INFECTION AND IMMUNITY, Nov. 1991, p. 4255–4258
0019-9567/91/114255-04$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 59, No. 11

Inhibition of Chemotaxis of Neutrophil Leukocytes to Interleukin-8 by Endotoxins of Various Bacteria

L. P. BIGNOLD,[1]* S. D. ROGERS,[1] T. M. SIAW,[1] AND J. BAHNISCH[2]

Department of Pathology, University of Adelaide, G.P.O. Box 498, Adelaide, South Australia 5001,[1] and Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000,[2] Australia

Received 8 March 1991/Accepted 6 August 1991

The effects of endotoxins from various bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Shigella flexneri*, *Salmonella typhosa*, and *Pseudomonas aeruginosa*) on chemotaxis of neutrophil leukocytes to formyl peptide and interleukin-8 were tested in an improved chemotaxis assay involving a "sparse-pore" polycarbonate (Nuclepore) membrane in a Boyden-type chamber. The possible chemotactic activity of the endotoxins themselves were tested by the same technique. In addition, the effects of these substances on random motility of neutrophils were tested with a corresponding assay involving similar chambers fitted with membranes of standard pore density. Possible activation of the complement system of serum by each endotoxin was tested with sheep erythrocyte assays and the maximum endotoxin concentration (100 μg/ml) used in the chemotaxis and motility assays. All endotoxins inhibited chemotaxis of neutrophils to interleukin-8. No endotoxin affected chemotaxis to formyl peptide or was itself chemotactic for neutrophils. Endotoxin of *S. flexneri* inhibited random motility of neutrophils, while the others had no such effect. Endotoxins of *K. pneumoniae* and of *P. aeruginosa* produced moderate and marked inhibition, respectively, of total complement, as measured by hemolysis of sheep erythrocytes, without affecting the levels of C3c and C4 in these assays. Endotoxins of the other bacteria had no demonstrable effect in any of these assays of complement activation. These results suggest that chemotaxis to interleukin-8 may be mediated by cellular mechanisms different from those involved in chemotaxis to formyl peptide. Furthermore, the presence of these endotoxins could be significant for the suppression of neutrophil accumulation in inflammatory lesions mediated by interleukin-8.

The possible roles of endotoxins (lipopolysaccharide mainly of cell wall origin; 29) both in the accumulation of neutrophil leukocytes in infective lesions and in the behavior of these cells in the circulation in septicemia have long been controversial (3, 24, 28, 44). In experimental animals, injection of endotoxin into the skin provokes local tissue leukocytosis after a delay of up to 1 h, but intravenous injection of endotoxin generally produces neutropenia (13). In addition, endotoxins have been reported to inhibit the accumulation of neutrophils in inflammatory sites (31, 41).

In vitro, endotoxins have been reported to have various effects on neutrophil chemotaxis and motility. Early work (3) and later studies (10, 14, 23) indicated that the migration of neutrophils decreases in the presence of endotoxins. There is little evidence that endotoxins are themselves chemotactic (3, 24, 28, 44), but endotoxins have been reported to induce chemotactic activity in plasma by the activation of complement (16, 25, 36).

Endotoxins also have been reported to inhibit chemotaxis of neutrophils to certain other chemotaxins. Issekutz and coworkers (20, 21) found that endotoxins inhibit chemotaxis to C5a but not to culture supernatants of *Escherichia coli*, and other reports (18, 19) have supported the finding that endotoxins can inhibit chemotaxis to complement-derived chemotaxins. However, Haslett et al. (17) and Nitzan et al. (26) provided evidence that endotoxins can inhibit chemotaxis of human neutrophils to the synthetic bacterial product *N*-formyl-methionyl-leucyl-phenylalanine (FMLP).

