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Effects of Pyocyanine, a Phenazine Dye from *Pseudomonas aeruginosa*, on Oxidative Burst and Bacterial Killing in Human Neutrophils

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The effects of pyocyanine (phenazinium, 1-hydroxy-5-methyl-hydroxide, inner salt) on oxidative burst in human polymorphonuclear leukocytes were studied by several different approaches. In a cell- and enzyme-free system, pyocyanine oxidized NADPH. The reduced pyocyanine could be measured by its reaction with ferricytochrome *c*. It was shown by this assay that resting as well as phorbol myristate acetate- or zymosan-stimulated granulocytes reduced pyocyanine. The effect was independent of mitochondria, as cytoplasts were similarly active. Measurement of the hexose monophosphate shunt in intact granulocytes in the presence of pyocyanine indicated a concentration-dependent activation of the shunt without the generation of O_2^-, suggesting that pyocyanine oxidizes NADPH to $NADP^+$ when it enters granulocytes. Intracellular NADPH in granulocytes was indeed lowered by almost 40% after incubation with pyocyanine. It is by this shuttling of reduction equivalents, leading to the partial depletion of NADPH, that pyocyanine affects the observed concentration-dependent partial inhibition of the phorbol myristate acetate- and zymosan-stimulated generation of O_2^-. A further consequence was that the intracellular killing of *Staphylococcus aureus* was also partially suppressed, particularly at higher loads of granulocytes with bacteria. Phagocytosis was not inhibited by pyocyanine concentrations as high as 500 μM. Pyocyanine did not affect the intracellular killing of *Pseudomonas aeruginosa*. The possible relevance of these findings to the course of mixed hospital infections in immunocompromised patients is discussed.

Pseudomonas aeruginosa is frequently involved in nosocomial infections in patients with cystic fibrosis, in patients in burn and intensive care units, or in more general terms, in immunocompromised hosts. Besides a number of exoenzymes, *P. aeruginosa* bacteria liberate a blue dye-stuff, pyocyanine, which was noticed a century ago in dressings of infected wounds (11). Pyocyanine was recently shown to inhibit T-lymphocyte proliferation in mice (19) and humans (26) at the level of interleukin-2 receptor expression (19, 21) or interleukin-2 synthesis (21).

Pyocyanine can undergo redox reactions (7) and is an electron carrier (15). Hence, it theoretically might interfere with the oxidative burst (for reviews, see references 1 and 2) of phagocytes, which is an important defense mechanism against many microorganisms. We therefore studied the effects of pyocyanine on the oxidative burst and bacterial killing by human polymorphonuclear leukocytes (PMNs). Both processes were inhibited substantially by pyocyanine. Additional experiments in enzyme- and cell-free systems and with cytoplasts suggested that pyocyanine indeed acts as an electron carrier in this system, partially depleting the critical substrate NADPH intracellularly. Possible implications for the infectious processes in immunocompromised hosts are discussed. While this study was in process, Miller et al. (18) reported the partial inhibition of O_2^- generation by phorbol myristate acetate (PMA)-stimulated PMNs.

MATERIALS AND METHODS

Chemicals and enzymes. Cytochrome *c* (type III from horse heart), NAD^+, $NADP^+$, zymosan, PMA, superoxide dismutase (SOD) from bovine erythrocytes, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (Deisenhofen, Federal Republic of Germany). NADH, NADPH, glutamate dehydrogenase, and 6-phosphogluconate dehydrogenase were purchased from Boehringer GmbH, (Mannheim, Federal Republic of Germany). Pyocyanine was isolated from *P. aeruginosa* cultures as described previously (19). In more recent experiments, synthetic pyocyanine was also used.

