INFECTION AND IMMUNITY, Sept. 1991, p. 3316–3318
0019-9567/91/093316-03$02.00/0

Vol. 59, No. 9

Production of Leukotriene B_4 and 5-Hydroxyeicosatetraenoic Acid by Human Neutrophils Is Inhibited by *Pseudomonas aeruginosa* Phenazine Derivatives

MICHAEL MULLER* AND TANIA C. SORRELL

Department of Medicine, University of Sydney, and Department of Infectious Diseases and Microbiology, Westmead Hospital, Westmead, New South Wales 2145, Australia

Received 8 April 1991/Accepted 15 June 1991

Pyocyanin, a phenazine pigment produced by *Pseudomonas aeruginosa*, and its metabolite 1-hydroxyphenazine inhibited leukotriene B_4 and 5-hydroxyeicosatetraenoic acid production by up to 70% in human neutrophils stimulated with the calcium ionophore A23187 (5 μM). This potential anti-inflammatory effect was dose dependent and occurred at low concentrations (10 to 50 μM) that did not inhibit neutrophil viability.

Pseudomonas aeruginosa, although normally nonpathogenic for humans, is a significant opportunistic pathogen in the immunocompromised host (9, 14) and is associated with chronic, localized infection of the respiratory tract in individuals with cystic fibrosis and bronchiectasis (12).

The initial point of contact for bacteria with the host cellular defenses is the phagocytic cell, particularly the polymorphonuclear leukocyte (PMNL). Human PMNL have been shown to produce the proinflammatory mediators leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) in response to various stimuli (16). LTB_4 is rapidly metabolized by PMNL to 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (17). PMNL are also involved directly in the clearance of foreign organisms from the host (11).

Virulence factors of *P. aeruginosa* include exotoxin A, heat-stable hemolysin (a glycolipid), phospholipase C, alkaline protease, elastase, a cytotoxin, and exoenzyme S (13, 18). Phospholipase C at close to cytotoxic concentrations stimulates release of eicosanoids from PMNL in vitro (6). In vivo, pseudomonal extracellular enzymes are probably important in the pathogenesis of acute tissue damage. However, the role of these extracellular enzymes in chronic disease is uncertain, because their effect may be neutralized by the ability of the host to mount a specific antibody response (3). Pyocyanin (5-methyl-1-hydroxyphenazine), a phenazine pigment of low molecular weight, is produced in abundance by clinical isolates of *P. aeruginosa* and is present at sites of chronic pseudomonal infection (20). The effects of this pigment and its decomposition product, 1-hydroxyphenazine (1-OHP), on superoxide generation and other parameters of human PMNL function were investigated recently (7, 10, 15). We now present evidence that pyocyanin and 1-OHP cause the inhibition of products of the 5-lipoxygenase pathway.

Normal PMNL were separated from 25 ml of anticoagulated venous blood by centrifugation at 250 × *g* for 45 min through 20 ml of Mono-Poly resolving medium (Flow Laboratories, Australia). The PMNL layer was removed and washed with calcium- and magnesium-free Hanks balanced salt solution (HBSS). Contaminating erythrocytes were removed by hypotonic lysis. PMNL (93% purity or greater) were washed, resuspended in Hanks balanced salt solution at a concentration of 5×10^6 cells per ml, and labeled with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (0.5 μCi/ml; Amersham) at 37°C for 1 h. Excess label was removed, and the cells were resuspended in Hanks balanced salt solution containing calcium and magnesium.

Pyocyanin was prepared by the photochemical method of Knight et al. (5), and 1-OHP was prepared by the method of Armstrong et al. (1). The final purification of each was achieved by thin-layer chromatography on silica gel G with chloroform-methanol (9:1, vol/vol). Pyocyanin and 1-OHP were stored in methanol in the dark at −80 and −20°C, respectively. Before use, purified pigments were subjected to thin-layer chromatography on silica gel G with chloroform-methanol (1:1 and 9:1, vol/vol, respectively). Each pigment migrated as a single band in these systems. The UV spectra and extinction coefficients, measured in methanol with a Shimadzu UV-265 scanning spectrophotometer, were in good agreement with established values (19).

Pyocyanin or 1-OHP was preincubated with 1 ml of cell suspension at 37°C for 20 min with shaking. Both pyocyanin and 1-OHP were freely soluble at 37°C. After incubation with pyocyanin or 1-OHP (0 to 50 μM) for 30 min at 37°C, more than 95% of PMNL were shown to be viable by the trypan blue exclusion test. The PMNL were then stimulated for 5 min with the calcium ionophore A23187 (5 μM; Boehringer Mannheim). All reactions were terminated by the addition of 1 volume of ethanol.

After extraction by the method of Bligh and Dyer (2), arachidonic acid metabolites were separated by high-performance liquid chromatography with an Ultrasphere C18 5 μm column (250 by 4.6 mm; Beckman Instruments, Sydney, Australia). Metabolites were identified by comigration with authentic standards (Amersham). The mobile phase consisted of methanol-water-acetic acid (75:24.95:0.05, vol/vol/vol) (pH 5.4), and the flow rate was 1 ml/min. Fractions of 1 ml were collected, dried, and counted in a Packard scintillation counter.