Interleukin-8 (IL-8) (neutrophil-activating peptide or protein [1, 22]; monocyte-derived neutrophil chemotactic factor [15, 33, 45]; monocyte-derived neutrophil-activating peptide [35]; neutrophil-activating factor [4, 27, 38, 42]; and granulocyte chemotactic protein [39]) is a cytokine derived primarily from stimulated monocytes. IL-8 is a possible mediator of inflammation, since its biological activities (43) include the induction of blood granulocytosis when injected intravenously as well as local plasma leakage and rapid and sustained accumulation of neutrophils when injected intradermally (11, 12, 30, 40). In vitro in optimal concentrations, IL-8 is approximately equally as potent a chemotactic factor as are optimal concentrations of C5a, leukotriene B4, and formyl peptide (1). Unlike C5a, platelet-activating factor, and leukotrienes, however, IL-8 is resistant to degradation by plasma peptidases (27), enhancing the likelihood of its significance in human inflammatory responses (1). Little is known of the effects of endotoxins on chemotaxis of neutrophils to IL-8 in any assay system.

In our laboratory, an improved Boyden-type chamber method for the measurement of chemotaxis in vitro has recently been devised; it involves the use of a "sparse-pore" polycarbonate membrane between the two compartments of the chamber (5–7). The pores of the new membrane average approximately 48 μm apart, allowing neutrophils adherent to the membrane ample room to move without encountering a pore leading to the lower surface unless chemotactically attracted to a substance in the lower compartment diffusing from the pores. The new method allows a clear distinction between chemotactic movement and random movement of neutrophils and has the additional advantage of allowing concurrent studies of random motility to be carried out with the same substratum (polycarbonate membrane of standard pore density) in a chamber similar to that used in the chemotaxis assay (9). When one type of substratum is used throughout various assays, the problem of diverse properties of different substrata in different assays is avoided.

* Corresponding author.

This improved method was used to determine the effects of endotoxins from a variety of bacteria on chemotaxis to IL-8 and to FMLP as well as to assess the effects of endotoxins on random motility of neutrophils. In addition, the possibility of the endotoxins themselves being chemotactic was investigated. The complement-activating activity of each endotoxin was tested by estimating the levels of C3c and C4 in treated human serum and by determining the ability of treated serum to induce hemolysis of sheep erythrocytes by an appropriate antibody after incubation for 1 h with each endotoxin (100 μg/ml).

Phenol-extracted endotoxins of *E. coli* serotype O111:B4 (catalog no. L-2630), *Klebsiella pneumoniae* 15380 (catalog no. L-4268), *Vibrio cholerae* serotype Inaba 569B (catalog no. L-5262), *Shigella flexneri* serotype 1A (catalog no. L-9018), *Salmonella typhosa* 14901 (catalog no. L-2387), and *Pseudomonas aeruginosa* serotype 10 (Habs) (catalog no. L-8643) and FMLP (catalog no. F-3506) were obtained from Sigma (St. Louis, Mo.). Recombinant human IL-8 was the kind gift of I. Lindley, Sandoz Forschungsinstitut, Vienna, Austria. Neutrophils were separated from the peripheral blood of healthy volunteers by the Ficoll-Hypaque method as previously described (8) with a final concentration of 9,000 cells per μl. The chambers for chemotaxis and random motility were of the micro-Boyden type and were incubated at 37°C for 20 min as previously described (9). Fixation, staining, and counting of the cells attached to the sparse-pore (for chemotaxis) and standard (for random motility) membranes were as previously described (9). The incubation medium for all experiments was Hanks solution with 10% (vol/vol) autologous fresh heparinized plasma (obtained as the supernatant after centrifugation in the neutrophil separation technique) and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.2. To test the effects of endotoxins on chemotaxis to either IL-8 (100 ng/ml) or FMLP (10^{-7} M), we placed chemoattractant with endotoxin (100 μg/ml) in the lower compartments of the chambers and cells with the corresponding endotoxin (100 μg/ml) in the upper compartments. The results were compared with those for chemoattractant without endotoxin in either compartment as a positive control. To test the effects on random motility, we placed medium with endotoxin in the lower compartments and cells with endotoxin in the upper compartments. The random motility of these cells was compared with the motility in medium without endotoxin in either compartment. To test the possible chemotactic activity of the endotoxins themselves, we placed endotoxin in medium in the lower compartments and cells in medium alone in the upper compartments. Migration was compared with migration in chambers with medium alone in each compartment.

