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Pyocyanin Production by *Pseudomonas aeruginosa* Induces Neutrophil Apoptosis and Impairs Neutrophil-Mediated Host Defenses In Vivo¹

Lucy Allen,* David H. Dockrell,† Theresa Pattery,§ Daniel G. Lee,¶ Pierre Cornelis,§ Paul G. Hellewell,* and Moira K. B. Whyte^{2‡}

Clearance of neutrophils from inflamed sites is critical for resolution of inflammation, but pathogen-driven neutrophil apoptosis can impair host defenses. We previously showed that pyocyanin, a phenazine toxic metabolite produced by *Pseudomonas aeruginosa*, accelerates neutrophil apoptosis in vitro. We compared wild-type and pyocyanin-deficient strains of *P. aeruginosa* in a murine model of acute pneumonia. Intratracheal instillation of either strain of *P. aeruginosa* caused a rapid increase in bronchoalveolar lavage neutrophil counts up to 18 h after infection. In wild-type infection, neutrophil numbers then declined steadily, whereas neutrophil numbers increased up to 48 h in mice infected with pyocyanin-deficient *P. aeruginosa*. In keeping with these differences, pyocyanin production was associated with reduced bacterial clearance from the lungs. Neutrophil apoptosis was increased in mice infected with wild-type compared with the phenazine-deficient strain or two further strains that lack pyocyanin production, but produce other phenazines. Concentrations of potent neutrophil chemokines (MIP-2, KC) and cytokines (IL-6, IL-1 β) were significantly lower in wild-type compared with phenazine-deficient strain-infected mice at 18 h. We conclude that pyocyanin production by *P. aeruginosa* suppresses the acute inflammatory response by pathogen-driven acceleration of neutrophil apoptosis and by reducing local inflammation, and that this is advantageous for bacterial survival. *The Journal of Immunology*, 2005, 174: 3643–3649.

Resolution of acute inflammation involves the programmed cell death (apoptosis) of invading inflammatory cells, predominantly neutrophils. There is evidence for active regulation of neutrophil life span and apoptosis at inflamed sites by host factors such as cytokines (1, 2), and also that genetic modification of key regulators of apoptosis may alter neutrophilic inflammation (3, 4). There is limited information, however, on the effects upon host defenses of alterations in the timing and extent of neutrophil apoptosis in vivo (4).

A number of pathogens induce inappropriate or premature apoptosis of host immune cells, particularly macrophages, depleting cell numbers and function and thus impairing host defense and favoring bacterial persistence (5). Induction of neutrophil apoptosis by bacterial pathogens has been less frequently described (5). *Pseudomonas aeruginosa*, an opportunistic pathogen causing pneumonia and often fatal infections in susceptible patient populations (6), has evolved a number of immunoevasive strategies by which bacterial factors affect host immunity (7). These include secretion of factors that impair neutrophil phagocytosis and acti-

vation (7) and also type III secretion system-dependent cytotoxicity (8). Importantly, *P. aeruginosa* generates highly diffusible pigmented toxic secondary metabolites, known as phenazines, that are critical for *P. aeruginosa* killing of *Caenorhabditis elegans* and of mice in septicemia models (9). We showed that the predominant phenazine pigment, pyocyanin, induces rapid apoptosis of human neutrophils, with a 10-fold acceleration of constitutive neutrophil apoptosis in vitro, but no apoptosis of epithelial cells or macrophages (10). Although pyocyanin is the major phenazine secreted by *P. aeruginosa*, 1-hydroxyphenazine and phenazine-1-carboxylic acid are also released (11). However, studies with a phenazine-deficient mutant confirmed the phenazine group of exotoxins has a net proapoptotic effect upon human neutrophils and showed the phenazine-deficient strain to have a significantly reduced proapoptotic effect (10). The in vitro effects of pyocyanin are observed at concentrations reported in sputum obtained from patients chronically colonized with *P. aeruginosa* (12).

We hypothesized that *P. aeruginosa* infection would provide an in vivo model of pathogen-driven neutrophil apoptosis and, specifically, that phenazine production, and in particular pyocyanin, is a critical determinant of outcome of *P. aeruginosa* infection of the lung. We established a murine model of acute *P. aeruginosa* infection to examine effects of wild-type, phenazine-deficient, and pyocyanin-deficient strains upon inflammatory cell numbers and apoptosis, bacterial clearance, and generation of chemokines and cytokines, and thus to probe the role of neutrophil apoptosis in regulation of host defense.

