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NOTES

Antilipopolysaccharide Factor from Horseshoe Crab, *Tachypleus tridentatus*, Inhibits Lipopolysaccharide Activation of Cultured Human Endothelial Cells

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Antilipopolysaccharide (anti-LPS) factor is a basic protein that is purified from the hemocyte lysate of the Japanese and American horseshoe crabs (*Tachypleus tridentatus* and *Limulus polyphemus*). Anti-LPS factor has previously been reported to inhibit LPS-mediated activation of limulus factor C, lyse endotoxin-sensitized erythrocytes, and inhibit the growth of some gram-negative bacteria. In this study, we examine the ability of anti-LPS factor purified from *T. tridentatus* to inhibit the activation of cultured human endothelial cells by LPS. Anti-LPS factor inhibited the stimulation of endothelial adhesiveness for neutrophils by LPS in a dose-dependent manner. Maximum inhibition was achieved when anti-LPS factor was mixed with LPS prior to addition to the endothelial cell monolayers. Anti-LPS factor inhibited endothelial cell activation by LPS derived from *Salmonella minnesota* Re and Rc mutants as well as from the wild type (smooth), suggesting that it recognizes the lipid A moiety of LPS.

Horseshoe crabs (*Limulus polyphemus* and *Tachypleus tridentatus*) are phylogenetically ancient creatures that have provided important insights into the evolution of coagulation and antibacterial systems (13). The vasculature of these crabs contains only one cell type, the hemocyte (also known as the amebocyte), and no circulating plasma coagulation factors (14). When hemocytes are exposed to endotoxin, they degranulate and form a gel (2). The exquisite sensitivity of the hemolymph coagulation system to levels of endotoxin as low as 10 pg/ml has led to its use in testing reagents and biologic fluids for the presence of endotoxin (7).

Iwanaga et al. have shown that coagulation of *T. tridentatus* hemolymph occurs via a sequence of hemocyte granule proteins in a manner analogous to the human coagulation cascade (13). Recently, all of the proteins in the pathway have been characterized and purified. During the purification of these proteins, a potent inhibitor of lipopolysaccharide (LPS)-induced coagulation was discovered (25). This protein, anti-LPS factor, contains 102 amino acids and has a molecular weight of 11,500. The amino-terminal region of anti-LPS factor is very basic, and the hydropathy profile of the entire protein suggests that it is an amphipathic molecule (1). The amino acid sequences of anti-LPS factor isolated from *L. polyphemus* and from *T. tridentatus* are nearly identical (19). Preliminary characterization of the biologic activities of the purified protein has revealed that anti-LPS factor lyses endotoxin-sensitized erythrocytes (21) and inhibits the growth of rough mutants of gram-negative bacteria (18).

Endogenous proteins that modulate the biological activity of endotoxin are important, not only because they provide insights into mechanisms of host defense against gram-negative bacteria, but also because they may suggest new therapeutic approaches to controlling the deleterious effects of gram-negative endotoxins. For example, proteins that bind LPS could perhaps be used to prevent LPS from interacting with the critical cellular mediators of endotoxicity.

Endothelial cells appear to be one of the important cellular targets of LPS (3, 5, 12). When exposed to LPS in vitro, endothelial cells become activated, as evidenced by increased adhesiveness for neutrophils (22, 24); enhanced production of prostacyclin (20); surface expression of tissue factor (4); and release of tissue plasminogen activator-inhibitor (6, 11), interleukin-1 (15, 17), and granulocyte-macrophage colony-stimulating activity (23). Since these proinflammatory and procoagulant effects of LPS on endothelial cells may contribute significantly to the pathophysiology of sepsis, we examined the ability of anti-LPS factor purified from *T. tridentatus* to inhibit the activation of human endothelial cells by LPS in vitro by using the induction of an endothelial adhesion molecule for neutrophils as our assay.

Anti-LPS factor purified from *T. tridentatus* was a generous gift of Sadaaki Iwanaga, Kyushu University, Fukuoka, Japan. It was suspended in phosphate-buffered saline (0.15 M sodium chloride, 0.01 M sodium phosphate [pH 7.4] at 1 mg/ml and diluted for cell assays. *Salmonella typhimurium*, *Escherichia coli* O55:B5, and *E. coli* J5 LPS were purchased from Sigma Chemical Co., St. Louis, Mo. *S. minnesota* Re and Rc LPS were purchased from List Biological Laboratories, Inc., Campbell, Calif.