Chemical synthesis of pyocyanine (compound 7). The chemical synthesis of pyocyanine is shown in Fig. 1. The original procedure of Surrey (27) was improved in three reaction steps. First, the very large excess of lead tetraacetate in the oxidation of pyrogallol monomethyl-ether (compound 1) was replaced by nearly equivalent amounts of tetrachloro-*ortho*-benzoquinone to afford the *ortho*-quinone (compound 2) in a 90% yield (10). Second, the coupling of compound 2 with *ortho*-phenylenediamine (compound 3) was performed as described previously (27), but for the cleavage of the methyl ether (compound 4) to the phenol (compound 5), we performed the reaction with boron tribromide at room temperature instead of using boiling hydrogen bromide in acetic acid. The third improvement concerned the isolation of the zwitterionic form (compound 7) from the methylated salt (compound 6). Pyocyanine is very water soluble, and stirring with potassium carbonate in dichloromethane followed by filtration over a short column of silica gel was used instead of an aqueous workup.

3-Methoxy-*ortho*-benzoquinone (compound 2). A solution of 10.00 g (71.43 mmol) of bisphenol (compound 1) in 30 ml of diethyl ether and 18.45 g (75 mmol) of tetrachloro-*ortho*-benzoquinone in 150 ml of diethyl ether were mixed and stirred for 6 h at 0°C. The solution was filtered to afford

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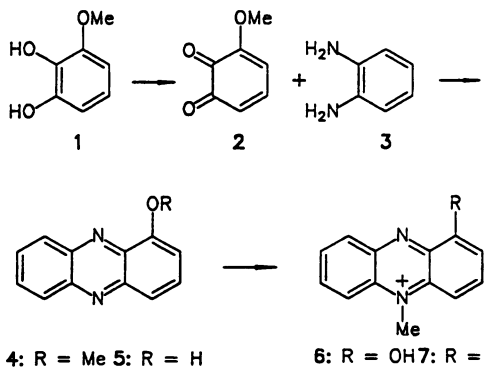

FIG. 1. Chemical synthesis of pyocyanine. For details and compound definitions, see the text. Me, Methyl.

8.90 g (90%) of compound 2, which was immediately reacted with compound 3 to afford compound 4 (27).

Ether cleavage of compound 4. A solution of 9.96 g (47.43 mmol) of 1-methoxyphenazine (compound 4) in 100 ml of anhydrous dichloromethane was treated with 150 ml of a 1 M solution of boron tribromide in dichloromethane at −70°C. The solution was then stirred for 15 h at room temperature and poured into ice water. The organic phase was dried (Na_2SO_4) and filtered over a short column of silica gel (CH_2Cl_2). The less polar fraction was crystallized from diethyl ether to afford 6.48 g (70%) of compound 5.

Pyocyanine (compound 7). A total of 276 mg (1 mmol) of compound 5 was heated with 2 ml of dimethyl sulfate for 2 h at 120°C. Excess reagent was removed at reduced pressure, and the residue was stirred for 4 h under nitrogen with 2 g of powdered potassium carbonate and 10 mg of 18-crown-6 in 30 ml of dichloromethane. The solution was filtered and passed through a column (2 by 5 cm) of silica gel. Elution with dichloromethane–30% diethyl ether (ca. 30 ml) removed traces of starting material, and elution with dichloromethane–25% methanol afforded the dark blue pyocyanine that was recrystallized from chloroform (183 mg, 63%).

Isolation of PMNs. PMNs were isolated from fresh blood of healthy donors. The bulk of erythrocytes was precipitated by sedimentation with 6% Dextran T 500 (Pharmacia, Uppsala, Sweden) as described by Markert et al. (14). PMNs in the supernatant were sedimented through a Lymphoprep gradient as described by Boyum (4). The remaining erythrocytes in the pellet were lysed with NH_4Cl (24). The PMNs obtained were over 99% viable, as judged by trypan blue exclusion.

Reduction of pyocyanine by PMNs. The reduction of pyocyanine by PMNs was performed in a 5-ml stoppered tube equipped with a side arm (28). PMNs were suspended at 1.5 × 10^6 in 1 ml of physiological saline at 37°C, 50 μM pyocyanine was added, and the tube was partially evacuated to about 4,000 Pa (30 mm Hg).