Materials and Methods

Bacteria

Two strains of *P. aeruginosa*, wild-type (PA14) and a phenazine-deficient but otherwise genetically identical strain (Δ *phnAB*), were the kind gift of F. Ausubel (Massachusetts General Hospital, Boston, MA) and were

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described previously (9). The $\Delta phnAB$ strain is deficient in the anthranilate-synthase complex, a key step in the production of the *Pseudomonas* quinolone signal molecule, which is necessary to induce phenazine synthesis (13, 14), and has pyocyanin production $\sim 10\%$ of the wild type (9). Two further strains, *phzM* and *phzS*, again isogenic mutants of PA14, are deficient in the *phzM* and *phzS* genes, respectively, and thus do not produce any pyocyanin (15, 16). All four bacterial strains showed growth curves with identical kinetics, with no alteration in time to mid log phase growth (data not shown). Bacteria were stored at -70°C , and frozen aliquots were thawed and grown to mid log phase in Luria-Bertani broth (Oxoid) with 10% FCS (Autogen Bioclear). The concentration of bacteria in mid log phase growth was determined by measurement of absorbance at 600 nm compared with a standard absorbance curve based on known numbers of CFU. Bacteria were washed twice in PBS, and 1×10^7 CFU were resuspended in 30 μl of PBS. The PA14 wild-type strain retained phenazine production at all time points. Bacteria in mid log phase were used for infection because of previous reports that *P. aeruginosa* was most effective in inducing epithelial cell apoptosis in vivo when instilled in this growth phase (17).

Mice

Specific pathogen-free 8- to 12-wk-old C57BL6 mice (Harlan) weighing 25–30 g were used in all experiments. All experiments were in accordance with the Home Office Animal (Scientific Procedures) Act 1986 and following ethical review by the Animal Care and Welfare Committee of the University of Sheffield.

Instillation of bacteria

Mice were anesthetized with an i.p. injection of a mixture containing 200 mg/kg ketamine hydrochloride (Willows Francis Veterinary), 10 mg/kg xylazine hydrochloride (Bayer), and 0.02 mg/kg atropine sulfate (Phoenix Pharmaceuticals). Intratracheal (i.t.)³ instillation of 30 μl solutions containing bacteria was performed, as previously described (18). Direct instillation of bacteria into the lung via the endotracheal route has previously been associated with delivery of $>95\%$ of the infecting inoculum into the lung (19).

Bronchoalveolar lavage

Mice were killed by overdose of i.p. sodium pentobarbitone (Loveridge). Bronchoalveolar lavage (BAL) was performed, as described, using four 1.0-ml aliquots of 10 U/ml ice-cold heparinized saline (Leo Laboratories), which were then pooled (20). A total of 10 μl of BAL was diluted in 90 μl of 3% acetic acid for a hemocytometer total cell count. Cytospin preparations (Cytospin 3; Thermo Shandon) were made from each BAL sample (100 μl), stained with Diff-Quick (Merck), and assessed by blinded reviewers. Differential cell counts, including the proportion of neutrophils that were apoptotic, were then calculated using the fractions for each leukocyte population estimated by analysis of cytospin preparations. The remainder of the BAL fluid was centrifuged ($300 \times g$ for 6 min), supernatant was stored at -70°C , and the cell pellet was resuspended for flow cytometry.

Detection of apoptosis

Assessment of apoptosis by morphological criteria was as previously described (21), determining the proportion of cells with light microscopic features of apoptosis on duplicate cytospins stained by Diff-Quick (counting >300 cells per slide). Apoptosis was also assessed by flow cytometry, detecting externalization of phosphatidylserine, which correlates well with other features of apoptosis (10, 22), and costaining with To-Pro3 to distinguish late-apoptotic or necrotic cells by failure of the latter to exclude this vital dye (19). A total of 1×10^6 cells was washed, preincubated in 10 μl of binding buffer containing 10 μg of mouse IgG, washed, then resuspended in 100 μl of PBS containing 0.5 μg of FITC-1A8 or isotype control (BD Pharmingen), and incubated for 15 min at 4°C . Cells were then washed and incubated with a 1/20 dilution of annexin V-PE in annexin V-binding buffer (BD Pharmingen) for 30 min at 4°C . As a negative control, a parallel aliquot of cells was stained in the presence of 10 mM EDTA. Following a final wash, the cells were resuspended in 400 μl of binding buffer containing a 1/10,000 dilution of the vital dye To-Pro3 (Molecular Probes). Cells were analyzed on a dual-laser FACSCalibur flow cytometer (BD Pharmingen), using FL1 for 1A8, FL2 for annexin V-PE, and FL4 for To-Pro3. Ten thousand events were recorded and analyzed using CellQuest software (BD Pharmingen). The neutrophil population was identified as 1A8-positive, early apoptotic cells as annexin V-positive/To-Pro3 negative, and late apoptotic cells as annexin V-positive/To-Pro3-positive. These two populations were combined to give the total number of apoptotic cells.