Complement studies consisted of nephelometric measurements of the levels of binding of appropriate antibodies (Behring Diagnostics, Sydney, New South Wales, Australia; catalog no. OSAP15 and OSAO15) to C3c and C4 and estimates of hemolysis of sheep erythrocytes induced by treated serum (Quantiplate; Kallestad Inc., Chaska, Minn.).

Statistical comparisons were made with a paired two-tailed *t* test.

The results of the investigation of the effects of endotoxins on chemotaxis to IL-8 and FMLP are shown in Table 1. Chemotaxis (percentage of cells passing through the sparse-pore membrane) to IL-8 and chemotaxis to FMLP in the absence of endotoxin were 24.3% ± 4.3% and 32.2% ± 16.1%, respectively. In the presence of the various endotoxins the chemotactic responses to IL-8 were all significantly ($P < 0.05$) lower than those in the presence of IL-8 alone and ranged between 9.2% ± 8.4% and 15.7% ± 19.7%. No endotoxin, however, had any statistically significant effect on chemotaxis of the cells to FMLP.

TABLE 1. Effects of endotoxins on chemotaxis of neutrophils to FMLP and IL-8

Endotoxin	% of cells passing through a sparse-pore membrane (mean ± SE for triplicate experiments) to:	
	FMLP	IL-8
None	32.2 ± 16.1	24.3 ± 4.3
E. coli	36.0 ± 18.3	15.7 ± 19.7[a]
K. pneumoniae	42.0 ± 14.7	9.8 ± 7.0[a]
V. cholerae	37.4 ± 5.6	14.3 ± 14.5[a]
S. flexneri	23.8 ± 6.5	9.2 ± 8.4[a]
S. typhosa	35.7 ± 16.8	9.4 ± 8.0[a]
P. aeruginosa	43.9 ± 23.2	10.6 ± 7.7[a]

[a] Result was significantly ($P < 0.05$) different from that for IL-8 in the absence of endotoxin.

The results of the study of the effects of endotoxins on random motility and the possible chemotactic activity of the endotoxins themselves are shown in Table 2. The random motility of neutrophils through a standard polycarbonate membrane in the chambers in the presence of *S. flexneri* endotoxin (25.3% ± 18.6% of cells passed through the membrane) was significantly ($P < 0.05$) lower than that in the absence of endotoxin (70.1% ± 15.7% of cells passed through the membrane) in medium alone, but none of the other endotoxins had a significant effect on the random motility of the cells. No significant chemotactic response (percentage of cells passing through the sparse-pore membrane in the chambers) to any endotoxin in the lower compartment was detected.

In the studies of complement activation (Table 3), endotoxins of *K. pneumoniae* and *P. aeruginosa* moderately and markedly reduced total hemolytic complement levels in serum, respectively, but without changing C3c and C4 levels, as measured by sheep erythrocyte assays. No other endotoxin had any effect on the parameters of complement activation tested by these methods.

The present results concerning the lack of effect of endotoxins on chemotaxis to formyl peptide are difficult to reconcile with previous reports of a corresponding positive

TABLE 2. Chemotactic potency and effects on random motility of endotoxins[a]

Endotoxin	Chemotactic potency (% of cells passing through a sparse-pore membrane)	Random motility (% of cells passing through a standard membrane)
FMLP (positive control)	36.6 ± 1.0	ND
None (negative control)	4.4 ± 3.5	70.1 ± 15.7
E. coli	7.3 ± 9.9	73.2 ± 6.4
K. pneumoniae	11.9 ± 8.0	43.8 ± 20.6
V. cholerae	7.0 ± 5.0	55.9 ± 21.6
S. flexneri	8.3 ± 8.7	25.3 ± 18.6[b]
S. typhosa	15.4 ± 13.6	79.6 ± 11.9
P. aeruginosa	11.5 ± 10.4	51.2 ± 19.3

[a] Results are means ± standard errors for triplicate experiments.
[b] Result was significantly ($P < 0.05$) different from that in the absence of endotoxin.

