In Vitro Effect of Synthetic Pyocyanine on Neutrophil Superoxide Production

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Received 28 July 1986/Accepted 17 November 1986

Pyocyanine, a low-molecular-weight phenazine pigment produced by Pseudomonas aeruginosa, has previously been shown to strongly inhibit human lymphocyte blastogenesis. We now report that synthetic pyocyanine can also affect the generation of superoxide by human peripheral blood polymorphonuclear leukocytes (PMNs) in a dose-dependent manner. Superoxide production by PMNs stimulated with phorbol myristate acetate (PMA) was measured in the presence and absence of pyocyanine, phenazine, and trifluoperazine, a phenothiazine of similar chemical structure to the phenazine pigments. Pyocyanine at 50 μM inhibited superoxide production to 28.9 ± 2.8% of PMA control values, whereas at the lower concentration of 1 μM, the production of superoxide was significantly enhanced (203 ± 31.7% of PMA control values). Phenazine, the tricyclic parent compound of pyocyanine, had only a minor effect. Trifluoperazine had a marked inhibitory effect on superoxide generation at concentrations above 1 μM. None of the compounds induced superoxide generation in the absence of PMA. Pyocyanine at all concentrations, unlike phenothiazines, had very little effect on the release of neutrophil granule enzymes. The effect of P. aeruginosa phenazine pigments on polymorphonuclear phagocytes is of significance, since inhibition of host PMN function at sites of infection could result in ineffective bacterial killing, whereas enhanced PMN function could lead to greater tissue damage. These two possibilities are not mutually exclusive and may coexist on local pyocyanine concentrations.

Pseudomonas aeruginosa secretes a number of toxins including phenazine pigments. The major phenazine pigment produced by approximately 50% of all P. aeruginosa clinical isolates is pyocyanine (1-hydroxy-5-methylphenazine), which has an intense blue color at neutral pH (13). The potential pathogenic role of pyocyanine is controversial (17, 18). Nevertheless, pyocyanine has been shown to alter cell function in several systems. Its antibiotic properties have been attributed to the nonenzymatic oxidation of NADH with the production of superoxide anion (10). In vitro pyocyanine inhibits human lymphocyte proliferation (26). In further pursuit of P. aeruginosa pathogenic mechanisms, we investigated the effect of pyocyanine on superoxide generation and lysosomal degranulation by polymorphonuclear leukocytes (PMNs). Since the phenazine tricyclic ring structure has similarities to phenothiazines, these studies were performed in parallel with trifluoperazine (TFP), a potent phenothiazine known to inhibit both of these neutrophil functions (8, 23). However, the activity of pyocyanine was dissimilar in that only superoxide production was inhibited, and at concentrations less than 5 μM, pyocyanine actually enhanced superoxide production by phorbol ester-activated neutrophils.

MATERIALS AND METHODS

Leukocyte isolation. Venous blood was obtained from normal adult volunteers in accordance with the guidelines established by the Institutional Review Board of University Hospitals of Cleveland. Peripheral blood mononuclear cells were removed from heparinized (2 U/ml) whole blood by standard Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) discontinuous gradient centrifugation (29). Mixed granulocyte populations were prepared from the resulting erythrocyte-granulocyte pellet by additional sedimentation on dextran. Equal volumes of cell pellet, Hanks balanced salt solution (HBSS) without calcium and magnesium (pH 7.4) (GIBCO Laboratories, Grand Island, N.Y.), and 4.5% dextran T-500 (Pharmacia Fine Chemicals) were gently mixed and allowed to settle. Granulocytes were harvested, and residual erythrocytes were removed by hypotonic lysis. Cells were washed three times in cold HBSS without calcium and magnesium and resuspended at a density of 2 × 10⁷ PMNs per ml. This cell population contained >95% PMNs by standard Wright stain procedure; viability was >95% as determined by acridine orange-ethidium bromide fluorescence microscopy (14).

Preparation and characterization of pyocyanine. Pyocyanine was prepared by the photooxidation of phenazine methosulfate followed by sequential chloroform and aqueous extractions and crystallization from water as described by Knight et al. (13). Pyocyanine obtained by this method comigrated with authentic pyocyanine in thin-layer chromatography. The dark blue crystals yielded a melting point of 132 to 133°C which agrees with reported values for pure pyocyanine (13, 22, 28). Acid and alkaline solutions gave the same UV-visible spectra as that reported for pyocyanine (32). A molar extinction coefficient of Em = 3.1 × 10⁴ at 310 nm was determined with freshly recrystallized pyocyanine in water at pH 7.0. Residual phenazine methosulfate contamination was less than 6% based on sulfur content by elemental analysis and on proton nuclear magnetic resonance analysis. This synthesized pyocyanine has been shown to be equivalent in its inhibition of lymphocyte proliferation to the pyocyanine extracted from P. aeruginosa culture supernatants (26).