Quantitative bacteriology

The lungs were isolated and removed using sterile technique, and then homogenized in 1 ml of sterile PBS. Homogenate was inoculated on replicate blood agar plates, and bacterial numbers in lungs were determined by the surface viable count method (19). Bacteremia was similarly assessed, inoculating peripheral blood obtained from cardiac puncture.

ELISA

KC, MIP-2, and IL-6 in BAL fluid were measured by Duoset ELISA Development System Kit (R&D Systems) with a limit of detection of 15 pg/ml. Levels of processed IL-1 β present in BAL fluid were determined using an IL-1 β ELISA kit (Dr. S. Poole, National Institute for Biological Standards and Control, Potter's Bar, U.K.) with a limit of detection of 10 pg/ml mature IL-1 β .

Statistics

Parametric data were analyzed for statistical variance using a two-way ANOVA, followed by Bonferroni's posttest for multiple comparisons. Nonparametric data were assessed using the Mann-Whitney *U* test. Results were considered significant if $p < 0.05$.

Results

Infection with wild-type *P. aeruginosa* results in reduced neutrophilic inflammation compared with the phenazine-deficient strain

To determine the in vivo effect of phenazine production on neutrophilic inflammation in the lung, we studied an acute *P. aeruginosa* infection model similar to previous studies (23, 24). Either PA14 or $\Delta phnAB$ *P. aeruginosa* was instilled i.t. (time 0 h), then BAL was performed at time points up to 72 h following instillation, and total and differential cell counts were obtained. As previously described (23, 24), *P. aeruginosa* induced rapid migration of inflammatory cells into the lung (Fig. 1A). No differences between the two strains could be detected at 4 or 18 h following instillation. At 30 and 48 h after instillation, there were significantly more cells in the group infected with the phenazine-deficient strain, but in both groups the acute inflammatory response was resolving by 72 h. The differences in cell counts were due to increased neutrophils in mice receiving the phenazine-deficient strain (Fig. 1B). For comparison, neutrophil numbers in control mice, lavaged with PBS, are typically $<0.3 \times 10^5$ at all time points (4). There were no significant differences in macrophage numbers (Fig. 1C).

Clearance of wild-type *P. aeruginosa* is less efficient than clearance of the phenazine-deficient strain

Bacteria of both strains were detected in lung homogenates at time points up to 72 h following instillation. The majority of mice (80%) had cleared the phenazine-deficient strain at 72 h, whereas wild-type bacteria could still be isolated from 57% of mice 72 h after infection (Fig. 2A). No bacteremia was detected with either strain at 48 h. There was a more rapid reduction in numbers of phenazine-deficient bacteria that was significant at 48 h ($p < 0.05$), but not at 72 h, in which there was an outlier sample (Fig. 2B).

Phenazine-producing *P. aeruginosa* is associated with accelerated neutrophil apoptosis

We hypothesized that promotion of neutrophil apoptosis by the phenazine-producing strain could contribute to the observed difference in numbers of inflammatory neutrophils between the two strains of *P. aeruginosa*. Neutrophil apoptosis in BAL was assessed by two methods, morphology (4) and annexin V/To-Pro3 staining and flow cytometry (19). Morphologic assessment (Fig. 3A) showed infection with the wild-type strain was associated with increased numbers of apoptotic neutrophils at 18 h (Fig. 3B), and flow cytometry also showed a significant increase

³ Abbreviations used in this paper: i.t., intratracheal; BAL, bronchoalveolar lavage.

