further during infective exacerbations.

The molecular mechanisms underpinning *P. aeruginosa* virulence have been the subject of recent studies. In two experimental models of *P. aeruginosa* killing, the nematode *C. elegans* and murine septicemia, phenazine pigments secreted by *P. aeruginosa* were found to be important mediators of death of these organisms (10). Evidence from the *C. elegans* model showed that so-called “fast killing” of these nematode worms by *P. aeruginosa*, leading to death of the organism in 4–24 h, is largely mediated by the production of phenazines. *C. elegans* mutants with altered sensitivity to oxidative stress showed some resistance to fast killing, implying a role for oxidative stress in phenazine-induced death (10). Hassan and Fridovich (39) had previously described a mechanism of pyocyanin toxicity, whereby electron flow from biological pathways is diverted to increase intracellular oxygen radical generation, resulting in cell death. Under aerobic conditions in vitro, pyocyanin treatment results in the formation of the reactive oxygen species superoxide and hydrogen peroxide (40, 41). *P. aeruginosa* is itself insensitive to pyocyanin, and thus escapes free radical injury (42). We found that pyocyanin induces rapid ROI production in resting neutrophils, detectable within 5 min, that persists over a 5-h period in culture. ROI generation is observed at concentrations of pyocyanin that also accelerate apoptosis. Pyocyanin enhances superoxide generation by both resting and FMLP-stimulated neutrophils (14, 43), at concentrations in the range 12.5–25 μM. In studies of superoxide generation by contact-activated neutrophils, the duration of superoxide production was prolonged at 20 min by addition of pyocyanin, in keeping with our results (44). Pyocyanin-induced ROI generation is believed to be independent of host cell NADPH oxidases (41). We were able to study a single patient with chronic granulomatous disease, lacking a functional NADPH oxidase complex, and demonstrate that pyocyanin-induced death occurred at equivalent rates to those of control neutrophils (R. A. Lawson and M. K. B. Whyte, unpublished observations).

Oxidative stress has previously been implicated in neutrophil apoptosis, with ROI implicated in death of PMA-activated neutrophils (23) and in Fas-mediated apoptosis (45). In addition, constitutive neutrophil apoptosis is delayed under hypoxic conditions (46) and in neutrophils from chronic granulomatous disease patients (45). ROI production is not, however, inevitably linked to acceleration of apoptosis. Certain stimuli, e.g., FMLP, are known to generate significant ROI production by neutrophils (3), but have no significant effect upon apoptosis (4, 24). Recent studies suggest this may depend upon whether ROI production is predominantly intracellular or extracellular, with the latter having little effect upon apoptosis (47). Our experiments show that pyocyanin induces intracellular ROI production, but also that this persists over a period of hours, exposing the cell to sustained oxidative stress. These observations, together with the observed inhibition of pyocyanin-induced death by the antioxidants DPI and NAC, suggest that ROI production is implicated in the acceleration of neutrophil apoptosis.

Pyocyanin treatment of respiratory epithelial cells results in depression of intracellular levels of the adenine nucleotides, cAMP and ATP, at concentrations (50–200 μM) causing impairment of cAMP synthesis (36). Ciliary beat slowing in the presence of pyocyanin was prevented by elevation of cAMP levels either indirectly, using forskolin or 3-isobutyl-1-methylxanthine, or directly using dbcAMP (36). We investigated whether pyocyanin treatment of human neutrophils reduced [cAMP], and whether induction of neutrophil apoptosis by pyocyanin could be prevented by pharmacological elevation of [cAMP]. We confirmed that pyocyanin, at concentrations that are proapoptotic to neutrophils, causes a significant reduction in [cAMP], levels that precede the morphological appearances of apoptosis, but occurs later than ROI generation. Treatment of neutrophils with a synthetic cAMP analogue, dbcAMP, was able to inhibit pyocyanin-induced apoptosis. Other studies of treatment of neutrophils with cAMP analogues have shown delay of constitutive neutrophil apoptosis (37) and also of apoptosis induced either by ligation of the Fas death receptor or by treatment with cycloheximide (38). The mechanisms
by which cAMP elevation inhibits apoptosis are unclear, but include the downstream activation of type 1 cAMP-dependent protein kinases (38), and may also, in part, be due to suppression of ROI generation (48).