TABLE 3. Effects of endotoxins (100 μg/ml) on complement fractions of fresh human serum

Endotoxin	Amt (g/liter) of:		Hemolysis (U/ml)
	C3c	C4	
None	0.79	0.31	180
E. coli	0.74	0.28	180
K. pneumoniae	0.85	0.29	99
V. cholerae	0.77	0.28	164
S. flexneri	0.86	0.29	148
S. typhosa	0.75	0.28	180
P. aeruginosa	0.93	0.30	<23

effect (18, 26). However, methodological differences, especially the improved technique and possibly the different substratum used in our chemotaxis assay, may have been of significance. The lack of apparent activation of the complement system by all endotoxins except those of *K. pneumoniae* and *P. aeruginosa* may have been related to the dose of endotoxin used (100 μg/ml), since much higher doses on the order of milligrams per milliliter) have been required to activate the complement system of serum in previous studies (24). Alternatively, the sheep erythrocyte assays used in the present study may have been insensitive to changes in the activation of the alternative complement pathway (32).

The inhibitory effect of all endotoxins on chemotaxis to IL-8 but not to FMLP may indicate different mechanisms of induction of chemotaxis by the two agents. Chemotaxis to FMLP is mediated by the FMLP receptor, transduced through GTP-binding membrane proteins (2). The receptor for IL-8 is distinct from the receptor for FMLP (4, 34), and the IL-8 receptor or its corresponding transduction mechanism may be sensitive to inactivation by endotoxins. However, the inhibitory effect of endotoxins on chemotaxis to IL-8 also appears to parallel the inhibitory effect of these substances on chemotaxis to C5a (18, 20, 21). It is conceivable that separate mechanisms govern chemotaxis to endogenous mediators and to formyl peptide (37). The further elucidation of the mechanisms of effects of endotoxins on neutrophils will necessarily involve the study of both the control mechanisms and the transduction mechanisms associated with these receptors.

This work was supported by the Australian Research Council.