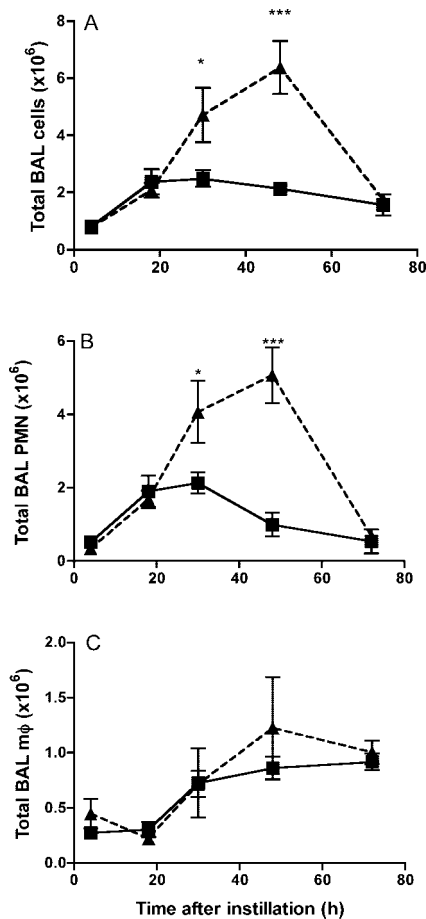


FIGURE 1. Phenazine production by *P. aeruginosa* reduces inflammatory cell accumulation in the lungs. Total and differential cell counts from cytospin preparations of BAL at 4, 18, 30, 48, and 72 h following infection with wild-type (solid lines) or phenazine-deficient (dotted lines) strains of *P. aeruginosa*, as described in *Materials and Methods* (mean \pm SEM of 7–9 mice at each time point). **A**, Total cell counts increased following infection with each strain, but cell numbers were significantly greater at 30 h ($p < 0.05$) and 48 h ($p < 0.001$) in the phenazine-deficient strain. **B**, Total neutrophil counts were significantly different between wild-type and phenazine-deficient strains at 30 h ($p < 0.05$) and 48 h ($p < 0.001$). **C**, Total macrophage counts increased following infection, but with no significant differences between the two strains.

at 18 h (Fig. 3, *C* and *D*). The total load of apoptotic neutrophils was also significantly greater as compared with mice infected with the phenazine-deficient strain (Fig. 3*E*). There was good correlation of morphologic and annexin V-binding results, as in previous studies (25, 26).

Because the Δ *phnAB* strain of *P. aeruginosa* still retains 10% of wild-type pyocyanin production in vitro, we also examined two further strains of *P. aeruginosa*, *phzM* and *phzS*, that are completely deficient in pyocyanin production (15). We showed that, at 18 h when induction of neutrophil apoptosis was maximal in the wild-type infection, there was a significant reduction in neutrophil apoptosis in mice infected with either the *phzM* or *phzS* strains, detected by both morphology (Fig. 4*A*) and flow cytometry (Fig. 4*B*).

Wild-type P. aeruginosa induces reduced chemokine and cytokine production in the lung

Phenazine production by *P. aeruginosa* might reduce pulmonary neutrophilia not only by acceleration of apoptosis, but also by reducing recruitment to the lungs. A number of chemokines and

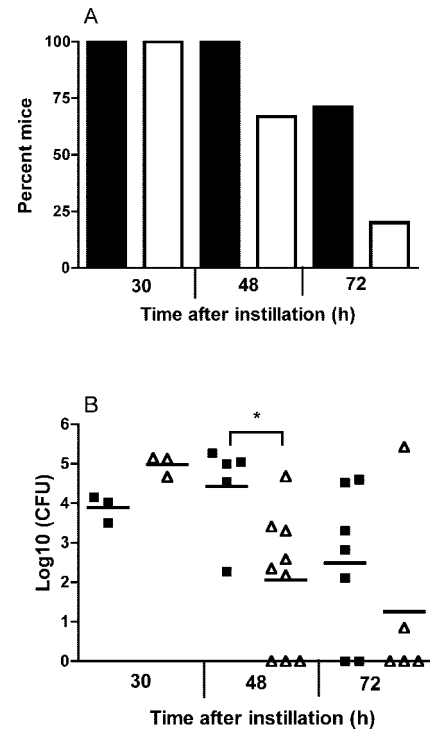


FIGURE 2. Microbiologic outcome of infection with wild-type or phenazine-deficient strains of *P. aeruginosa*. **A**, The percentage of mice with detectable bacteria in the lung following i.t. instillation of wild-type (■) compared with phenazine-deficient *P. aeruginosa* ($n = 5-9$ in each group) was the same at 30 h. Thereafter, a greater proportion of mice infected with the phenazine-deficient strain had successfully cleared the infection. **B**, Bacteria in lung homogenates were measured at various time points following i.t. instillation of wild-type (squares) or phenazine-deficient (triangles) strains of *P. aeruginosa*. At 48 h, numbers of bacterial CFU were significantly lower in mice infected with the phenazine-deficient rather than the wild-type *P. aeruginosa* strain ($p < 0.05$) (mean \pm SEM, $n = 5-9$). This trend was also clearly seen at 72 h, but did not reach statistical significance.

