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Preliminary Characterization of *Pseudomonas aeruginosa* Peptide Chemotactins for Polymorphonuclear Leukocytes

PATRICIA A. FONTÁN,[1]* CLAUDIA R. AMURA,[1] VERÓNICA E. GARCÍA,[1] M. CRISTINA CERQUETTI,[1] AND DANIEL O. SORDELLI[1,2]

Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 P-12, 1121 Buenos Aires, Argentina,[1] and Department of Pediatrics, Georgetown University, Washington, D.C. 20007[2]

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In a previous report, we showed that supernatants of *Pseudomonas aeruginosa* cultures exhibit chemotactic activity for polymorphonuclear leukocytes (PMNL). In this study, *P. aeruginosa* chemotactins were isolated, purified, and partially characterized. The organisms were cultured in Vogel-Bonner defined medium, and cultures were stopped in late log phase. Chemotactins withstood heating, remained unaltered after acid or alkali treatment in a pH range from 4 to 10, and resisted digestion by trypsin or carboxypeptidase, but chemotactic activity was decreased by 73% after incubation with pronase. Only 2% of the total chemotactic activity of culture supernatants could be extracted with chloroform. Chemotactins with molecular sizes <3 kDa constituted the largest contribution to the chemotactic activity of culture supernatants. Pretreatment of PMNL with 10^{-5} M formylmethionyl-leucyl-phenylalanine (FMLP) inhibited chemotaxis towards FMLP and *P. aeruginosa* culture supernatants but not towards complement component C5a. In conclusion, the total chemotactic activity for PMNL of *P. aeruginosa* culture supernatants was due, almost exclusively, to chemotactins that have properties similar, if not identical, to those exhibited by formylmethionyl peptides.

Pseudomonas aeruginosa pneumonia is characterized by extensive tissue damage, which appears to be induced not only by bacterial exoproducts but also by secretory components of polymorphonuclear leukocytes (PMNL) and mononuclear phagocytes (6). There are resident phagocytes in the lung, the alveolar macrophages, which may secrete reactive oxygen species and lysosomal enzymes in response to a stimulus of bacterial origin (19). Inflammatory cells, however, seem to be quantitatively more important in the production of lung damage. The numerous PMNL that migrate from the circulation in response to chemotactins released at the site of infection are clearly associated with tissue damage (10). Previous experiments in an animal model have shown that *P. aeruginosa* deposited in the lungs induces significant migration of PMNL and mononuclear phagocytes (16). Although host-derived chemotactins may be regarded as the sole inducers of inflammatory cell migration, it has been demonstrated that *P. aeruginosa* releases exoproducts with chemotactic activity for PMNL (15). These chemotactins exhibit their action even in the absence of the host's humoral factors, such as complement components (15). This study was aimed at the preliminary characterization of chemotactins released by *P. aeruginosa*.

MATERIALS AND METHODS

Chemotactins. The synthetic chemotactic peptide formylmethionyl-leucyl-phenylalanine (FMLP) was obtained from Sigma Chemical Co., St. Louis, Mo. Chemotactins from *P. aeruginosa* and *Escherichia coli* were obtained by culturing the bacteria in Vogel-Bonner defined medium (15) at 37°C with continuous agitation at 200 rpm in an orbital air shaker until late log phase was reached. Bacteria were pelleted by centrifugation at 5,000 × *g* and 4°C for 10 min, and then the supernatant fluid was decanted, filtered through a 0.2-μm-pore-size filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and either stored at −20°C until used or lyophilized. Complement component C5a was obtained by treating 5 ml of normal human serum with 5 μg of heat-killed *E. coli* for 1 h at 37°C. Killed bacteria were removed by centrifugation at 5,000 × *g* for 10 min, and the supernatant fluid was stored at −20°C until used.