Phenazine was purchased from Sigma Chemical Co. (St. Louis, Mo.), and carrier-free TFP (10-[3-(4-methylpipera-
zine-1-yl)-propyl-2-trifluoromethylphenothiazine) was a gift of Smith Kline & French Laboratories (Philadelphia, Pa.).

Continuous assay of superoxide generation. Measurement of superoxide-dependent cytochrome c reduction (25) was performed in a continuous recording Beckman double-beam spectrophotometer. Sample and reference cuvettes contained 10^6 PMNs and 1.2 ng of ferricytochrome c (Sigma) in HBSS containing calcium and magnesium (pH 7.4) (GIBCO) in a total volume of 1.0 ml. The reference cuvette in addition contained 10 µg of superoxide dismutase (Sigma). Measurement of the superoxide dismutase-inhibitable reduction of ferricytochrome c reflects the amount of superoxide generated by PMNs. The A550 was recorded continuously at 37°C for 2 to 5 min to obtain a preactivation base line, whereupon the activating agent was added to each cuvette and the response followed for 10 min. Control levels of superoxide generation in normal PMNs were induced by using optimal (1 µg/ml) and suboptimal (10 ng/ml) final concentrations of phorbol myristate acetate (PMA; Sigma) as the activating agent. The effect of various concentrations of phenazine pigments on superoxide generation was evaluated by observing the extent of each pigment in the presence or absence of PMA.

The rate of superoxide production was calculated by dividing the linear rate of the change of the A550 (∆A550) by the micromolar extinction coefficient for the reduction of ferricytochrome c (εΜ = 0.021) (20). Results are expressed as nanomoles of O_2^- generated per minute per 10^6 PMNs.

A cell-free superoxide-generating system was used to control for any interference of pyocyanin with the reduction of cytochrome c by acting as an acceptor for electrons from O_2^- . Continuous cell-free superoxide production was measured as described above by monitoring cuvettes containing acetaldehyde (2.0 mM; Eastman Kodak Co., Rochester, N.Y.) and xanthine oxidase (10 mU/ml; type II; Sigma) in the presence and absence of phenazine pigment (12). Reference cuvettes contained superoxide dismutase as above.

Cytochemical evaluation of neutrophil enzymes. PMN granule enzyme release and membrane enzyme activity were qualitatively evaluated by cytochemical staining procedures for myeloperoxidase and alkaline phosphatase (Sigma histochemical kits). PMNs were incubated for 20 min at 37°C in the presence and absence of PMA or phenazine compounds or both. Cytotoxic effects of the phenazine compounds were determined by assessing cell viability of part of each sample. The remainder of each cell suspension was cyt centrifuged (Shandon Southern Cytospin), and slide preparations were stained for myeloperoxidase and alkaline phosphatase activity. Results are presented as scores from 1+ to 4+ based on the percentage of positive-staining cell in the sample (11, 19).

Determination of neutrophil granule enzyme exocytosis. Lysozyme, an enzyme constituent of both azurophilic and specific granules, was measured as an indicator of neutrophil degranulation in these studies. PMNs were incubated at a density of 5 x 10^6 PMNs per ml in HBSS without calcium or magnesium in the presence and absence of phenazine compound or PMA or both for 30 min at 37°C. These studies were performed in the absence of cytochalasin B, a compound that enhances degranulation in response to some soluble stimuli. Samples were centrifuged, and the cell-free supernatant was assayed for lysozyme activity. Lysozyme activity was determined in a turbidimetric assay by measuring the rate of lysis of Micrococcus lylodeikicus at pH 6.2 (Worthington Diagnostics, Freehold, N.J.) (16). Total enzymatic content of granules was determined by assaying the activity of neutrophil extract prepared by freeze-thawing a pellet (5 x 10^6 PMNs) three times in 0.1% Triton X-100. Results are expressed as the percentage of total enzyme released.

Statistical analysis. Results are presented as the mean ± standard deviation. Significance was established at P < 0.05 by an unpaired, two-sided Student's t test.

