Effect of Pyocyanine, a Pigment of *Pseudomonas aeruginosa*, on Production of Reactive Nitrogen Intermediates by Murine Alveolar Macrophages

JUDD SHELLITO, STEVE NELSON, AND RICARDO U. SORENSEN

Section of Pulmonary and Critical Care Medicine, Departments of Medicine and Pediatrics, Louisiana State University Medical Center, New Orleans, Louisiana 70112

Received 12 December 1991/Accepted 10 June 1992

In this study we investigated the effect of pyocyanine, a pigment produced by *Pseudomonas aeruginosa*, on production of reactive nitrogen intermediates by macrophages. We found that addition of pyocyanine to cultures of murine alveolar macrophages inhibited the capacity of these cells to produce reactive nitrogen intermediates (measured as nitrite) in a dose-dependent manner without altering cell viability, cytokine-induced Ia expression, or production of tumor necrosis factor.

Pyocyanine (PYO), a phenazine pigment released by most clinical strains of *Pseudomonas aeruginosa* (22), has important modulatory effects on cellular immune functions of the host. The present study was undertaken to characterize the effects of PYO on macrophage-derived reactive nitrogen intermediates (RNI). RNI are important in macrophage antimicrobial function directed against mycobacteria, fungi, protozoa, and some bacteria (12, 20). We chose to study macrophages from lung tissue because chronic pulmonary infections with *P. aeruginosa* are an important clinical problem, because PYO has been detected in the sputum of chronically infected patients (24), and because alveolar macrophages are important in host defense against *P. aeruginosa* infection (7, 13, 21).

Cells were lavaged from the lungs of virus-free BALB/c mice (Simonsen Laboratories, Gilroy, Calif.) as previously described (3). Lavaged cells were adjusted in RPMI 1640 with high glucose, 25 mM N-2-hydroxyethylpiperazone-N'-2-ethanesulfonic acid (HEPES), 100 µg of gentamicin per ml, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 5% heat-inactivated fetal bovine serum (all from GIBCO) to a concentration of 2.5 × 10⁵ cells per ml. Lavaged cells consisted of more than 95% alveolar macrophages and were more than 90% viable by trypsin blue exclusion.

PYO was prepared by photooxidation of phenazine methosulafate followed by sequential chloroform and aqueous extractions and crystallization from water as previously described (11). The purity of this compound was confirmed by UV-visible spectroscopy and thin-layer chromatography and was equivalent to PYO extracted from *P. aeruginosa* culture supernatants in terms of inhibition of lymphocyte proliferation (16).

Cells to be assayed for RNI were added to 96-well microtiter plates at 2.5 × 10⁵ cells per well in complete medium containing various concentrations of PYO. The cells were stimulated to release RNI with added murine recombinant gamma interferon (IFN-γ) (8 × 10⁶ U/mg; Genentech, South San Francisco, Calif.). Preliminary experiments indicated that a well concentration of 12.5 ng of IFN-γ per ml produced maximal release of RNI (data not shown). Control wells contained no added PYO or IFN-γ. The cells and added materials were cultured for 24 to 72 h at 37°C in 5% CO₂ in air. At termination of the cell culture, culture supernatants were aspirated from individual culture wells for RNI assay. In some experiments, cells were cultured with various concentrations of PYO for 24 h and the cell monolayers were washed three times with warmed complete medium to remove the added PYO. The cells were then cultured for an additional 24 to 72 h in medium with and without added IFN-γ before RNI assay.

To assay RNI as release of nitrite, we added 100-µl samples of culture supernatants to microtiter wells containing 200 µl of Greiss reagent (0.1% N-1-naphthylethylenediamine, 1.0% sulfanilamide, 5% phosphoric acid [all from GIBCO]), incubated the microtiter plate at room temperature for 10 min, and then measured the absorbance at 540 nm in a microplate reader. The concentration of nitrite in each sample was calculated by using sodium nitrite as a standard. The background nitrite concentrations in each medium were calculated and subtracted from the experimental values. Preliminary experiments established that unstimulated alveolar macrophages did not release detectable RNI after 24 to 72 h of culture and that PYO at the concentrations used for these studies did not interfere with the absorbance assay (data not shown).

Ia antigen was assayed by enzyme-linked immunosorbent assay (ELISA) as previously described (3) on alveolar macrophages cultured for 48 h with 12.5 ng of IFN-γ per ml in complete medium containing various concentrations of PYO. In the absence of IFN-γ, murine alveolar macrophages express little surface Ia (<0.2 absorbance unit per culture) (3). Tumor necrosis factor (TNF) was assayed by ELISA (Genzyme Corp., Cambridge, Mass.) in culture supernatants of cells incubated for 24 to 48 h with 2 µg of Escherichia coli lipopolysaccharide (GIBCO) per ml and various concentrations of PYO. The addition of PYO alone to cultures of alveolar macrophages did not induce Ia antigen expression or the release of detectable TNF (data not shown).

Alveolar macrophages stimulated with IFN-γ released RNI progressively for up to 72 h of in vitro culture (Fig. 1). When PYO was present in the cell cultures along with IFN-γ, concentrations of RNI in the culture supernatants were decreased (Fig. 1). Decreased RNI levels were seen as early as 24 h into the culture, but significant differences in

* Corresponding author.
comparison with cells cultured without PYO were not evident until 48 h and were also evident at 72 h. Significant inhibition was noted at a PYO concentration of 25 μM, with complete inhibition present at a concentration of 50 μM.