Chemotactin characterization. Chemotactin heat stability was investigated by assaying the chemotactic activity of *P. aeruginosa* culture supernatant after incubation for 15 min in boiling water. Chemotactin resistance to acid and basic hydrolysis was tested after incubation of culture supernatant at room temperature for 15 min at a pH that varied from 2 to 10. Culture supernatants were filtered through a Centricon 30 membrane filter (Amicon Corp., Lexington, Mass.) at 2,500 × *g* and 4°C. The filtrate was then passed through a Centricon 10 membrane filter, and the new filtrate was passed through a Centricon 3 filter. All four fractions (molecular mass less than 30 kDa, mass greater than 10 kDa but less than 30 kDa, mass greater than 3 kDa but less than 10 kDa, and mass less than 3 kDa) were reconstituted to the original volume with fresh Vogel-Bonner culture medium and tested for chemotactic activity. The lipid fraction was extracted by treating 25 ml of culture supernatant five times with 5-ml volumes of chloroform. These five volumes were pooled together, and the chloroform was evaporated at 80°C. The residue was dissolved in Hanks balanced salt solution without Ca^{2+} or Mg^{2+} (HBSSw) and further diluted to the original concentration in HBSSw. Chemotactic activity was assessed in both the culture supernatant (after chloroform extraction) and the 1× chloroform extractive. Aliquots of culture supernatant were treated with pronase (0.2 mg/ml), carboxypeptidase (0.1 unit/ml), or trypsin (0.01 mg/ml) for 30 min at 37°C, and the enzyme digests were tested for chemotactic activity.

Isolation and purification of chemotactic fractions. One liter of culture supernatant was lyophilized and then extracted

* Corresponding author.

three times with a total of 500 ml of an n-butanol–glacial acetic acid mixture (10:1, vol/vol). In order to desalt the chemotactic material, the combined extracts were evaporated under vacuum at 45°C and the residue was dissolved in water and applied to a 20-by-2-cm Dowex 50 × 2 column (Sigma), 200–400 mesh, H^+ form. *P. aeruginosa* peptide chemotactins (PAPCs) were eluted with 0.2 M ammonium acetate buffer containing 0.2 M pyridine, pH 5. The solvents were removed by means of a rotary evaporator (7). The residue was dissolved in water and passed through Centriprep 3 (Amicon). The fraction with a molecular mass less than 3 kDa was lyophilized for further analysis. Silica gel G plates (Fisher Scientific) with 0.25-mm-thick layers containing a fluorescent additive were used. Lyophilized material was applied to the thin-layer chromatography (TLC) plate by using a minimum amount of developing solvent. Ascending development was allowed to continue until a scored point 15 cm from the origin was reached by using the acidic system n-butanol–glacial acetic acid–water (10:1:3, vol/vol/vol). After being dried, the plates were monitored under UV light. One of them was treated with ninhydrin to detect peptide fractions (13). Corresponding areas of plates run simultaneously were scraped off, eluted with NH_4OH, and lyophilized. The lyophilized material was reconstituted with phosphate buffer solution, pH 7.4, and assayed for chemotactic activity.

Human cells. Ten milliliters of blood from healthy human volunteers was collected in 0.5 ml of 5% EDTA and diluted with saline. The PMNL-rich plasma fraction was obtained by sedimentation in 6% dextran (molecular weight, 266,000; Sigma), and erythrocytes were hypotonically lysed with distilled water at 4°C. The PMNL-rich fraction was washed twice with HBSSw, and PMNL were suspended to a density of 10^7 cells per ml in HBSSw. Over 90% of the cells were PMNL, with 99% viability; the remaining 10% were mononuclear cells.

Chemotaxis assay. Chemotaxis was assayed by using a Boyden multi-microwell assembly (5). The lower wells were filled with 25 μl of chemoattractant and covered with a polyvinylpyrrolidone-free polycarbonate filter (pore diameter, 3 μm; Nuclepore, Bethesda, Md.). Fifty microliters of a suspension containing 10^7 PMNL per ml was added to each one of the upper wells (5×10^5 cells per well). After incubation under 90% humidity and 5% CO_2 for 1 h at 37°C, the filter was removed and the cells remaining on the upper surface of the filter were gently scrubbed off with a rubber policeman. The cells were fixed and stained with Diff-Quick (Dade Diagnostics, Inc., Aguada, P.R.). The number of cells retained in the filter was estimated spectrophotometrically with a microELISA plate reader, after extraction of the dye with chloroform and 0.1 N HCl, as described in a previous report (4).