RESULTS

Effect of phenazine compounds on superoxide production by isolated PMNs. The effect of phenazones, pyocyanin, and TFP on the rate of induced superoxide production is shown in Fig. 1. At 50 µM pyocyanin inhibited the PMA-induced superoxide generation by PMNs. However, at 1 µM pyocyanin enhanced superoxide generation more than twofold above the PMA-induced base line of 8.1 nmol of O_2^- per min per 10^6 PMNs. Inhibition by pyocyanin at 50 µM is not due to a cytotoxic effect, since PMNs incubated in the presence of this concentration of pyocyanin remained viable (>95%). Phennaline, the parent compound of pyocyanin, had minimal effect on superoxide production. Since the synthetic pyocyanin might contain as much as 5% by weight

![FIG. 1. Effect of tricyclic compounds on PMN superoxide generation. PMNs (10^6) were incubated with various concentrations of phenazine (PHE), pyocyanin (PYO), or TFP and activated with 1 µg of PMA per ml as described in the text. Results are expressed as a percentage of the nanomoles of O_2^- per minute per 10^6 PMNs generated by PMNs in the presence of these compounds compared with that generated in the absence of the compound.](http://iai.asm.org/content/56/4/1215/F1.large.jpg)
phenazine methosulfate, a 5 \mu M concentration of this synthetic precursor was tested for activity; none was observed (data not shown). However, TFP markedly inhibited the PMA-induced superoxide response at concentrations above 1 \mu M. None of the concentrations of phenazine, pyocyanine, or TFP tested induced superoxide production when added alone.

Since all three compounds could modify PMN function only in the presence of PMA, a submaximal concentration of PMA (10 ng/ml) was also investigated. At a concentration of 50 \mu M for each compound, there was no significant difference in the inhibition observed with 1 \mu g or 10 ng of PMA per ml. Thus, a final concentration of 1 \mu g/ml was used in all subsequent experiments.

**Activity of pyocyanine in a cell-free superoxide-generating system.** Depending on the pH of the environment, pyocyanine can act as an electron donor or an electron acceptor, raising the possibility that it could compete with cytochrome c for interaction with superoxide anions. Therefore, we examined its effect on superoxide-dependent cytochrome c reduction in the cell-free acetaldehyde-xanthine oxidase system. The data in Table 1 show that none of the compounds, pyocyanine, phenazine, or TFP, at either 50 or 1 \mu M, significantly decreased the amount of superoxide anion from the base-line value of 1.42 \pm 0.20 nmol/min. Therefore, pyocyanine does not act by scavenging superoxide radicals.

**Cytochemical evaluation of PMNs incubated with phenazine compounds.** Viability by acridine orange-ethidium bromide fluorescence microscopy was >95% for pyocyanine, phenazine, or TFP at either 50 or 10 \mu M concentrations under the same incubation conditions as those used for the superoxide assay. Therefore, the effects of these compounds on the inhibition of \( \mathrm{O}_2^- \) generation are not due to cytotoxicity but rather to a more direct effect on the PMN \( \mathrm{O}_2^- \) generating system.

Cytocentrifuged preparations of PMNs were incubated with either 50 or 10 \mu M concentrations of pyocyanine, phenazine, or TFP with or without PMA to evaluate their effect on neutrophil membrane and lysosomal enzyme activity. The absence or decrease in activity of granule enzyme markers such as myeloperoxidase and alkaline phosphatase would indicate lysosomal degradation as well as loss of neutrophil functions. Cytochemical detection of myeloperoxidase and alkaline phosphatase activities demonstrated that PMNs incubated with pyocyanine or phenazine gave the same number and intensity of cells staining as control samples. Although PMN viability in the presence of TFP was always >95%, cytochemical data could not be obtained because of major loss of cell morphology. These results suggested that the concentrations of pyocyanine that had marked effects on superoxide generation had little if any effect on cell viability and on other cell functions such as degranulation.

**Effect of phenazine compounds on neutrophil enzyme degranulation.** To confirm the results obtained by cytochemical analysis of neutrophil lysosomal enzymes, we measured the release of lysozyme into the incubation medium under the same assay conditions as for both cytochemical analysis and superoxide generation. Since lysozyme is present in both azurophilic and specific granules, its measurement in cell-free supernatants is an indication of exocytosis by both granule populations. The effect of 1 to 50 \mu M concentrations of pyocyanine or phenazine alone on lysozyme release was not significantly different from the control value of 5.68 \pm 1.82\% of total enzyme activity (Fig. 2), indicating that these compounds neither enhanced nor inhibited granule enzyme release. However, TFP at a concentration of 50 \mu M induced degranulation above the control value. PMA alone enhanced degranulation of control cells from 5.68 to 9.02\%. This effect was not additive with any of the three phenazine compounds tested.