Human umbilical vein endothelial cells were isolated as previously described (27). Cells were cultured in RPMI 1640 medium (Whittaker MA Bioproducts, Walkersville, Md.) containing 10 mM *N*-2-hydroxyethylpiperazine-N'-2-ethane-

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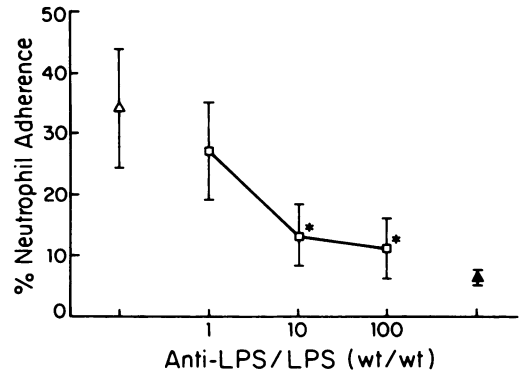

FIG. 1. Dose-dependent inhibition by anti-LPS factor of LPS-induced stimulation of endothelial cell adhesiveness for neutrophils. *E. coli* LPS (100 ng/ml) was incubated with anti-LPS factor (10 ng/ml to 10 μg/ml) for 30 min at room temperature. The mixture was then added to endothelial cells, and the adherence assay was performed after a 4-h incubation. Ratios by weight of anti-LPS factor to LPS are expressed on the abscissa. Adherence induced by LPS alone (△) or medium alone (▲) is indicated. Values represent the means ± SE of three experiments. Symbol: *, $P < 0.05$ compared with LPS alone by paired *t* test.

sulfonic acid (HEPES), 20% fetal calf serum (Hyclone Sterile Systems, Logan, Utah), 20 μg of endothelial cell-derived growth factor per ml (16), and 90 μg of heparin (Sigma) per ml. (26).

The neutrophil adherence assay was performed by the method of Gamble et al. (10). Human neutrophils were purified by standard techniques of Ficoll-Hypaque gradient centrifugation, dextran sedimentation, and hypotonic lysis of contaminating erythrocytes. This procedure resulted in a cell population containing more than 90% neutrophils and being greater than 95% viable by trypan blue dye exclusion. The isolated neutrophils were labeled with ^{51}Cr as described previously (9) and suspended in RPMI 1640 medium at 2,000/μl. First- or second-passage endothelial cells were plated at confluent density in 48-well plates. After overnight incubation, growth medium was removed, and 250-μl portions of RPMI 1640 medium containing 5% heat-inactivated normal calf serum and various concentrations of LPS or anti-LPS factor were added to triplicate wells for 4 h at 37°C. After the period of stimulation, the monolayers were washed once with phosphate-buffered saline. Radiolabeled neutrophils were then added in 250 μl of RPMI 1640 medium (5×10^5 cells). After a 30-min incubation, supernatant medium and nonadherent neutrophils were decanted, and each well was washed once with phosphate-buffered saline. Adherent, radiolabeled neutrophils were solubilized with 1 M ammonium hydroxide, and the entire material from each well was analyzed for radioactivity in a gamma counter. Adherence was determined as a percent of total ^{51}Cr added: percent adherence = (^{51}Cr counts per minute in NH_4OH lysate/total ^{51}Cr counts per minute added) × 100.

When anti-LPS factor and LPS were mixed and incubated at room temperature for 30 min before being added to endothelial cells, the induction of an endothelial cell adhesiveness for neutrophils was inhibited in a dose-dependent manner (Fig. 1). Ratios of anti-LPS factor to LPS (wt/wt) of 10:1 or greater significantly decreased LPS-stimulated endothelial adhesiveness for neutrophils. At a 20:1 ratio, anti-LPS factor inhibited LPS-stimulated neutrophil binding by 78 ± 9% (mean ± standard error [SE] of six experiments; $P < 0.05$). Anti-LPS factor was not toxic to endothelial cells at concentrations up to 10 μg/ml for 24 h as assessed by phase-contrast microscopy, ^{51}Cr release, and trypan blue dye exclusion (data not shown).

TABLE 1. Inhibition by anti-LPS factor of stimulation of endothelial cell adhesiveness for neutrophils by rough and smooth LPS[a]

Species	% Neutrophil adherence[b]	
	Medium	Anti-LPS factor
None (medium alone)	2 ± 0	2 ± 1
E. coli 055:B5	41 ± 2	14 ± 3[c]
S. typhi	39 ± 8	9 ± 2[c]
S. minnesota Re	22 ± 3	6 ± 2[c]
S. minnesota Rc	39 ± 3	4 ± 0[c]
E. coli J5	40 ± 5	18 ± 3[c]

[a] Endothelial cell monolayers were pretreated with medium alone or medium containing LPS (200 ng/ml) with or without anti-LPS factor (4 μg/ml). LPS and anti-LPS factor were mixed at room temperature for 30 min before being added to endothelial cells.