RESULTS

P. aeruginosa culture supernatant (1:1) exhibited chemotactic activity of a magnitude similar to that exhibited by *E. coli* culture supernatant (0.069 versus 0.052 optical density units; difference not significant). Lyophilized culture supernatants of *P. aeruginosa* were reconstituted to 1/10 of the original volume with HBSSw. Maximum chemotactic activity was observed at the 1:32 dilution of the concentrated culture supernatant with HBSSw, whereas maximum chemotactic activity of FMLP was seen at the 10^{-7} M concentration (Fig. 1). Both dose-response curves followed a similar pattern, with decreased chemotactic activity at a high chemotactin concentration. FMLP diluted in fresh Vogel-Bonner medium to 10^{-7} M displayed chemotactic activity similar to that of 10^{-7} M FMLP in HBSSw, indicating that the culture medium did not contain any PMNL chemotaxis inhibitor (data not shown). Similar optical density readings were obtained in chemotaxis experiments in which PMNL were confronted with either fresh Vogel-Bonner medium or HBSSw. These results showed that the Vogel-Bonner medium did not have chemotactic activity for PMNL.

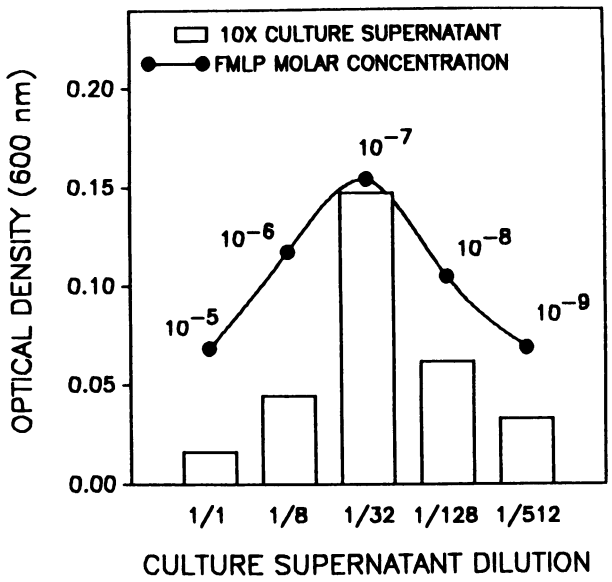

FIG. 1. Chemotactic activity of *P. aeruginosa* culture supernatants for PMNL. PMNL chemotaxis is expressed as the optical density produced by the dye extracted from the filters. Progressive dilutions of 10× culture supernatants are shown on the abscissa. The control curve showing the chemotactic activity of FMLP has been arbitrarily superimposed on the dose-response curve of culture supernatants in such a way that the peaks of both curves coincide. Random migration was subtracted from each sample, and each bar or point represents only chemotactic activity for PMNL. The result of a representative experiment is depicted. Three different culture supernatants were tested, and experiments with each culture supernatant were repeated three times with similar results.

Chemotactins withstood heating and remained unaltered after acid or alkali treatment in a pH range from 4 to 10. Chemotactins resisted digestion with trypsin and with carboxypeptidase, but chemotactic activity was decreased by 73% after incubation with pronase (Fig. 2). Chloroform extractives reconstituted with fresh medium to the original culture supernatant volume had a chemotactic activity equivalent to 2% of that exhibited by the 1:1 supernatant (Fig. 2). Ultrafiltration through Centricon 30, Centricon 10, and Centricon 3 filters showed that the fraction composed of components with molecular sizes smaller than 3 kDa contributed most to chemotactic activity (Fig. 3).