Cytochrome *c* reduction assay and stimulation of O_2^- generation by zymosan and PMA. Cytochrome *c* reduction was measured in microdilution plates by using an enzyme-linked immunosorbent assay reader as described by Rajkovic and Williams (23). Briefly, 240 μM ferricytochrome *c* was added to 2 × 10^6 PMNs in modified physiological saline (modified phosphate-buffered saline, which was 0.137 M NaCl, 0.01 M sodium and potassium phosphate buffer [pH 7.4] containing 2.7 mM KCl, 0.95 mM $CaCl_2$, 0.41 mM $MgSO_4$, and 5.5 mM glucose). Where indicated, 300 U of SOD was included. The reaction was started by adding pyocyanine (at the indicated concentrations), the stimulants (at 1 mg of opsonized zymosan per ml and 400 nM PMA), or both to give a final volume of 150 μl. Measurements were done in triplicate. A reference solution of dithionite-reduced cytochrome *c* (horse heart type III; Sigma), with an extinction coefficient (by the specification of the supplier) of 29.5 mmol $liter^{-1}$ cm^{-1} and with a known concentration, was determined with a spectrophotometer (Zeiss) and was used to calibrate the enzyme-linked immunosorbent assay reader to an optical density at 546 nm of 0.646 corresponding to 100 μM reduced cytochrome *c*. O_2^- was calculated from the SOD-inhibitable fraction of reduced cytochrome *c*.

Determination of pyridine nucleotides. NADPH and NADH were extracted from 10^7 PMNs with 0.5 ml of 2 M KOH. The pH was adjusted to 7.8 with 1 ml of 1 M triethanolamine hydrochloride. $NADP^+$ and NAD^+ were extracted from an equal number of cells with 1 ml of 2 M $HClO_4$, followed by neutralization with 240 μl of 10 M KOH. Pyridine nucleotides were then determined by fluorimetry as described previously (22).

Bacteria. *Staphylococcus aureus* Oxford was obtained from H. Reichenbach (Gesellschaft für Biotechnologische Forschung), and *P. aeruginosa* was obtained from G. Schmidt (Forschungsinstitut Borstel). Bacteria were grown aerobically overnight at 30°C in the following liquid medium: 0.5% casein peptone, 0.5% Proteose Peptone (Difco Laboratories, Detroit, Mich.), 0.1% beef extract, and 0.1% yeast extract.

Opsonization of zymosan and bacteria. Zymosan (at 10 mg per ml of modified phosphate-buffered saline) was sonicated to facilitate suspension. An equal volume of human serum was added, and the suspension was incubated for 30 min at 37°C. Zymosan was washed and suspended in modified phosphate-buffered saline. Bacteria were suspended at 10^8/ml in human serum and incubated for 30 min at 37°C. They were spun down, washed, and suspended in Hanks balanced salt solution containing 1% gelatin and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5).

Preparation of cytoplasts. Cytoplasts were prepared as described by Roos and Voetman (25) by centrifuging cytochalasin *b* (Sigma)-treated PMNs in a Ficoll 70 (Pharmacia) step gradient. The cytoplasts were washed free of cytochalasin *b* prior to use.

Measurement of CO_2 liberated by the hexose monophosphate shunt. Activation of the hexose monophosphate shunt (HMPS) was measured as described previously (17). Briefly, 3.75 × 10^6 PMNs in 1.5 ml of modified phosphate-buffered saline containing 2.5 μCi of D-[1-^{14}C]glucose or D-[6-^{14}C]glucose (specific activity, 55.6 mCi/mmol; Amersham Buchler) were incubated at 37°C in 25-ml Erlenmeyer flasks equipped with a center well which contained 200 μl of 10% KOH. Stimulation was started by adding pyocyanine (at the indicated concentrations), PMA (at 400 nM), or opsonized zymosan (at 1 mg/ml). The reaction was stopped, and CO_2 was liberated by the addition of 500 μl of 1 N HCl. The radioactive CO_2 trapped in the KOH was measured in a liquid scintillation counter.