cytokines critical for neutrophil recruitment and activation were therefore measured in BAL. KC and MIP-2, structural and functional homologues of human IL-8 (27) and Gro- α (28), are major neutrophil chemokines in mice. Infection by both strains of *P. aeruginosa* elevated BAL concentrations of KC and MIP-2 at 4 h, but was more prolonged in the phenazine-deficient strain (Fig. 5, *A* and *B*). IL-6 has a critical role in neutrophil recruitment to the lung via interaction with pulmonary endothelial cells, stimulation of local chemokine production, notably IL-8, and up-regulation of ICAM-1 on endothelial cells (29, 30). IL-6 levels were markedly increased by the wild-type strain at 4 h, but with the phenazine-deficient strain the peak concentration was not reached until 18 h (Fig. 5*C*). IL-1 β , although not essential for neutrophil recruitment to the lung (31), has a number of proinflammatory and antiapoptotic effects upon neutrophils (32). IL-1 β concentrations (Fig. 5*D*) were increased in mice infected with the phenazine-deficient, but not the wild-type strain at 18 h. Thus, in mice infected with the phenazine-deficient strain, there was a marked increase in neutrophil chemokines and cytokines at 18 h that preceded the rise in neutrophil numbers (Fig. 1*B*) that was not seen in mice infected by the wild-type strain.

Discussion

A central role for neutrophil apoptosis in resolution of inflammation is widely accepted, with neutrophil apoptosis a result of the

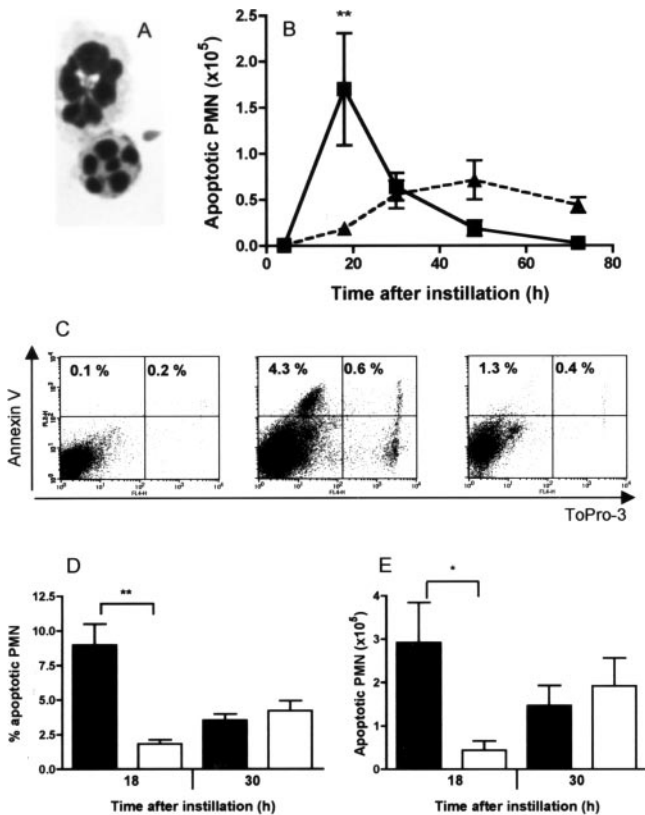


FIGURE 3. Neutrophil apoptosis is accelerated in mice infected with wild-type *P. aeruginosa* compared with the $\Delta phnAB$ strain. *A*, Photomicrograph of a BAL cytospin showing an apoptotic neutrophil (arrowed) and a nonapoptotic neutrophil. *B*, Neutrophil apoptosis was assessed by cytospin morphology (mean \pm SEM, $n = 5-7$) following instillation of wild-type (solid line) or phenazine-deficient (dotted line) strains of *P. aeruginosa*. *C*, Neutrophil apoptosis was also assessed by flow cytometry. Representative dot plots show annexin V and To-Pro3 staining 18 h after instillation of *P. aeruginosa* (from right to left) following EDTA pretreatment (negative control), wild-type, or phenazine-deficient strain instillation. Numbers of early apoptotic cells (annexin V⁺/ToPro3⁻) are shown in the upper left panel, and late apoptotic cells (annexin V⁺/ToPro3⁺) are shown in the upper right panel (*D*). The percentage of apoptotic neutrophils was significantly lower in the phenazine-deficient strain-infected mice at 18 h (**, $p < 0.01$) as were (*E*) the total numbers of apoptotic neutrophils (*, $p < 0.05$). At 4, 48, and 72 h, the numbers of cells were too few for reliable estimation of apoptosis by flow cytometry.