Preincubation of PMNL for 5 min with FMLP induced a dose-related decrease in the PMNL chemotactic response to nondiluted culture supernatant and to 10^{-7} M FMLP (Fig. 4). Preincubation for more than 5 min led to PMNL clumping. Abolishment of chemotactic activity towards 10^{-7} M FMLP or PAPCs was attained by preincubation of the cells with 10^{-5} M FMLP. When PMNL were preincubated with PAPCs (molecular size, <3 kDa) and exposed to 10^{-7} M FMLP or C5a in a chemotaxis assay, a priming effect was

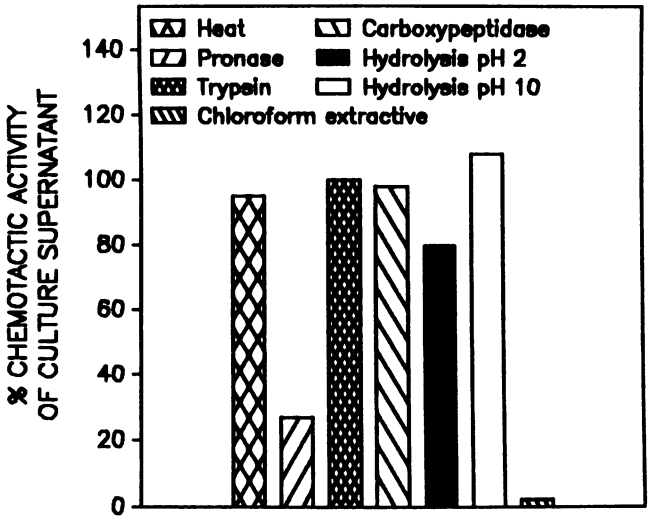

FIG. 2. Chemotactic activity of *P. aeruginosa* culture supernatant after heat, acid, or alkali treatment and after enzymatic digestion. Controls for supernatants treated with the enzymes were enzyme solutions of the same final concentration in HBSSw. The activity of the chloroform extractive reconstituted to the original volume with fresh medium is also shown. Random migration was subtracted from each sample, and each bar represents only chemotactic activity for PMNL. The result of a representative experiment is depicted. The experiments were repeated three times with similar results.

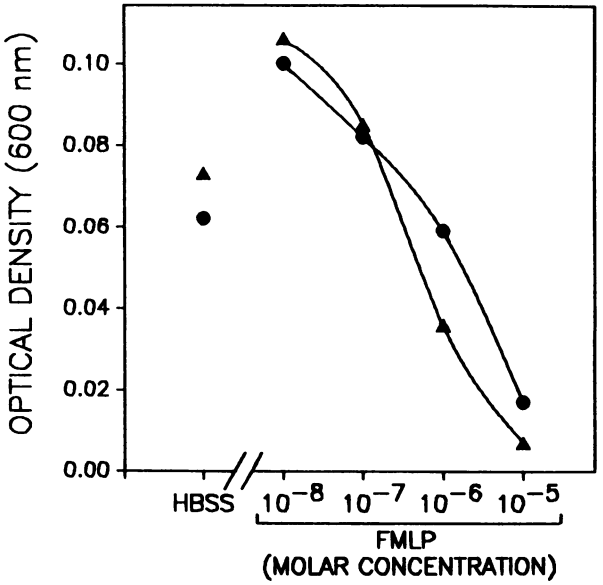

FIG. 4. Chemotactic responses of PMNL preincubated with different concentrations of FMLP. Undiluted *P. aeruginosa* culture supernatant (●) and 10^{-7} M FMLP (▲) were used as chemoattractants. Random migration was subtracted from each sample, and each bar represents only chemotactic activity for PMNL. The result of a representative experiment is depicted. The experiment was repeated four times with similar results.