Phagocytosis assay. The phagocytosis assay was done as described by Leijh et al. (12). Briefly, PMNs and preopsonized bacteria (at a ratio of 1/400) were incubated at 37°C in HEPES-buffered Hanks balanced salt solution containing 1% gelatin. At various times fractions were taken and diluted with ice cold, HEPES-buffered Hanks balanced salt solution with gelatin, and the PMNs were sedimented at 200 × *g* in the cold. The remaining bacteria in the supernatant were

determined by limiting dilution (16) for *P. aeruginosa* or by counting the number of colonies on nutrient agar (Difco) for *S. aureus*.

Bacterial killing. Bacterial killing was done as described by Leijh et al. (12). PMNs and bacteria were mixed in different ratios, and phagocytosis was allowed to proceed for 5 min at 37°C. PMNs from the fractions were spun down and washed at 200 × *g* in the cold and incubated in HEPES-buffered Hanks balanced salt solution at 37°C for the indicated times. The PMNs were then lysed in distilled water, and the surviving bacteria were determined as described above.

RESULTS

Reduction of pyocyanine to leukopyocyanine by PMNs, and measurement of reduced pyocyanine with the cytochrome *c* reduction test. Before we studied the effects of pyocyanine on the oxidative burst, we wanted to test and understand the effect of pyocyanine on resting PMNs. The addition of 50 μM pyocyanine to an aerated suspension of PMNs caused no visible effect. However, pyocyanine was decolorized within 90 min after partial evacuation of the culture vessel. When the suspension was exposed to air, the blue pyocyanine color returned immediately. This indicates that PMNs, like *Pseudomonas* bacteria (7), can reduce pyocyanine to leukopyocyanine, which was spontaneously reoxided by molecular oxygen.

Hassan and Fridovich (9) had shown in a cell- and enzyme-free system that pyocyanine can be reduced by NADH, and on reoxidation in air it gives rise to O_2^-. These investigators measured O_2^- by the SOD-inhibitable fraction of Nitro Blue Tetrazolium reduction, which amounted to greater than 80% of the total. In order to test whether pyocyanine could act as an electron carrier between NADPH and cytochrome *c*, we measured cytochrome *c* reduction in such a system, replacing NADH with NADPH, the substrate for O_2^- generation by PMNs (1). Cytochrome *c* reduction by NADPH proceeded nonenzymatically and was catalyzed by pyocyanine in a dose-dependent manner (data not shown). The process was not inhibitable by SOD, which was in agreement with similar findings of Nishikimi et al. (20) with phenazine methosulfate, indicating that the reaction of the reduced phenazine compounds is faster with ferricytochrome *c* than it is with molecular oxygen:

$$\text{NADPH} + \text{H}^+ + \text{Pyo} \rightarrow \text{NADP}^+ + \text{PyoH}_2 \quad (1)$$

$$\text{PyoH}_2 + \text{Fe}^{3+}\text{cyt } c \rightarrow \text{Pyo} + 2\text{Fe}^{2+}\text{cyt } c + 2\text{H}^+, \quad (2)$$

where Pyo is pyocyanine, and cyt *c* is cytochrome *c*. This allowed us to measure the reduction of pyocyanine by resting and stimulated PMNs by the cytochrome *c* reduction test. SOD was added to destroy the interfering O_2^- generated by stimulated PMNs. The results (Fig. 2A) indicate that pyocyanine is reduced even by unstimulated cells and that opsonized zymosan did not influence this process. PMA, however, led to an increased reduction velocity but did not alter the extent of the reaction.