REFERENCES

1. **Baggiolini, M., A. Walz, and S. L. Kunkel.** 1989. Neutrophil-activating peptide/interleukin 8, a novel cytokine that activates neutrophils. J. Clin. Invest. **84:**1045–1049.
2. **Becker, E. L.** 1987. The formyl-peptide receptor of the neutrophil. A search and conserve operation. Am. J. Pathol. **129:**16–24.
3. **Bennett, I. L., and L. E. Cluff.** 1957. Bacterial pyrogens. Pharmacol. Rev. **9:**427–475.
4. **Besemer, L., A. Hujber, and B. Kuhn.** 1989. Specific binding, internalization, and degradation of human neutrophil activating factor by human polymorphonuclear leukocytes. J. Biol. Chem. **264:**17409–17415.
5. **Bignold, L. P.** 1987. A novel polycarbonate (Nuclepore) membrane demonstrates chemotaxis unaffected by chemokinesis in the Boyden chamber. J. Immunol. Methods **105:**275–280.
6. **Bignold, L. P.** 1988. Kinetics of chemoattraction of polymorphonuclear leukocytes towards N-formyl peptide studied using a novel polycarbonate (Nuclepore) membrane in the Boyden chamber. Experientia **44:**518–521.
7. **Bignold, L. P.** 1988. Review. Measurement of chemotaxis of polymorphonuclear leukocytes in vitro: the problems of control of gradients of chemotactic factors, of control of the cells and of separation of chemotaxis from chemokinesis. J. Immunol. Methods **108:**1–18.
8. **Bignold, L. P., and A. Ferrante.** 1987. Mechanism of separation of polymorphonuclear leukocytes from whole blood by the one-step Hypaque-Ficoll method. J. Immunol. Methods **96:**29–33.
9. **Bignold, L. P., S. D. Rogers, and D. G. Harkin.** 1990. Effects of plasma proteins on adhesion, spreading, polarisation in suspension, random motility and chemotaxis of neutrophil leukocytes on polycarbonate (Nuclepore) filtration membrane. Eur. J. Cell Biol. **53:**27–34.
10. **Bryant, R. E., R. M. Des Prez, and D. E. Rogers.** 1967. Studies on human leukocyte motility. II. Effects of bacterial endotoxin on leukocyte migration adhesiveness and aggregation. Yale J. Biol. Med. **40:**192–204.
11. **Colditz, I., R. Zwahlen, B. Bewald, and M. Baggiolini.** 1989. In vivo inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. Am. J. Pathol. **134:**755–760.
12. **Colditz, I. G., R. D. Zwahlen, and M. Baggiolini.** 1990. Neutrophil accumulation and plasma leakage induced in vivo by neutrophil-activating peptide-1. J. Leukocyte Biol. **48:**129–137.
13. **Cybulsky, M. I., M. K. W. Chan, and H. Z. Movat.** 1988. Acute inflammation and microthrombosis induced by endotoxin, interleukin-1 and tumour necrosis factor and their implication in Gram negative infection. Lab. Invest. **58:**365–378.
14. **Dahinden, C., C. Galanos, and J. Fehr.** 1983. Granulocyte activation by endotoxin 1. Correlation between adherence and other granulocyte functions and role of endotoxin structure on biologic activity. J. Immunol. **130:**857–862.
15. **Djeu, J. Y., K. Matsushima, J. J. Oppenheim, K. Shiotsuki, and D. K. Blanchard.** 1990. Functional activation of human neutrophils by recombinant monocyte-derived neutrophil chemotactic factor/IL-8. J. Immunol. **144:**2205–2210.
16. **Gewurz, H., H. S. Shin, and S. E. Mergenhagen.** 1968. Interactions of the complement system with endotoxin polysaccharide: consumption of each of the six terminal complement components. J. Exp. Med. **128:**1049–1057.
17. **Haslett, C., L. A. Guthrie, M. M. Kopaniak, R. B. Johnston, and P. M. Henson.** 1985. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am. J. Pathol. **119:**101–110.
18. **Henricks, P. A. J., M. E. van der Tol, R. M. W. N. Thyssen, B. S. van Asbeck, and J. Verhoef.** 1983. *Escherichia coli* lipopolysaccharides diminish and enhance cell functions of human polymorphonuclear leukocytes. Infect. Immun. **41:**294–301.
19. **Hermanowicz, A., A. Hermel, A. Nowak, Z. Sliwinski, Z. Narwarska, D. Borys, and B. Banas.** 1986. Function of phagocytes in experimentally induced colitis in rabbits. II. The Schwartzman phenomenon in the colon. Int. Arch. Allergy Appl. Immunol. **80:**245–252.
20. **Issekutz, A. C., and W. D. Biggar.** 1977. Influence of serum-derived chemotactic factors and bacterial products on human neutrophil chemotaxis. Infect. Immun. **15:**212–220.
21. **Issekutz, A. C., M. Ng, and W. D. Biggar.** 1979. Effect of cyclic adenosine 3′,5′ monophosphate antagonists on endotoxin-induced inhibition of human neutrophil chemotaxis. Infect. Immun. **24:**434–440.
22. **Larsen, C. G., O. Anderson, E. Appella, J. J. Oppenheim, and K. Matsushima.** 1989. The neutrophil-activating protein (NAP-1) is also chemotactic for T-lymphocytes. Science **243:**1464–1466.
23. **Martin, S. P., and S. H. Chaudhuri.** 1952. Effect of bacteria and their products on migration of leukocytes. Proc. Soc. Exp. Biol. Med. **81:**286–288.
24. **Morrison, D., and J. R. Ulevitch.** 1978. The effects of bacterial endotoxins on host mediation systems. Am. J. Pathol. **93:**525–617.
25. **Morrison, D. C., and L. F. Kline.** 1977. Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharide (LPS). J. Immunol. **118:**362–368.