The paradigm of neutrophil killing of bacteria is based on the observation that bacterial products, as well as cytokines induced by them, delay apoptosis of neutrophils, extending their life span in tissues and increasing their destructive potential (8). When the bacteria are eradicated, the stimulus for prolonged neutrophil survival is removed and the inflammatory response desolves, with neutrophils dying by apoptosis. A number of pathogens, however, dysregulate this process and evade host defenses by premature induction of neutrophil apoptosis. Burkhodleria cepacia hemolysin, a pore-forming toxin, induces DNA fragmentation and apoptosis of neutrophils (49), and Escherichia coli also induces neutrophil apoptosis (50). Among viruses, both influenza A (51) and the respiratory syncytial virus (52) accelerate neutrophil apoptosis, with influenza A-mediated induction of apoptosis being associated with increased expression of Fas ligand (51).

P. aeruginosa is the major pathogen in cystic fibrosis lung disease and other forms of bronchiectasis; successful eradication of the organism is rare, and infection leads to progressive lung disease that is ultimately fatal (6, 7). It is also an increasingly prevalent opportunistic microorganism. P. aeruginosa has evolved a number of virulence mechanisms to evade uptake by host phagocytes, predominantly neutrophils, including inhibition of mucociliary clearance and alterations to ion transport within the respiratory epithelium (reviewed in Ref. 53). P. aeruginosa produces a number of phenazine pigments and, of these, pyocyanin is generated in the largest quantities. Phenazines are responsible for the blue-green color of the sputum that is characteristic of infected patients (54). Pyocyanin release is not the only mechanism by which P. aeruginosa can induce death of human neutrophils. Dachex et al. (55) recently reported that a clinical strain of P. aeruginosa, CHA, is able to induce a form of necrosis in human neutrophils via type III secretion-dependent mechanisms. Nonetheless, the induction of apoptosis by pyocyanin is remarkable both for the rapidity and extent of apoptosis and because this soluble and highly diffusible toxin is capable of having widespread effects on inflammatory neutrophil exudates (7).

Neutrophil apoptosis is classically regarded as a form of cell death that will promote resolution of inflammation (8). Apoptosis impairs proinflammatory neutrophil functions (3) and leads to clearance by macrophages (1), with ingestion of apoptotic neutrophils triggering macrophage production of antiinflammatory cytokines (56) and suppressing generation of proinflammatory mediators (57). Since apoptotic death is not proinflammatory, induction of death by apoptosis rather than necrosis could confer further advantage to an invading pathogen, limiting further host responses. There is, however, evidence of extensive tissue injury in the context of pseudomonal infection (7). This might arise because of proinflammatory effects either of pyocyanin itself (e.g., generation of ROI) or of other pseudomonal cytotoxins, such as proteases, exotoxins, and LPS (7). There is also evidence, however, that excessive apoptosis may be detrimental (58). Where there is very extensive neutrophil apoptosis, the clearance of these cells by macrophages and other phagocytes may be overwhelmed, leading to secondary necrosis and thus to inappropriate release of proinflammatory neutrophil contents (1, 8). In addition, we have shown that higher concentrations of pyocyanin, which could exist locally in the lung (9), are able to induce necrotic death of neutrophils, suggesting this may also contribute to tissue damage. Studies of the effects of pyocyanin upon neutrophil life span and death in a model of pulmonary inflammation will attempt to dissect the relative importance of these mechanisms in vivo.

In summary, our data demonstrate that the P. aeruginosa pigment, pyocyanin, induces apoptosis of human peripheral blood neutrophils via generation of ROS and lowering of [cAMP], These effects occur at clinically relevant concentrations of pyocyanin. Induction of neutrophil apoptosis may thus be another powerful weapon in the armory of host defense evasion strategies employed by this organism. Further understanding of the cellular mechanisms of pyocyanin-induced death may give rise to therapeutic strategies aimed at preserving host responses to this serious infection.

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References