Influence of pyocyanine on O_2^- formation in resting and stimulated PMNs. Generation of O_2^- by zymosan- and PMA-stimulated PMNs, measured as the SOD-inhibitable fraction of reduced ferricytochrome *c*, was decreased by the addition of pyocyanine in a dose-dependent manner (Fig. 3A). These effects were not due to the general toxicity of pyocyanine, as doses up to 500 μM were not toxic and did not inhibit phagocytosis (see below). The extent of inhibition not only varied with the pyocyanine concentration but also depended on the donor of the PMNs, giving values among

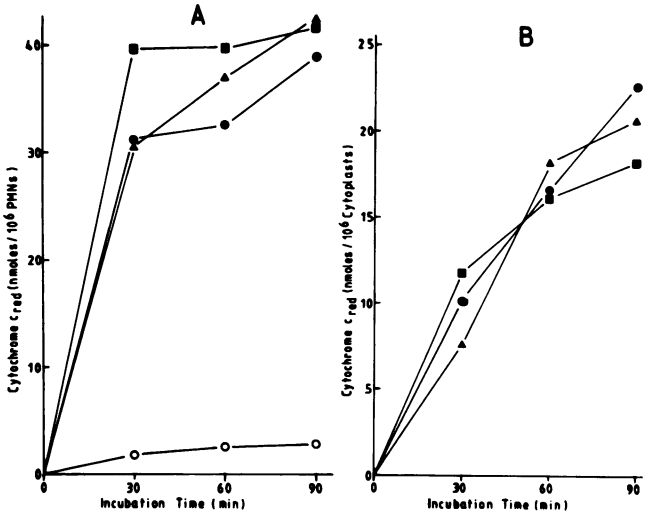

FIG. 2. Pyocyanine acts as an electron carrier between PMNs and ferricytochrome *c* (A) and cytoplasts and ferricytochrome *c* (B). Symbols: ■, PMA and pyocyanine (100 μM); ▲, zymosan and pyocyanine (100 μM); ●, pyocyanine (100 μM); ○, unstimulated PMNs without pyocyanine. SOD (300 U/ml) was added to all samples. Cytochrome *c* reduction of PMA- or zymosan-stimulated PMNs or cytoplasts without pyocyanine was completely inhibited by SOD (data not shown). Cytochrome *c* reduction was measured at 546 nm.

samples from different individuals in the range of 12 to 49% inhibition of PMA-inducible O_2^- release at 50 μM pyocyanine and in the range of 74 to 82% inhibition of zymosan-inducible O_2^- release at comparable pyocyanine concentrations. There was no influence of pyocyanine on the low spontaneous generation of O_2^- in resting PMNs. In some experiments we also measured (by the horseradish peroxidase-mediated, catalase-inhibitable oxidation of phenol red) the influence of pyocyanine on the generation of H_2O_2 by stimulated PMNs. As expected, the generation of H_2O_2 was inhibited to an extent similar to that of O_2^- (data not shown).

Effects of pyocyanine on cytoplasts. To narrow down the effects of pyocyanine on the enzymes and substrates relevant to the oxidative burst, and since earlier studies have indicated that pyocyanine interferes with succinic dehydrogenase (15), which is an integral component of the electron transport chain in mitochondria, we prepared cytoplasts from PMNs. Such cytoplasts are enucleated cells devoid of granules and mitochondria and can be activated to generate active oxygen species (25). Cytoplasts could reduce pyocyanine almost as well as intact PMNs could (Fig. 2B), and as with PMNs, PMA- as well as zymosan-stimulated O_2^- generation in cytoplasts was partially inhibited by pyocyanine (Fig. 3B). This indicates that the effects of pyocyanine on oxidative burst are not due to interference with electron transport in the mitochondria.