defervescence of antiapoptotic bacterial factors and host cytokines that follows successful bacterial eradication (1, 2). This process may be subverted by pathogens, with premature induction of apoptosis permitting evasion of host defenses (5). Chronic infection with *P. aeruginosa* is a major cause of pulmonary damage and mortality in patients with cystic fibrosis (33), and acute infection is frequently observed in ventilator-associated pneumonia, in which *P. aeruginosa* pneumonia is associated with much higher mortality than other pathogens (34, 35). Production of phenazines is largely confined to *Pseudomonas* and *Streptomyces* spp. and *Burkholderia cepacia* (36). *P. aeruginosa*, however, is the only organism known to produce the specific phenazine pyocyanin (36, 37) that we have previously shown to accelerate neutrophil apoptosis in vitro (10). We therefore studied *P. aeruginosa* lung infection both as a model in which to study pathogen-driven neutrophil apoptosis and also to determine whether phenazine production plays a critical role in outcome of infection in vivo.

Mice infected with wild-type *P. aeruginosa* showed increased neutrophil numbers up to 18 h following infection; thereafter,

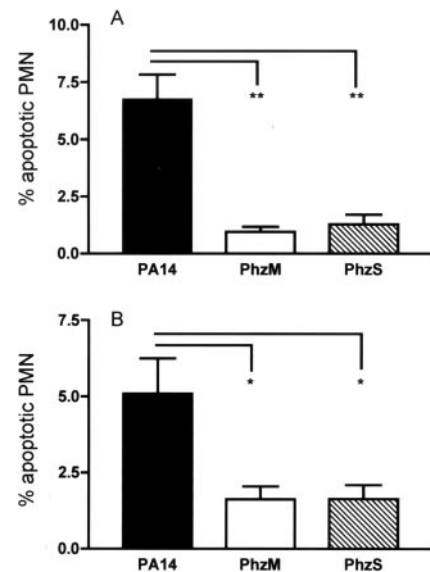


FIGURE 4. Neutrophil apoptosis is delayed in mice infected with two pyocyanin-deficient strains of *P. aeruginosa*. Neutrophil apoptosis was assessed (mean \pm SEM, $n = 5$) at 18 h following instillation of wild-type (■), *phzM* (□), or *phzS* (▨) strains of *P. aeruginosa*. *A*, Percentage of apoptosis by cytospin morphology was significantly reduced in the two pyocyanin-deficient strains (**, $p < 0.01$ in each case). *B*, Percentage of apoptosis by annexin V staining was also significantly reduced for these two strains (*, $p < 0.05$). Equivalent results were obtained for total numbers of apoptotic neutrophils by both morphology and flow cytometry.

numbers declined. In contrast, mice infected with a phenazine-deficient strain ($\Delta phnAB$) showed a continuing increase in neutrophil numbers up to 48 h after infection. The significance of these differences was emphasized by the observation that the greater neutrophil numbers in mice infected with phenazine-deficient strains were associated with a more rapid clearance of bacteria from the lungs. A less virulent strain would be anticipated to be more rapidly cleared, but the fact it was associated with greater neutrophil numbers from 18 to 48 h reflects the significant role that neutrophils play during this period in aiding bacterial clearance, and that this important host response is inhibited by bacterial phenazine production. The difference in neutrophil numbers was, at least in part, due to acceleration of neutrophil apoptosis by phenazines, with mice infected with wild-type *P. aeruginosa* having increased numbers of apoptotic cells at 18 h. Mice infected with the phenazine-deficient strain showed lower levels of neutrophil apoptosis at 18 h, concomitant with the continued rise in total neutrophil numbers up to 48 h. At 48 and 72 h after infection, there were higher levels of neutrophil apoptosis in the $\Delta phnAB$ -infected mice, although the differences were not statistically significant. This is explained by neutrophil apoptosis not being pathogen driven in these mice, but instead occurring at later time points, following bacterial clearance, and permitting resolution of the inflammatory response. Because the $\Delta phnAB$ strain of *P. aeruginosa* produces $\sim 10\%$ of the pyocyanin of the wild-type strain (9), we confirmed the delay of neutrophil apoptosis in mice infected with two further strains of *P. aeruginosa* that produce no pyocyanin (10, 15, 16). Annexin V staining produced consistently higher values for percentage of neutrophil apoptosis than morphologic counts, in keeping with previous studies of human BAL (38) and with annexin V binding being an earlier marker of apoptosis (39).