[b] Values represent means ± SE from triplicate wells.

[c] $P < 0.05$ by the two-tailed, unpaired *t* test.

Inhibition of LPS-stimulated endothelial adhesiveness for neutrophils by anti-LPS factor was consistently greater when anti-LPS factor and LPS were mixed prior to addition to endothelial cells. In three separate experiments, incubation of anti-LPS factor (4 μg/ml) with LPS (200 ng/ml) for 30 min at room temperature prior to addition to endothelial cells reduced adherence by 59 ± 1% compared with 24 ± 5% when anti-LPS factor and LPS were added simultaneously (mean ± SE; $P < 0.05$). Pretreatment of endothelial cells with anti-LPS factor did not prevent subsequent activation of endothelial cells by LPS when the anti-LPS factor was removed by washing prior to the addition of LPS (data not shown). These results suggest that anti-LPS factor prevents endothelial cell activation by LPS by interacting with LPS rather than with the endothelial cells.

Although premixing of LPS with anti-LPS factor produced the greatest effect, significant inhibition of LPS-stimulated endothelial cell activation was still observed when anti-LPS factor was added to the endothelial cells at the same time as LPS. Notably, anti-LPS factor was inhibitory even when added to the endothelial cells up to 1 h after LPS. In six experiments, pretreatment of endothelial cells with LPS (200 ng/ml) alone for 4 h resulted in neutrophil adherence of 27 ± 7% (mean ± SE). Addition of anti-LPS factor (4 μg/ml) to the incubation medium 1 h before, at the same time as, or 1 h after addition of LPS resulted in adherence values of 17 ± 5, 16 ± 6, and 17 ± 5%, respectively (mean ± SE of six experiments; $P < 0.05$ compared with LPS alone). Addition of anti-LPS factor to endothelial cells after a 2-h exposure to LPS, however, did not consistently prevent LPS-induced neutrophil adherence. Deacylated LPS has also been shown to inhibit LPS-induced endothelial adhesiveness for neutrophils when added up to 1 h after exposure to LPS (22). Together, these results suggest that maximal stimulation of some endothelial cell responses requires prolonged exposure to LPS.

Anti-LPS factor inhibited endothelial cell stimulation by LPS derived from several members of the family *Enterobacteriaceae* (Table 1). Both rough and smooth forms of LPS were inhibited equally, suggesting that anti-LPS factor binds to the lipid A or 2-keto-3-deoxyoctulosonic acid core regions, which are the least variable components of the gram-negative bacterial cell wall (8). Cationic and hydrophobic,

not covalent, forces appear to mediate the molecular interactions of anti-LPS factor with LPS, which probably occur via the very amphipathic NH_2-terminal region of anti-LPS factor (18).

In summary, our studies demonstrate that anti-LPS factor purified from the horseshoe crab *T. tridentatus* inhibits the activation of cultured human endothelial cells by LPS. These findings extend the biologic activities of this protein and demonstrate that some proteins that interact with lipid A can prevent activation of critical effector cells by LPS.