Effects of pyocyanine on HMPS and the intracellular content of NADPH and NADH. The effects of pyocyanine described above are most easily explained by assuming that it enters the PMNs and oxidizes intracellular NADPH to $NADP^+$, thus partially depleting the substrate for the O_2^--generating system. Pyocyanine induced the appearance of $NADP^+$, which should then lead to activation of the HMPS in otherwise unstimulated cells. That this is indeed the case is shown in Fig. 4. Pyocyanine-induced CO_2 release from [6-^{14}C]glucose was less than 10% of that from [1-^{14}C]glu-

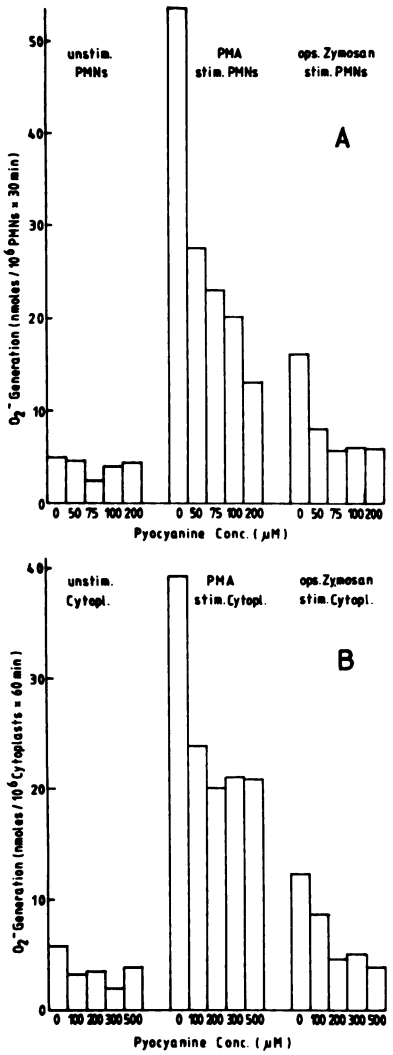

FIG. 3. Concentration dependence of the effects of pyocyanine on spontaneous and stimulated O_2^- generation in PMNs (A) and cytoplasts (cytopl.) (B). O_2^- values were calculated from the SOD-inhibitable fraction of ferricytochrome *c* reduction.

cose, indicating that pyocyanine did not unspecifically stimulate glucose oxidation by mitochondria.

Pyocyanine at a 100-μM concentration caused stimulation of the HMPS to the same extent as zymosan did, with both stimuli being additive. Stimulation of the HMPS by PMA was apparently maximal and, thus, was not further increased by pyocyanine. The effects of pyocyanine were dose dependent (Fig. 5). Interestingly, two different levels of maximal HMPS activity were reached by stimulation with pyocyanine with or without zymosan, on the one hand, and by PMA, on the other. Since the HMPS might have been stimulated by mechanisms other than an intracellular increase of $NADP^+$, pyridine nucleotides were measured directly in PMNs after incubation with 400 μM pyocyanine for 1 h. The content of reduced pyridine nucleotides was indeed diminished from 1.6 to 1.0 nmol of NADH/10^8 PMNs and from 1.7 to 1.1 nmol of NADPH/10^8 PMNs; there were concomitant increases of NAD^+ and $NADP^+$.

Effects of pyocyanine on phagocytosis and bacterial killing. The experiments described above showed that pyocyanine partially inhibits the generation of phagocytosis-induced

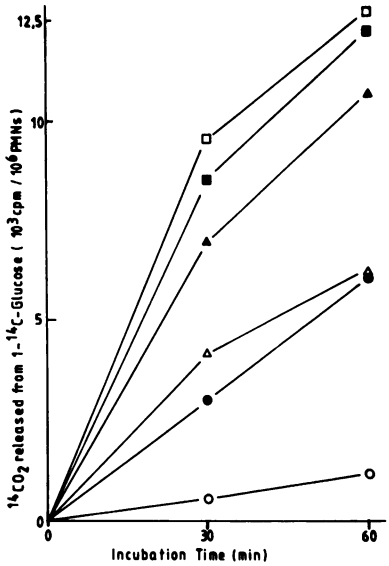

FIG. 4. Kinetics of the effects of pyocyanine on the HMPS in resting and stimulated PMNs. Symbols: ○, resting PMNs, △, zymosan-stimulated PMNs; □, PMA-stimulated PMNs. Closed symbols indicate results for PMNs as described for open symbols, but in the presence of 100 μM pyocyanine. The HMPS was estimated by measuring the $^{14}CO_2$ liberated from 1-^{14}C-labeled glucose.