These studies provide important in vivo evidence that delayed clearance of bacteria is associated with accelerated neutrophil apoptosis in mice infected with the wild-type strain of *P. aeruginosa*,

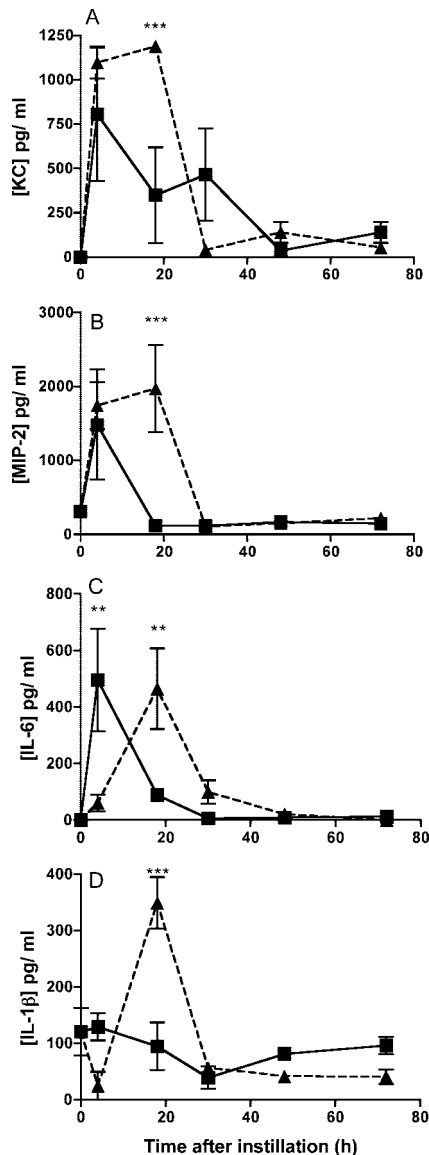


FIGURE 5. Proinflammatory chemokine and cytokine levels in BAL of mice infected with wild-type and phenazine-deficient strains of *P. aeruginosa*. Values represent the mean \pm SEM of the concentration (pg/ml) of A, KC; B, MIP-2; C, IL-6; and D, IL-1 β in BAL fluid 4, 18, 30, 48, and 72 h after infection. Either three or four mice were studied at each time point shown. All of the chemokines and cytokines were detectable by 4 h following infection. At 18 h, however, there were significantly greater levels of all four cytokines in mice infected with the phenazine-deficient strain (**, $p < 0.01$ for KC and IL-6, and ***, $p < 0.001$ for MIP-2 and IL-1 β).

supporting the concept that pyocyanin-induced apoptosis of neutrophils benefits *P. aeruginosa*, rather than being a mechanism of host defense. Studies by Lau et al. (40) have demonstrated that *phzM* and *phzS* strains of *P. aeruginosa* are cleared more rapidly from the lungs in a similar murine pneumonia model, confirming the role of pyocyanin as a bacterial virulence factor and adding to the previous observation that the Δ *phnAB* strain causes reduced mortality in a sepsis model (9). The *phzM* and *phzS* mutants do not have reduced production of phenazine precursors of pyocyanin (41). The same phenotype of accelerated neutrophil apoptosis was shared by all three mutant strains, and it is therefore pyocyanin that has a key role in accelerating neutrophil death, supporting our previous in vitro observations (10).

Acceleration of apoptosis by pyocyanin appears to be relatively specific for neutrophils, with no acceleration of monocyte-derived macrophages or epithelial cells in vitro (10). The mechanisms of this neutrophil specificity are the subject of ongoing studies. Pyocyanin-induced neutrophil apoptosis is, however, associated with production of reactive oxygen intermediates (10) that may have a specific role in induction of neutrophil apoptosis (26, 42), and a central role for oxidative stress in pyocyanin-induced killing was identified by Mahajan-Miklos et al. (9). Recent work by Ran et al. (43), in which a yeast library was screened for mutants with altered pyocyanin susceptibility, isolated a number of mutations in the vacuolar ATPase, an enzyme that is inactivated by region of interest, further identifying a possible mechanism of pyocyanin-induced cell death.

P. aeruginosa infection also induces epithelial cell apoptosis via Fas-Fas ligand interactions on the epithelial surface as a mechanism of host protection (24). Phenazines do not, however, induce significant apoptosis of pulmonary epithelial cells (10), and pyocyanin-induced neutrophil apoptosis is independent of Fas ligation (M. Whyte, unpublished findings). Phenazines exert other effects on pulmonary epithelial cells in vitro, including inhibition of ciliary beat frequency (44), which could in theory impair inflammatory cell clearance from the airway. It is unlikely, however, that this contributed to our findings because cell numbers lavaged from the lung were higher in the phenazine-deficient than in the wild-type strain, arguing against a phenazine-induced delay of neutrophil clearance.