The effect of PYO on alveolar macrophage RNI persisted even after the PYO had been washed from the cultured cells (Fig. 2). In these experiments, alveolar macrophages were cultured with various amounts of PYO for 24 h and then washed to remove the pigment from the cells. Culture medium with and without IFN-γ was then added, and the cells were cultured for an additional 24 to 48 h. Decreased release was noted at 24 h of culture, but significant suppression was not observed until 48 h. Partial suppression was evident at 5 μM PYO, with complete suppression present at 50 μM.

PYO did not alter the viability of cultured alveolar macrophages. The percentage of cells excluding trypan blue exceeded 90% at both time points and at 0, 5, 25, and 50 μM PYO (data not shown). The addition of PYO to concentrations that inhibited IFN-γ-induced release of RNI had no effect on IFN-γ-induced expression of Ia determinants (Fig. 3). Similarly, the addition of 5 to 50 μM PYO to alveolar macrophages stimulated with 2 μg of lipopolysaccharide per ml had no effect on TNF release at either 24 or 48 h of culture (data not shown).

The results of these experiments indicate that PYO suppresses RNI in cultures of alveolar macrophages over a dose range from 5 to 50 μM. At the highest concentration of PYO, suppression of RNI is 100%. Sorensen et al. have previously measured concentrations of PYO as high as 30 μM in the sputum of patients infected with P. aeruginosa (17). Therefore, the concentrations of PYO used here are relevant to clinical infections with this pathogen.

The ability of P. aeruginosa to release these concentrations of PYO into infected tissue and to suppress production of RNI by alveolar macrophages may be a means of evading host defenses against infection. RNI have been recognized to be a component of cellular defense of mice against infection with Mycobacterium tuberculosis (5), Toxoplasma gondii (1), Leishmania major (10), and Cryptococcus neoformans (8). RNI have also been implicated in human macrophage inhibition of Mycobacterium avium (6) but not Cryptococcus neoformans (4). P. aeruginosa is susceptible to growth inhibition by nitric oxide in vitro (14), and the addition of P. aeruginosa organisms to macrophages in vitro stimulates the production of RNI (9). Collectively, these data suggest that alveolar macrophages might use RNI to inhibit the growth of P. aeruginosa and that suppression of RNI by PYO might contribute to the establishment of bacterial infection. However, the importance of RNI in the killing of P. aeruginosa by alveolar macrophages has yet to be established.

The results of these experiments further indicate that the suppressive effect of PYO on RNI release persists even after the PYO has been washed from the cells. Available data indicate that PYO modulates a number of cellular functions by altering cellular respiration, probably at the level of the mitochondria (2, 19). PYO has also been shown to directly inactivate nitric oxide, the precursor of RNI, in aqueous solution (23). We believe that direct inactivation of nitric oxide is unlikely in our washed cells, because previous experiments with malignant cell lines demonstrated that

**FIG. 1.** Effect of PYO on release of RNI by alveolar macrophages. Lavaged alveolar macrophages were incubated either alone or with various concentrations of PYO for 24, 48, and 72 h. All cultures were also treated with 12.5 ng of IFN-γ per ml to stimulate production of RNI. Culture supernatants were then aspirated and assayed for RNI as nitrite concentration. Each bar represents the mean and standard error of the mean for two to seven mice. Symbols: *), P < 0.01; †, P < 0.001 (in comparison with no added PYO).

**FIG. 2.** Release of RNI by alveolar macrophages after removal of extracellular PYO. Lavaged alveolar macrophages were incubated with various concentrations of PYO for 24 h. The cell monolayers were then washed three times with warmed medium and stimulated with 12.5 ng of IFN-γ per ml. RNI was assayed in the culture supernatants at 24 and 48 h after the addition of IFN-γ. Each bar represents the mean and standard error of the mean for two to seven mice. Symbols: *, P < 0.01; †, P < 0.001 (in comparison with no added PYO).

**FIG. 3.** Effect of PYO on alveolar macrophage expression of Ia determinants. Lavaged alveolar macrophages were incubated either alone or with various concentrations of PYO for 48 h. All cultures were also treated with 12.5 ng of IFN-γ per ml to induce Ia expression. The cell monolayers were then washed, fixed, and assayed for Ia by ELISA. Each bar represents the mean and standard error of the mean for four to six experiments, except for 5 and 25 μM PYO, for which n = 1. There are no significant differences between the measured values.
washing reduced intracellular concentrations of PYO below levels shown to inhibit RNI in this study (15). However, when PYO is continually present in the assay system (Fig. 1), direct inactivation of RNI may also occur. Additional studies are necessary to define the exact mechanism(s) by which PYO modulates macrophage RNI.

Finally, the results of these experiments indicate that PYO does not alter the viability of alveolar macrophages and does not interfere with their ability to synthesize Ia glycoproteins in response to IFN-γ or to release TNF in response to lipopolysaccharide. This means that PYO is not toxic to cells but influences cellular function in a selective manner, depending on both the specific function studied and the concentration of PYO. The lack of an effect of PYO on Ia induction suggests that the mechanism of its effect is not interference with extracellular IFN-γ or with the binding of IFN-γ to its receptor on the cell membrane. The ability of P. aeruginosa cells (through PYO) to selectively modulate macrophage function may allow the bacteria to suppress host antimicrobial functions (RNI production) without compromising production of tissue-destructive factors (i.e., TNF) which may promote persistence of infection (18).

This work was supported by Public Health Service grant HL29246 (to J.S.) and by grant Z0739 from the National Cystic Fibrosis Foundation (to S.N.).

We thank Warren Summer and Stanley Greenberg for helpful advice and review of the manuscript. We thank Gerald Lane for technical assistance.

REFERENCES