extracellular O_2^-. We next studied the effects of pyocyanine on the intracellular killing of *S. aureus*, which, in contrast to killing of *P. aeruginosa*, is largely dependent on the generation of O_2^- in the phagosomes (13). It was first established that pyocyanine, at a maximal concentration of 500 μM and after a preincubation time of 2 h, did not inhibit the phagocytosis of staphylococci or pseudomonads or of fluorescein-conjugated zymosan (data not shown). The extent of inhibition of intracellular killing of staphylococci at constant

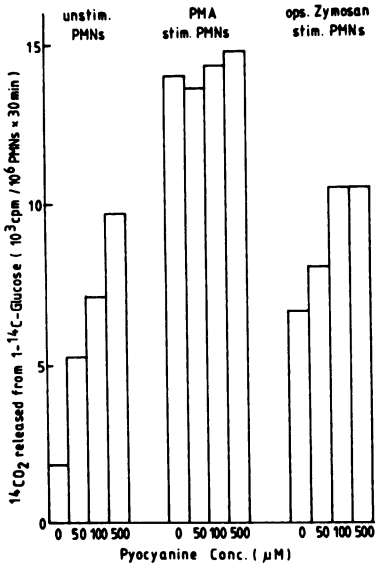

FIG. 5. Concentration dependence of the effects of pyocyanine on the HMPS in resting and stimulated PMNs. The HMPS was estimated as described in the legend to Fig. 4.

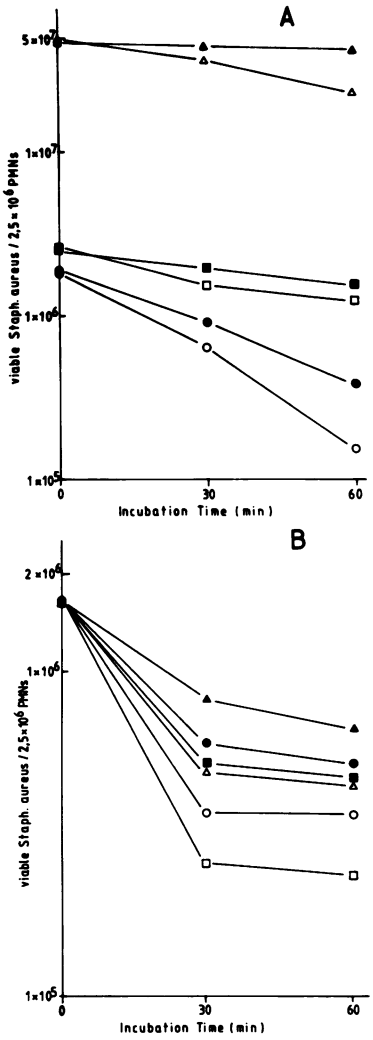

FIG. 6. Effects of pyocyanine on intracellular killing of *S. aureus* by PMNs. (A) Effects of the following different ratios of bacteria/PMNs: 100/1 (△), 10/1 (□), 1/1 (○). Open symbols indicate that no pyocyanine was present, and closed symbols indicate the presence of 100 μM pyocyanine. (B) Pyocyanine concentration dependency at a bacterium/PMN ratio of 5/1 with pyocyanine at 0 μM (□), 50 μM (○), 75 μM (△), 100 μM (■), 200 μM (●), and 500 μM (▲). The zero time point was after 5 min of phagocytosis. Each point represents the mean of colony counts from two parallel cultures. The deviation from the mean was less than 15%. Note the log scale of numbers of bacteria.

pyocyanine concentrations depended on the ratio of bacteria/PMNs (Fig. 6A), and at a constant ratio of bacteria/PMNs, it was dependent on the pyocyanine concentration (Fig. 6B). As expected, intracellular killing of *P. aeruginosa*, which is not dependent on the generation of active oxygen species (13), was not affected by pyocyanine (data not shown).