observed (0.014 versus 0.047 and 0.069 versus 0.100 for FMLP and C5a, respectively, in separate experiments), similar to that seen when PMNL were preincubated with 10^{-8} M FMLP and further exposed to 10^{-7} M FMLP (Fig. 4). A similar priming pattern was observed when PMNL were preincubated with PAPCs (molecular size, >3 kDa) (0.014 versus 0.029 and 0.069 versus 0.084 for FMLP and C5a, respectively, in separate experiments; random migration was subtracted from each sample, and each number represents only chemotactic activity for PMNL). Chemo-

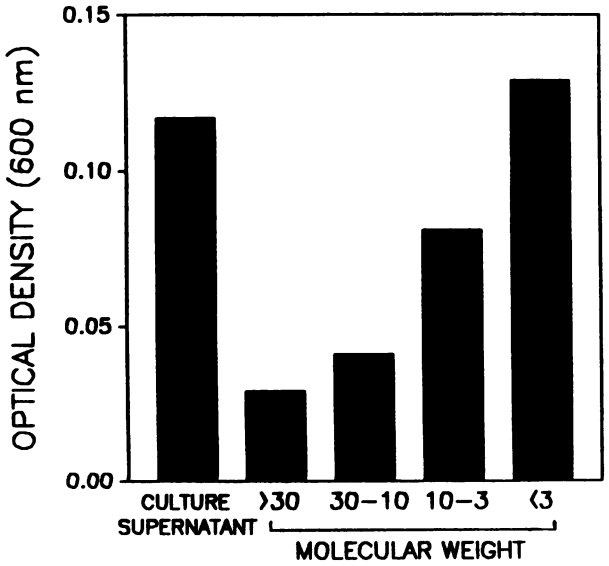

FIG. 3. Chemotaxis induced by different fractions of *P. aeruginosa* culture supernatant obtained by ultrafiltration. Random migration was subtracted from each sample, and each bar represents only chemotactic activity for PMNL. The result of a representative experiment is shown. The experiment was repeated four times with similar results.

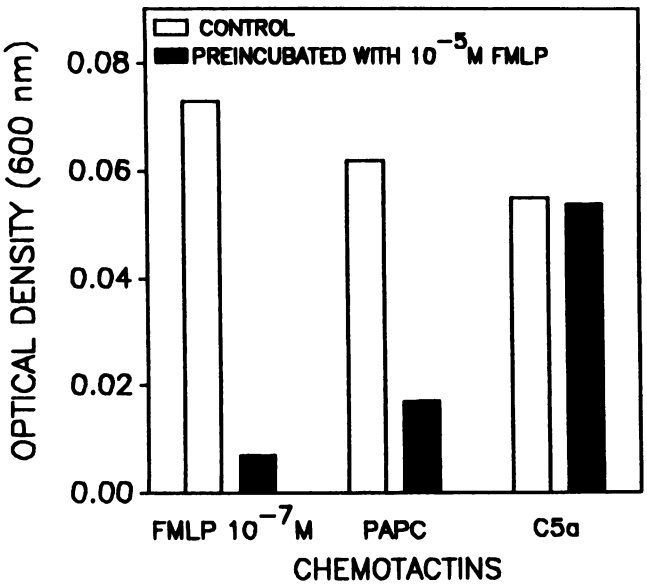

FIG. 5. Chemotactic response of PMNL preincubated with 10^{-5} M FMLP. Undiluted *P. aeruginosa* culture supernatant, 10^{-7} M FMLP, and C5a were used as chemoattractants. Random migration was subtracted from each sample, and each bar represents only chemotactic activity for PMNL. The result of a representative experiment is depicted. The experiment was repeated three times with similar results.