### DISCUSSION

Pyocyanine is the major phenazine pigment produced by *P. aeruginosa* (15, 17). In an extensive work published in 1961, Liu et al. (18) found pyocyanine to be nontoxic for
HeLa cells in vitro and nonlethal for mice in vivo. Liu (17) concluded that P. aeruginosa pigments play no part in human pathogenicity. Nevertheless, since pyocyanine has been shown to alter cellular function in several systems, more detailed studies of the effect of pyocyanine as a model compound for P. aeruginosa phenazine pigments on cell-mediated immunity and on phagocyte function are necessary (24). Here we show data indicating that high concentrations of synthetic pyocyanine inhibit the generation of $O_2^-$ by activated PMNs, while lower concentrations enhance superoxide generation.

Inhibition or enhancement of respiration by pyocyanine has been previously described in several other systems. Pyocyanine inhibited the uptake of molecular oxygen by mouse monocytes (3), HeLa cells (4), isolated mouse liver mitochondria (1, 2), cultured BHK cells (27), and intact guinea pig peritoneal macrophages (27). The experiments determining oxygen consumption by mouse liver mitochondria indicated that pyocyanine decreased respiration by inhibiting electron transport. It was shown to act at the site of ubiquinone-cytochrome b in the electron transport chain. This same mechanism of action might be extended to the inhibitory effect of pyocyanine on the generation of superoxide anion by human peripheral PMNs. When PMNs, which are end-stage leukocytes relatively poor in mitochondria, are stimulated by particulate or soluble inflammatory mediators, they undergo a respiratory burst characterized by increased oxygen consumption and increased hexose-monophosphate shunt activity. More than 90% of the consumed $O_2$ can be accounted for by the generation of superoxide anion with the subsequent dismutation to $H_2O_2$ (9). The enzyme responsible for this activity in PMNs, an NADPH oxidase, is localized in the plasma membrane. There is growing evidence that this oxidase is composed of several subunits. The oxidase component that generates $O_2^-$ is associated with the plasma membrane and appears to contain a flavin-dependent component, a b-type cytochrome, and possibly a ubiquinone (6, 21). Therefore, high concentrations of pyocyanine may inhibit PMA-induced $O_2^-$ generation by also interfering at the same ubiquinone-cytochrome b site of the electron transport chain as described for mouse liver mitochondria (1, 2).

Pyocyanine differs in its effect from TFP, a known strong inhibitor of superoxide generation by PMNs at concentrations above 1 μM (23). At these concentrations, the action of phenothiazines is attributed to interactions with the neutrophil membrane, rather than to a specific inhibition of calmodulin (23). Further studies are required to elucidate the mode of action of these two compounds.

A model for enhancement of superoxide generation by pyocyanine has been defined by Hassan and Fridovich (10) who showed that oxygen uptake by Escherichia coli was enhanced in the presence of 50 to 100 μM pyocyanine. In our studies, at concentrations less than 5 μM, pyocyanine acts synergistically with PMA to increase production of $O_2^-$. The mechanism of pyocyanine enhancement, other than the possible nonenzymatic oxidation of NADPH (10), is not apparent and may be entirely different from the mechanism of inhibition at higher concentrations. The higher concentrations of pyocyanine may be sufficient to interfere with electron transport and produce an overall inhibitory effect. Alternatively, a desensitization to superoxide-inducing stimuli, such as PMA or immune complexes, by down regulation of specific receptors for the stimulating agents may account for a decrease in superoxide generation (5). Regardless of exact mechanisms, these observations provide an interesting mechanistic basis for possible induction of tissue damage by P. aeruginosa.

Generation of toxic oxygen species by activated PMNs plays an important role in assisting the host to effectively eliminate invading microorganisms, although superoxide and its reactive derivatives can also produce tissue injury (7, 30, 31). The concentration-dependent enhancement or inhibition of PMN superoxide production reported here suggests a possible dual pathophysiological role for pyocyanine. At high concentrations, as might be found in close promixity to the bacteria, the ability of PMNs to kill the bacteria may be weakened by the inhibition of superoxide production, thus prolonging the infection. In the local environment, but at a distance from the bacteria, low concentrations of pyocyanine may enhance a superoxide production by PMNs activated by soluble factors and thus lead to increased tissue damage. Further investigation of these effects of pyocyanine should assist in defining the potential role of this and other phenazine pigments in chronic P. aeruginosa infections.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-19970 from the National Institutes of Health.

We appreciate the secretarial assistance of Claire Svet.

LITERATURE CITED