DISCUSSION

This study was done to clarify the inhibitory effects of pyocyanine on PMNs, including (i) its reaction with resting and stimulated PMNs, (ii) its influence on the generation of activated oxygen species, and (iii) the possible importance of pyocyanine in nosocomial infections.

Our data are best explained by assuming that an electron carrier mechanism was mediated by the passage of pyocyanine through the plasma membrane, by which reduction equivalents were shuttled out of the cells, causing a partial depletion of NADPH and NADH. The reduced leukopyocyanine was then reoxidized externally under aerobic conditions.

We demonstrated the reduced pyocyanine by its reaction with ferricytochrome *c*, a reaction that proceeded distinctly faster than that with molecular oxygen. Mitochondria were not vital for the intracellular reduction of pyocyanine, as this reaction also proceeded in cytoplasts which are enucleated PMNs devoid of mitochondria. We also measured the pyocyanine-induced decrease in intracellular NADPH (37%) and a concomitant increase in $NADP^+$. As would be expected, this led to stimulation of the HMPS, as indicated below:

$$2NADP^+ + \text{glucose 6-phosphate} + H_2O \xrightarrow{\text{HMPS}} 2NADPH + 2H^+ + \text{ribulose-5-phosphate} + CO_2 \quad (3)$$

Baehner et al. (3) have observed a similar stimulation of the HMPS in PMNs by the phenothiazine compound methylene blue.

The partial depletion of intracellular NADPH cannot be compensated for by reaction 3, and thus, a decrease in the generation of O_2^- follows because of the lack of the substrate of the O_2^--generating enzyme. This was observed in PMA- as well as zymosan-stimulated PMNs. By taking the various levels of responsiveness of PMNs from different donors into account, the inhibitory effect of pyocyanine was usually more pronounced on phagocytosis-stimulated PMNs than on PMA-stimulated cells, with the zymosan stimulation being a closer approximation to a physiological stimulus. In contrast to a recent report by Miller et al. (18), we did not observe any stimulation of O_2^- production at low doses of pyocyanine.

As a consequence of the pyocyanine-induced partial inhibition of O_2^- generation, we observed a partial inhibition of intracellular killing of *S. aureus*. Whereas phagocytosis was not influenced by pyocyanine concentrations as high as 500 μM, inhibitory effects on intracellular killing were noted at 50 μM and increased with higher concentrations. The importance of investigating the killing capacity of PMNs at different bacterium/PMN ratios has been pointed out by Clawson and Repine (5), who showed that the killing capacity reaches a peak at a load of 100 bacteria per PMN. Pyocyanine partially inhibited the killing of *S. aureus* at this and lower ratios. In contrast, no effects of pyocyanine on the intracellular killing of *P. aeruginosa* were noted, although these bacteria were well phagocytized. Our findings are compatible with the data of Mandell (13), who reported that PMNs are also able to kill *P. aeruginosa* under anaerobic conditions, i.e., without the oxidative burst.

Our findings may be of clinical relevance, since the local concentration of pyocyanine near *P. aeruginosa*-infected tissues may reach the 100-μM range, as was shown for sputum specimens from patients with cystic fibrosis and bronchiectasis (29). As pointed out above, pyocyanine did not affect phagocytosis or the killing of *P. aeruginosa* bacteria. However, it may well aggravate the situation in cases of mixed infections involving *S. aureus* and other microorganisms against which the oxidative burst is effective. Thus, for example, blood samples from over one-half of the patients from burn units yielded multiple isolates, including *P. aeruginosa* (6). Since *P. aeruginosa* predominantly infects patients who are in a critical state of health, with either low counts of PMNs or PMNs with already impaired

functions (8), further partial inhibition of the oxidative burst, like that shown here, may become decisive for the course of such mixed infectious.

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