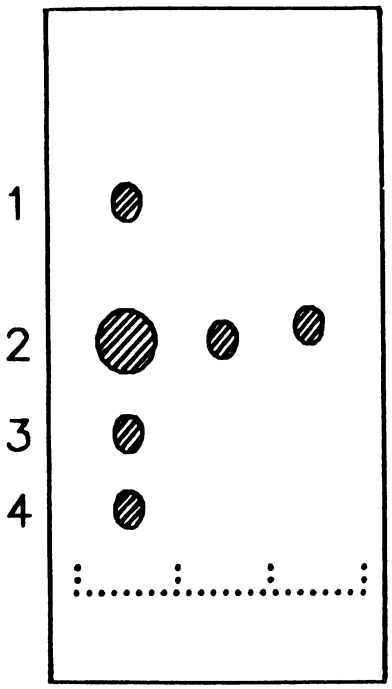

FIG. 6. Diagram of the acidic TLC of *P. aeruginosa* crude supernatant (left lane, spots 1 to 4), the <3-kDa fraction obtained by Centricon 3 ultrafiltration (middle lane), and FMLP (right lane). The spots indicate ninhydrin-reactive areas. Corresponding areas of duplicate plates were scraped off and washed with buffer. Chemotactic activity was detected in eluates from spots 1 and 2, whereas eluates from spots 3 and 4 carried no chemotactic activity. TLC of culture supernatants was repeated three times, and chemotaxis experiments were repeated twice with similar results.

taxis of PMNL to C5a was not affected by preincubation of PMNL with 10^{-5} M FMLP (Fig. 5). Preincubation of PMNL with C5a did not modify the chemotactic response of these cells to PAPCs (molecular size, <3 kDa and >3 kDa) (data not shown).

Desalted culture supernatants and culture supernatants subjected to ultrafiltration through a Centricon 3 membrane were subjected to TLC. The spot layout obtained after ninhydrin treatment is depicted in Fig. 6. Areas corresponding to those reacting with ninhydrin, from duplicate plates, were scraped off and assayed for chemotactic activity. Eluates from spots 1 and 2 exhibited chemotactic activity, whereas those from spots 3 and 4 did not display detectable activity (Fig. 6). A single spot was revealed in the lane where the <3-kDa fraction was loaded, and the eluate from that spot displayed chemotactic activity for PMNL. The <3-kDa fraction and FMLP migrated approximately the same distance, suggesting that these chemotactins have similar partition coefficients under the experimental condition utilized. Although the peptides extracted from spot 2 may resemble chemotactins with molecular sizes of <3 kDa and FMLP, the identity of the ninhydrin-reactive spot 1 remains to be elucidated.

DISCUSSION

We have previously shown that *P. aeruginosa* and *Staphylococcus aureus* release chemotactins in culture and that *P. aeruginosa* culture supernatants were able to recruit PMNL into the lower respiratory tract when introduced into mice as an aerosol (15). In the present study, we showed that these chemotactins have several features in common with the low-molecular-weight peptides described by Schiffman et al. in their classical study with *E. coli* (13). Amino acid sequence analysis of PAPCs was not addressed in the present study, but there is strong evidence that low-molecular-weight formylmethionyl peptides are responsible for most chemotactic activity of *P. aeruginosa* culture supernatants. Formylmethionyl chemotactic peptides are released not only by *E. coli* but also by other bacteria (12) and are chemotactic for PMNL from most animal species investigated (2, 8). Our results indicate that chemotactic activity is due not to a single chemotactin but to a group of formylmethionyl peptides with a different molecular weight. Chemotactic activity of PAPCs, but not of C5a, was abolished by preincubating the PMNL with FMLP. This finding strongly suggests that the FMLP receptor is involved in the chemotactic response of PMNL to PAPCs. Other, chemically different, chemotactins are known to be released by *P. aeruginosa*, such as the rhamnolipids described by Shryock et al. (14). The relative contribution of lipid chemotactins to PMNL migration in vitro, however, was only 2% of the total chemotactic activity of culture supernatants, which suggested that rhamnolipids may have little significance in the in vivo recruitment of PMNL to the lungs.