26. **Nitzan, D. W., W. Pruzanski, S. Saito, and N. Ranadive.** 1985. Modulation of locomotor activity of polymorphonuclear leukocytes by cationic substances and cationic lysosomal fractions from human neutrophils. Inflammation **9:**375–387.
27. **Peveri, P., A. Walz, B. Dewald, and M. Baggiolini.** 1988. A novel neutrophil-activating factor produced by human mononuclear phagocytes. J. Exp. Med. **167:**1547–1560.
28. **Proctor, R. A.** 1985. Effects of endotoxins on neutrophils, p. 244–259. *In* L. J. Berry (ed.), Handbook of endotoxin, vol. 3. Elsevier, Amsterdam.
29. **Raetz, C. R. H.** 1990. Biochemistry of endotoxins. Annu. Rev. Biochem. **59:**129–170.
30. **Rampart, M., J. Van Damme, L. Zonnekeyn, and A. G. Herman.** 1989. Granulocyte chemotactic protein induces plasma leakage and neutrophil accumulation in rabbit skin. Am. J. Pathol. **135:**21–25.
31. **Rosenbaum, J. T., K. T. Hartiala, R. O. Webster, E. L. Howes, and I. M. Goldstein.** 1983. Antiinflammatory effects of endotoxin. Inhibition of rabbit polymorphonuclear leukocyte responses to complement (C5)-derived peptides in vivo and in vitro. Am. J. Pathol. **113:**291–299.
32. **Ruddy, S.** 1986. Complement, p. 175–184. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), American Society for Microbiology, Washington, D.C.
33. **Samanta, A. K., J. J. Oppenheim, and K. Matsushima.** 1989. Identification and characterization of specific receptors for monocyte-derived neutrophil chemotactic factor (MDNCF) on human neutrophils. J. Exp. Med. **169:**1185–1189.
34. **Samanta, A. K., J. J. Oppenheim, and K. Matsushima.** 1990. Interleukin-8 (monocyte-derived neutrophil chemotactic factor) dynamically regulates its own receptor expression on human neutrophils. J. Biol. Chem. **265:**183–189.
35. **Schröder, J.-M., U. Mrowietz, E. Morita, and E. Christophers.** 1987. Purification and partial biochemical characterisation of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. J. Immunol. **139:**3473–3483.
36. **Sveen, K.** 1978. The importance of C5 and the role of the alternative complement pathway in leukocyte chemotaxis induced in vivo and in vitro by *Bacteroides fragilis* lipopolysaccharide. Acta. Pathol. Microbiol. Scand. Sect. B **86:**93–100.
37. **Ternowitz, T., T. Herlin, and K. H. Fogh.** 1987. Human monocyte and polymorphonuclear leukocyte chemotactic and chemokinetic responses to leukotriene B4 and FMLP. Acta Pathol. Microbiol. Scand. Sect. C **95:**47–54.
38. **Thelen, M., P. Peveri, P. Kernen, V. von Tscharner, A. Walz, and M. Baggiolini.** 1988. Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. FASEB J. **2:**2702–2706.
39. **Van Damme, J., J. Van Beeumen, R. Conings, B. Decock, and A. Billiau.** 1989. Purification of granulocyte chemotactic peptide/interleukin-8 reveals N-terminal sequence heterogeneity similar to that of β-thromboglobulin. Eur. J. Biochem. **181:**337–344.
40. **Van Damme, J., J. Van Beemen, G. Opendakker, and A. Billiau.** 1988. A novel, NH_2-terminal sequence-characterised human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis-promoting activity. J. Exp. Med. **167:**1364–1376.
41. **Verghese, M. W., and R. Snyderman.** 1981. Differential antiinflammatory effects of LPS in susceptible and resistant mouse strains. J. Immunol. **127:**288–293.
42. **Walz, A., P. Peveri, H. Aschauer, and M. Baggiolini.** 1987. Purification and amino acid sequencing of Naf, a novel neutrophil-activating factor produced by monocytes. Biochem. Biophys. Res. Commun. **149:**755–761.
43. **Westwick, J., S. W. Li, and R. D. Camp.** 1989. Novel neutrophil-stimulating peptides. Immunol. Today **10:**146–147.
44. **Wilson, M. E.** 1985. Effects of bacterial endotoxins on neutrophil function. Rev. Infect. Dis. **7:**404–418.
45. **Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim, and E. J. Leonard.** 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc. Natl. Acad. Sci. USA **84:**9233–9237.