KC and MIP-2 concentrations were increased at early time points following infection by either strain and, in mice infected with the phenazine-deficient strain, a further rise in levels preceded an increase in BAL neutrophil counts. KC and MIP-2 are major neutrophil chemoattractants (27, 28), and it is probable, therefore, that ongoing neutrophil recruitment contributed to the increased neutrophil numbers in BAL fluid with the phenazine-deficient strain. BAL fluid from the lungs of cystic fibrosis patients chronically colonized with *P. aeruginosa* contains high concentrations of the human KC counterpart, IL-8 (30). Both IL-8 (45) and Gro- α (46) also inhibit neutrophil apoptosis via CXCR2 receptors, although only at high concentrations. Chmiel et al. (23) failed to detect an increase in KC or MIP-2 following *P. aeruginosa* infection of wild-type mice, but the earliest time point examined was 48 h, when levels had also returned to baseline in our experiments. Previous studies have suggested phenazines enhance the production of chemokines by host cells in vitro, in contrast to our findings in vivo. Denning et al. (47) found pyocyanin increased IL-8 release from human airway epithelial cells in vitro, as did phenazine-1-carboxylic acid (11), while Lauredo et al. (48) showed pyocyanin, but not 1-hydroxyphenazine, caused a modest increase in IL-8 production by sheep alveolar macrophages in vitro, from 40 to 80 pg/ml. However, we observed greater increases in cytokine levels with the phenazine-deficient than with the wild-type strain of *P. aeruginosa*. The in vivo situation will reflect the contributions of a number of IL-8-producing cell types. For example, pulmonary epithelial cells are an important source of IL-8 in *P. aeruginosa* infection (49), yet also undergo *P. aeruginosa*-induced apoptosis (24). The accelerated neutrophil apoptosis in infection with the wild-type strain would also reduce the direct and indirect contributions of the neutrophil population to KC and MIP-2 production (50, 51). Overall, our findings show the phenazine-deficient strain induces greater KC and MIP-2 levels, suggesting that in the in vivo situation, in which many host and microbial features interact, phenazine production reduces induction of neutrophil chemoattractants, and thus neutrophil recruitment.

IL-1 β levels were elevated at 18 h in mice infected with the phenazine-deficient, but not the wild-type strain, and IL-1 β concentrations are also elevated in patients with chronic *P. aeruginosa* infection (52). IL-1 β is a major upstream activator of chemokine release (32), but neutrophil apoptosis in the lung was unaltered in IL-1 β -deficient compared with wild-type mice in models of acute lung injury (31). IL-1R1-deficient mice show both improved clearance of bacteria by 24 h and reduced neutrophil numbers in BAL (53), and elevated levels of IL-1 β were associated with sepsis in mice unable to generate ceramide-enriched lipid rafts (54). In our studies, however, enhanced levels of IL-1 β at 18 h were associated with subsequent bacterial clearance.

Measurements of IL-6 in BAL in our experiments reveal altered kinetics, with peak levels of IL-6 at 4 h in wild-type and 18 h in phenazine-deficient strain-infected mice. IL-6^{-/-} mice challenged with intrapulmonary LPS showed elevated MIP-2 levels and increased neutrophilic inflammation, implying a role for IL-6 in down-regulation of chemokines (55), although in a peritoneal inflammation model this was found not to be a direct effect of IL-6 (56). IL-6 is also proapoptotic for neutrophils, both in vitro (57) and in vivo (56), and plays an important role in resolution of neutrophilic inflammation (58). Overall, therefore, the early increase in IL-6 in mice infected with wild-type *P. aeruginosa* is likely to favor rapid neutrophil removal and possibly impair further neutrophil influx.

Our studies do not conclusively address the relative contributions of reduced apoptosis and increased recruitment to the increased numbers of neutrophils in mice infected with the phenazine-deficient strain. There is, however, clear evidence that both these processes contribute to increased cell numbers, with enhanced recruitment supported by higher levels of relevant chemokines in mice infected with the phenazine-deficient strain.

In conclusion, studies of an in vivo model of acute *P. aeruginosa* pulmonary infection have provided evidence of the key role of neutrophils in effective clearance of *P. aeruginosa* from the lung and demonstrate that phenazine toxic metabolites, specifically pyocyanin, impair neutrophilic host defenses by a number of distinct, but possibly interrelated mechanisms. Phenazine production leads to reduced chemokine and cytokine production. This results in reduced neutrophil numbers and accelerated neutrophil apoptosis, which are associated with impaired bacterial clearance. These processes are of clinical relevance, because they demonstrate defects in neutrophil host defense, including pathogen-driven neutrophil apoptosis, in a notoriously problematic and persistent bacterial infection.

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