PMNL accumulation seems to be one of the major causes of pathogenesis during *P. aeruginosa* infection (10). Although bacterial oligopeptide chemotactins were described nearly two decades ago, little is known about the contribution of *P. aeruginosa* chemotactic peptides to the induction of inflammation and subsequent tissue damage. An interesting finding in this regard was made by Dunn et al., who showed that exposure of PMNL to either FMLP or a *P. aeruginosa* culture supernatant fraction with a molecular size of <0.5 kDa rendered these cells cytotoxic to epithelial cells in culture. These authors did not investigate the nature of the supernatant component but speculated that it may be related to a formylated peptide or family of peptides (3). Whether PAPCs are or contain FMLP is not known. PAPCs, however, share a number of characteristics with FMLP, including the capacity to induce PMNL chemotaxis. Unpublished data from our laboratory showed that other known FMLP-induced PMNL actions are also triggered by PAPCs. The potential mechanisms for tissue damage that PMNL display when the FMLP receptor is triggered lend support to the hypothesis that PAPCs may indeed be an important pathogenesis factor of *P. aeruginosa*. Stimulation of the FMLP high-affinity receptors (1) on PMNL in vivo induces increased adherence, diapedesis, emigration, and chemotaxis (11). Higher concentrations of FMLP trigger the low-affinity receptors (1) and induce phagocytosis, degranulation, and, ultimately, the oxidative burst (17). In addition, during PMNL-mediated pulmonary vascular injury, formylmethionyl peptides may act in concert with other components of *P. aeruginosa*, e.g., lipopolysaccharide, which can exert a potent priming effect of PMNL for an enhanced response of these cells to FMLP (9, 20, 21). Furthermore, lipopolysaccharide promotes the release of host components, e.g., tumor necrosis factors alpha and beta, which can prime PMNL to induce production of chlorinated oxidants after FMLP stimulation (18). In the light of these findings, it can be suggested that PAPCs play an important role in the induction of PMNL-mediated tissue damage during *P. aeruginosa* infection. The intricate regulatory mechanisms which control the equilibrium between antibacterial defense

and tissue damage during *P. aeruginosa* infection, however, remain obscure and merit further investigation.

In conclusion, we have demonstrated that *P. aeruginosa* secretes in culture chemotactins that have properties similar, if not identical, to those exhibited by formylmethionyl peptides, and that the total chemotactic activity for PMNL of *P. aeruginosa* culture supernatants was due, almost exclusively, to these chemotactins. Further studies are required to ascertain the amino acid sequence of *P. aeruginosa* formylmethionyl oligopeptides and the pathophysiological importance of these chemotactins in the induction of lung tissue damage during acute *P. aeruginosa* pneumonia.