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LITERATURE CITED

1. **Aketagawa, J., T. Miyata, S. Ohtsubo, T. Nakamura, T. Morita, H. Hayashida, T. Miyata, and S. Iwanaga.** 1986. Primary structure of Limulus anticoagulant anti-lipopolysaccharide factor. J. Biol. Chem. **261**:7357–7365.
2. **Bang, F. B.** 1979. Ontogeny and phylogeny of response to gram-negative endotoxins among the marine invertebrates, p. 109–123. *In* E. Cohen (ed.), Biomedical applications of the horseshoe crab (Limulidae). Alan R. Liss, Inc., New York.
3. **Bevilacqua, M. P., and M. A. Gimbrone, Jr.** 1987. Inducible endothelial functions in inflammation and coagulation. Semin. Thromb. Hemostasis **13**:425–433.
4. **Colucci, M., G. Balconi, R. Lorenzet, A. Pietra, D. Locati, M. B. Donati, and N. Semararo.** 1983. Cultured human endothelial cells generate tissue factor in response to endotoxin. J. Clin. Invest. **71**:1893–1896.
5. **Cotran, R. S.** 1987. New roles for the endothelium in inflammation and immunity. Am. J. Pathol. **129**:407–413.
6. **Emeis, J. J., and T. Kooistra.** 1986. Interleukin-1 and lipopolysaccharide induce an inhibitor of tissue-type plasminogen activator in vivo and in cultured endothelial cells. J. Exp. Med. **163**:1260–1266.
7. **Friberger, P.** 1985. The design of a reliable endotoxin test, p. 139–149. *In* J. W. Cape (ed.), Bacterial endotoxins: structure, biomedical significance and detection With the Limulus amebocyte lysate test. Alan R. Liss, Inc., New York.
8. **Galanos, C., O. Freudenberg, O. Luderitz, E. T. Rietschel, and O. Westphal.** 1979. Chemical, physiochemical and biologic properties of bacterial lipopolysaccharides, p. 321–332. *In* E. Cohen (ed.), Biomedical applications of the horseshoe crab (Limulidae). Alan R. Liss, Inc., New York.
9. **Gallin, J. T., R. A. Clark, and H. R. Kimball.** 1973. Granulocyte chemotaxis: an improved in vitro assay employing ^{51}Cr-labelled granulocytes. J. Immunol. **110**:233–240.
10. **Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas.** 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc. Natl. Acad. Sci. USA **82**:8667–8671.
11. **Hanss, M., and D. Collen.** 1987. Secretion of tissue-type plasminogen activator and plasminogen activator inhibitor by cultured human endothelial cells: modulation by thrombin, endotoxin, and histamine. J. Lab. Clin. Med. **109**:97–104.
12. **Harlan, J. M., L. A. Harker, G. E. Striker, and L. J. Weaver.** 1983. Effects of lipopolysaccharide on human endothelial cells in culture. Thromb. Res. **29**:15–26.
13. **Iwanaga, S., T. Morita, T. Miyata, T. Nakamura, and J. Aketagawa.** 1986. The hemolymph coagulation system in invertebrate animals. J. Pro. Chem. **5**:255–268.
14. **Levin, J., and F. B. Bang.** 1964. The role of endotoxin in the extracellular coagulation of Limulus blood. Bull. Johns Hopkins Hosp. **115**:265–274.
15. **Libby, P., J. M. Ordovas, K. R. Auger, A. J. Robbins, L. K. Birinyi, and C. A. Dinarello.** 1986. Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. Am. J. Pathol. **124**:179–185.
16. **Maciag, T., J. Cerudolo, S. Ilsley, P. R. Kelley, and R. Forand.** 1979. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc. Natl. Acad. Sci. USA **76**:5674–5678.
17. **Miossec, P., D. Cavender, and M. Ziff.** 1986. Production of interleukin 1 by human endothelial cells. J. Immunol. **136**: 2486–2491.
18. **Morita, T., S. Ohtsubo, T. Nakamura, S. Tanaka, S. Iwanaga, K. Ohashi, and M. Niwa.** 1985. Isolation and biological activities of Limulus anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). J. Biochem. **97**:1611–1620.
19. **Muta, T., T. Miyata, F. Tokunaga, T. Nakamura, and S. Iwanaga.** 1987. Primary structure of anti-lipopolysaccharide from American horseshoe crab, *Limulus polyphemus*. J. Biochem. **101**:1321–1330.
20. **Nawroth, P. P., D. M. Stern, K. L. Kaplan, and H. L. Nossel.** 1984. Prostacyclin production by perturbed bovine aortic endothelial cells in culture. Blood **64**:801–806.
21. **Ohashi, K., M. Niwa, T. Nakamura, T. Morita, and S. Iwanaga.** 1984. Anti-LPS factor in the horsehoe crab *Tachypleus tridentatus*. FEBS Lett. **176**:207–210.
22. **Pohlman, T. H., R. S. Munford, and J. M. Harlan.** 1987. Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide in vitro. J. Exp. Med. **165**:1393–1402.
23. **Quesenberry, P. J., and M. A. Gimbrone.** 1980. Vascular endothelium as a regulator of granulopoiesis: production of colony-stimulating activity by cultured human endothelial cells. Blood **56**:1060–1067.
24. **Schleimer, R. P., and B. K. Rutledge.** 1986. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. J. Immunol. **136**:649–654.
25. **Tanaka, S., T. Nakamura, T. Morita, and S. Iwanaga.** 1982. Limulus anti-LPS factor: an anticoagulant which inhibits the endotoxin-mediated activation of Limulus coagulation system. Biochem. Biophys. Res. Commun. **105**:717–723.
26. **Thornton, S. C., S. N. Mueller, and E. M. Levine.** 1983. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. Science **222**:623–625.
27. **Wall, R. T., L. A. Harker, L. J. Quadracci, and G. E. Striker.** 1978. Factors influencing endothelial proliferation in vitro. J. Cell. Physiol. **96**:203–213.