The uptake of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid by PMNL was 86.5 ± 4.7% (n = 5) after 1 h of incubation with the label. Production of LTB_4 and 5-HETE by PMNL incubated with either pyocyanin or 1-OHP was inhibited in a dose-dependent manner. With each pigment, the relative decreases in total LTB_4 (Fig. 1), ω-oxidation products (Fig. 2), and 5-HETE (Fig. 3) were comparable. This suggests that pyocyanin and 1-OHP act at a common point in the arachi-

* Corresponding author.

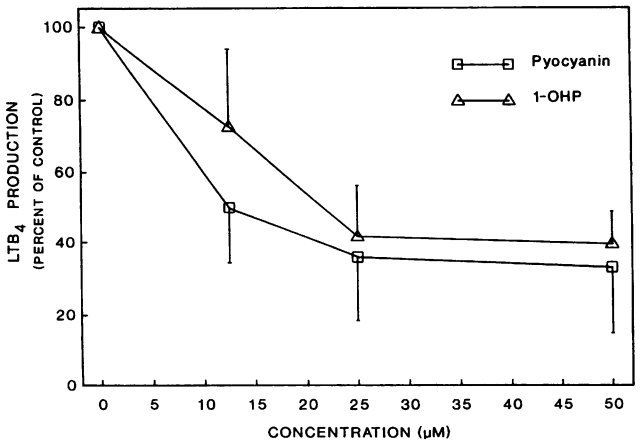

FIG. 1. Effect of pyocyanin and 1-OHP on LTB_4 production by PMNL stimulated for 5 min with A23187. Results are expressed as percentages of control values (means ± standard deviations of five experiments).

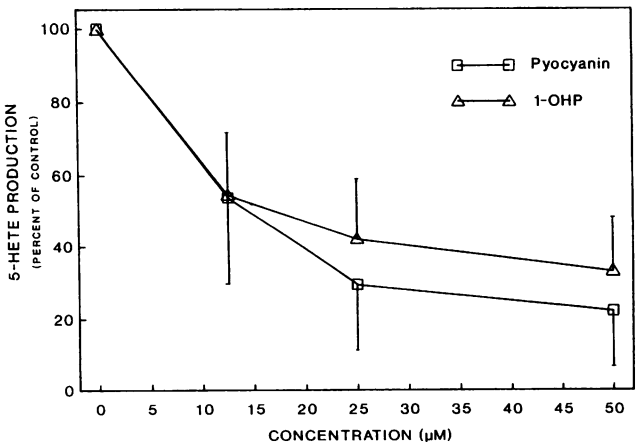

FIG. 3. Effect of pyocyanin and 1-OHP on 5-HETE production by PMNL stimulated for 5 min with A23187. Results are expressed as percentages of control values (means ± standard deviations of five experiments).

donic acid metabolic pathway, before or at the site of 5-lipoxygenase, and that either free arachidonic acid or 5-hydroperoxyeicosatetraenoic acid becomes the limiting substrate. Consequently, LTB_4 would become limiting and lead to the decreased production of ω-oxidation products. If the site of action of the phenazine pigments is at the 5-lipoxygenase step in the pathway, the observed inhibition may be due to inactivation of the enzyme by free radicals generated during the interaction of reduced phenazine pigments and molecular oxygen. Pyocyanin in the presence of NADH and molecular oxygen can generate superoxide (4). PMNL-derived superoxide dismutase may then convert the superoxide to hydrogen peroxide, which is known to irreversibly inactivate 5-lipoxygenase (8).

Substantial amounts of *P. aeruginosa* phenazine pigments have been identified in sputum samples from patients with either cystic fibrosis or bronchiectasis (20). The concentrations of phenazines used in this study were within the range detected in sputa from such patients and comparable to concentrations used in other studies. Pyocyanin has been described as proinflammatory due to its ability to stimulate superoxide production in human PMNL (15), although such stimulation has not been a consistent finding (7, 10). Although free radicals of PMNL origin are thought to be implicated in the progressive lung damage characteristic of cystic fibrosis, their influence may be offset by the anti-inflammatory effect of reduced LTB_4 production. LTB_4 is a major endogenous chemotaxin and hence is a potent proinflammatory mediator. Our data indicate that phenazine pigments have the potential to reduce the accumulation of PMNL at sites of pseudomonal infection. Such a reduction in PMNL numbers with a consequent reduction in phagocytic capacity may be relevant to the pathogenesis of chronic infection with *P. aeruginosa* in patients with cystic fibrosis or bronchiectasis.