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REFERENCES

1. **Allen, R. A., A. E. Traynor, G. M. Omann, and A. J. Jesaitis.** 1988. The chemotactic peptide receptor. A model for future understanding of chemotactic disorders. Hematol. Oncol. Clin. N. Am. **2**:33–59.
2. **Chadwick, V. S., D. M. Meller, D. B. Myers, A. C. Selden, A. Keshayarzian, M. F. Broom, and C. H. Hobson.** 1988. Production of peptides inducing chemotaxis in vitro and in vivo. Scand. Gastroenterol. **23**:121–128.
3. **Dunn, M. M., M. Dunne, and D. W. Kamp.** 1990. Polymorphonuclear leukocyte- and *P. aeruginosa*-induced damage to a human pulmonary epithelial cell line. J. Infect. Dis. **162**:172–177.
4. **Fontán, P. A., and D. O. Sordelli.** 1989. A spectrophotometric micromethod to assess polymorphonuclear leukocyte migration induced by bacterial chemotactins in a multi-microwell Boyden chamber assembly. J. Microbiol. Methods **10**:225–230.
5. **Harvath, L., W. Falk, and E. J. Leonard.** 1980. Rapid quantitation of neutrophil chemotaxis: use of polyvinylpyrrolidone-free membrane in a multi-well assembly. J. Immunol. Methods **37**:39–45.
6. **Henson, P. M., and R. B. Johnston, Jr.** 1987. Perspectives: tissue injury in inflammation. Oxidants, proteinases and cationic proteins. J. Clin. Invest. **79**:669–674.
7. **Hirs, C. H. W.** 1967. Separation of peptides. Methods Enzymol. **2**:361–365.
8. **Kermode, J. C., N. Muthukumaraswamy, and R. J. Freer.** 1988. Characteristics of binding of a potent chemotactic formyltetrapeptide, formyl-methionyl-leucyl-phenylalanyl-phenylalanine, to the receptors on rabbit neutrophil. J. Leukocyte Biol. **43**:420–428.
9. **Kharazmi, A., A. Fomsgaard, R. S. Conrad, C. Galanos, and N. Hoiby.** 1991. Relationship between chemical composition and biological function of *Pseudomonas aeruginosa* lipopolysaccharide: effect on human neutrophil chemotaxis and oxidative burst. J. Leukocyte Biol. **49**:15–20.
10. **Kindt, G. C., J. E. Gadek, and J. E. Weiland.** 1991. Initial recruitment of neutrophils to alveolar structures in acute lung injury. J. Appl. Physiol. **70**:1575–1585.
11. **Pike, M. C., M. S. Wicha, P. Yoon, L. Mayo, and L. A. Boxer.** 1989 . Laminin promotes the oxidative burst in human neutrophils via increased chemoattractant receptor expression. J. Immunol. **142**:2004–2011.
12. **Rot, A., L. E. Henderson, R. Sowder, and E. J. Leonard.** 1989. *Staphylococcus aureus* tetrapeptide with high chemotactic potency and efficacy for human leukocytes. J. Leukocyte Biol. **45**:114–120.
13. **Schiffman, E., H. V. Showell, B. A. Corcoran, P. A. Ward, E. Smith, and E. L. Becker.** 1975. The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. J. Immunol. **114**:1831–1837.
14. **Shryock, T. R., S. A. Silver, M. W. Banschbach, and J. C. Kramer.** 1984. Effect of *Pseudomonas aeruginosa* rhamnolipid on human neutrophil migration. Curr. Microbiol. **10**:323–328.
15. **Sordelli, D. O., M. C. Cerquetti, A. Morris Hooke, and J. A. Bellanti.** 1985. Effects of chemotactins released by *Staphylococcus aureus* and *Pseudomonas aeruginosa* on the murine respiratory tract. Infect. Immun. **49**:265–269.
16. **Sordelli, D. O., B. J. Zeligs, M. C. Cerquetti, A. Morris Hooke, and J. A. Bellanti.** 1985. Inflammatory responses to *Pseudomonas aeruginosa* and *Staphylococcus aureus* in the murine lung. Eur. J. Respir. Dis. **66**:31–39.
17. **Steinbeck, M. J., and J. A. Roth.** 1989. Neutrophil activation by recombinant cytokines. Rev. Infect. Dis. **11**:549–568.
18. **Test, S. T.** 1991. Effect of tumor necrosis factor on the generation of chlorinated oxidants by adherent human neutrophils. J. Leukocyte Biol. **50**:131–139.
19. **Ward, P. A., and J. Varani.** 1990. Mechanisms of neutrophil-mediated killing of endothelial cells. J. Leukocyte Biol. **48**:97–102.
20. **Worthen, G. S., C. Haslett, A. J. Rees, R. S. Gumbay, J. E. Henson, and P. M. Henson.** 1987. Neutrophil-mediated pulmonary vascular injury. Synergistic effect of trace amounts of lipopolysaccharide and neutrophil stimuli on vascular permeability and neutrophil sequestration in the lung. Am. Rev. Respir. Dis. **136**:19–28.
21. **Wright, G. G., P. W. Read, and G. L. Mandell.** 1988. Lipopolysaccharide releases a priming substance from platelets that augments the oxidative response of polymorphonuclear neutrophils to chemotactic peptide. J. Infect. Dis. **157**:690–696.