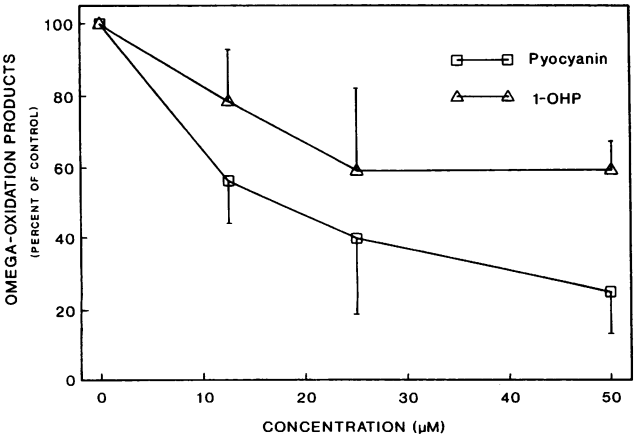

FIG. 2. Inhibition of the ω-oxidation products of LTB_4 from PMNL exposed to either pyocyanin or 1-OHP. PMNL were stimulated for 5 min with A23187. Results are expressed as percentages of control values (means ± standard deviations of five experiments).

This work was supported by Merck, Sharp and Dohme (Australia), Pty. Ltd.

REFERENCES

1. **Armstrong, A. V., D. E. S. Stewart-Tull, and J. S. Roberts.** 1971. Characterization of the *Pseudomonas aeruginosa* factor that inhibits mouse-liver mitochondrial respiration. J. Med. Microbiol. **4**:249–262.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
3. **Döring, G., W. Goldstein, A. Röll, P. O. Schiøtz, N. Høiby, and K. Botzenhart.** 1985. Role of *Pseudomonas aeruginosa* exoenzymes in lung infections of patients with cystic fibrosis. Infect. Immun. **49**:557–562.
4. **Hassan, H. M., and I. Fridovich.** 1980. Mechanism of the antibiotic action of pyocyanine. J. Bacteriol. **141**:156–163.
5. **Knight, M., P. E. Hartman, Z. Hartman, and V. M. Young.** 1979. A new method of preparation of pyocyanin and demonstration of an unusual bacterial sensitivity. Anal. Biochem. **95**:19–23.
6. **Meyers, D. J., and R. S. Berk.** 1990. Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent. Infect. Immun. **58**:659–666.
7. **Miller, K. M., D. G. Dearborn, and R. U. Sorensen.** 1987. In vitro effect of synthetic pyocyanine on neutrophil superoxide production. Infect. Immun. **55**:559–563.
8. **Mitsuda, H., K. Yasumoto, and A. Yamamoto.** 1967. Inactivation of lipoxygenase by hydrogen peroxide, cysteine and some other reagents. Agric. Biol. Chem. **31**:853–860.
9. **Morrison, A. J., and R. P. Wenzel.** 1984. Epidemiology of

infections due to *Pseudomonas aeruginosa*. Rev. Infect. Dis. **6**:S627–S642.
10. **Müller, P. K., K. Krohn, and P. F. Mühlradt.** 1989. Effects of pyocyanine, a phenazine dye from *Pseudomonas aeruginosa*, on oxidative burst and bacterial killing in human neutrophils. Infect. Immun. **57**:2591–2596.
11. **Pennington, J. E., M. G. Ehrie, and W. F. Hickey.** 1984. Host defense mechanisms against pneumonia due to *Pseudomonas aeruginosa*. Rev. Infect. Dis. **6**:S657–S666.
12. **Pier, G. B.** 1985. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. J. Infect. Dis. **151**:575–580.
13. **Pollack, M.** 1984. The virulence of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **6**:S617–S626.
14. **Pruit, B. A., R. B. Lindberg, W. F. McManus, and A. D. Mason.** 1983. Current approach to prevention and treatment of *Pseudomonas aeruginosa* infections in burned patients. Rev. Infect. Dis. **5**:S889–S897.
15. **Ras, G. J., R. Anderson, G. W. Taylor, J. E. Savage, E. Van Niekerk, R. Wilson, and P. J. Cole.** 1990. Proinflammatory interactions of pyocyanin and 1-hydroxyphenazine with human neutrophils in vitro. J. Infect. Dis. **162**:178–185.
16. **Sellmayer, A., Th. Strasser, and P. C. Weber.** 1987. Differences in arachidonic acid release, metabolism and leukotriene B_4 synthesis in human polymorphonuclear leukocytes activated by different stimuli. Biochim. Biophys. Acta **927**:417–422.
17. **Shak, S., and I. M. Goldstein.** 1984. ω-Oxidation is the major pathway for the catabolism of leukotriene B_4 in human polymorphonuclear leukocytes. J. Biol. Chem. **259**:10181–10187.
18. **Vasil, M. L.** 1986. *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. J. Pediatr. **108**:800–805.
19. **Watson, D., J. MacDermot, R. Wilson, P. J. Cole, and G. W. Taylor.** 1986. Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. Eur. J. Biochem. **159**:309–313.
20. **Wilson, R., D. A. Sykes, D. Watson, A. Rutman, G. W. Taylor, and P. J. Cole.** 1988. Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol phase toxicity for respiratory epithelium. Infect. Immun. **56**:2515–2517.