INFECTION AND IMMUNITY, Feb. 1990, p. 566–568
0019-9567/90/020566-03$02.00/0

Vol. 58, No. 2

Stimulation of Oxidative Burst in Human Monocytes by Lipoteichoic Acids

RACHEL LEVY,[1] MALAK KOTB,[2]* OFRA NAGAUKER,[1] GIPSY MAJUMDAR,[2] MICHAEL ALKAN,[1] ITZHAK OFEK,[2] AND EDWIN H. BEACHEY[2]

Clinical Biochemistry Unit and Infectious Disease Institute, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel,[1] and Veterans Administration Medical Center and University of Tennessee, Memphis, Tennessee 38104[2]

Received 25 July 1989/Accepted 25 October 1989

Lipoteichoic acid isolated from *Streptococcus faecalis* or *Streptococcus pyogenes* caused direct activation of the respiratory burst in human peripheral blood monocytes. This activity appears to be related to the ability of lipoteichoic acid to bind to the monocyte membrane and trigger the polarization of receptors (capping).

One of the most important host defense mechanisms against invading microbial pathogens is phagocytosis by professional phagocytes and the subsequent killing of the invading organisms by oxygen radicals (14, 19, 20). Although other antimicrobial activities of phagocytic cells, such as lysosomal enzymes (10) and cationic proteins (17, 26), have been reported, the highly toxic oxygen radicals are thought to play an important role in the inflammation and killing of endocytosed bacteria by phagocytic cells (14, 19, 20). Thus, bacterial products which stimulate oxidative burst in phagocytic cells may play an important role in the infectious process. Lipoteichoic acid (LTA) has been shown to be such a product (9, 25).

Most gram-positive bacteria contain LTA in their cytoplasmic membranes (2, 4, 16, 29, 30). LTA molecules are high-molecular-weight amphiphiles consisting of a hydrophobic glycolipid moiety and a hydrophilic chain composed of a glycerol phosphate polymer (15, 30). They are located on the surfaces of gram-positive bacteria as well as secreted to the extracellular milieu and are centrally involved in the binding of these bacteria to a variety of mammalian cells (1, 3, 4, 21, 23, 29, 30). In addition to being the adhesion factor for gram-positive bacteria (2), LTA may also play an important role in the pathogenesis of infectious diseases caused by these organisms (21). Several studies have demonstrated that LTA isolated from various organisms stimulates phagocytic cells (9, 11, 25, 28). The mechanisms by which LTA activates these cells have not been elucidated. In the present study, we examined the cell membrane-binding properties of LTA in relation to its ability to stimulate oxidative burst in human monocytes.

Peripheral blood was obtained from healthy adult donors. Mononuclear cells were separated on a Ficoll-Hypaque gradient (5). Monocytes were obtained from a Percoll gradient (27) and suspended in pyrogen-free Hanks balanced salt solution (HBSS). Polymorphonuclear leukocytes (PMN) were obtained by the dextran sedimentation method (22). The purity of each cell population was assessed by histochemical staining and light microscopy, and each was more than 95% homogeneous.

LTA from *Streptococcus faecalis* was purchased from Sigma Chemical Co. (St. Louis, Mo.) and purified by phenol-water extraction and anion-exchange chromatography on DEAE-Sephacel (8). This preparation retained alanine ester substitution and was in the form of sodium salt. The molar ratio of glycerol to phosphate was 1:1. LTA from *Streptococcus pyogenes* was extracted and purified as described (7). This LTA was essentially free of amino acids (except alanine) and of sugars (except glucose, 2 mol/mol of LTA) and had a glycerol/phosphate ratio of 1:1. LTA was freshly diluted in pyrogen-free HBSS before each experiment.

Antibodies to *S. pyogenes* were raised in New Zealand White rabbits as previously described (7). Deacylated LTA (polyglycerol phosphate; d-LTA) was obtained from the purified preparation of *S. pyogenes* LTA as described previously (23). The cell membrane binding by the LTA preparations was determined by their ability to sensitize erythrocytes as described previously (23) and was found to be 320 (i.e., a 1:32 dilution of 100 μg of LTA per ml gave positive hemagglutination with a 1:100 dilution of anti-LTA antibodies). The d-LTA was without any detectable sensitizing activity but possessed a potent antigenic activity, as determined by the hemagglutination inhibition assay (23).

The binding of LTA and of d-LTA to the monocytes and the PMN preparations was assessed by immunofluorescence techniques as previously described (7). Highly purified prep-

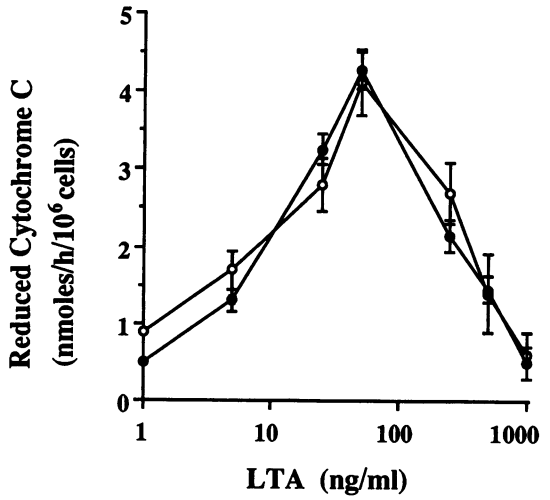

FIG. 1. Dose response curve for LTA action on cytochrome *c* reduction in monocytes LTA purified from *S. pyogenes* (○) or *S. faecalis* (●). The respiratory burst was measured for 1 h. Values are the means ± standard error of the mean (bars) of eight experiments.

* Corresponding author.

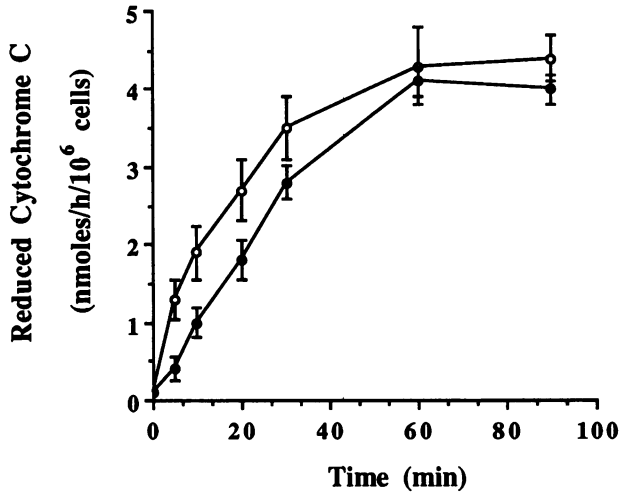

FIG. 2. Time course of the effect of LTA on cytochrome *c* reduction in monocytes. Effect of *S. pyogenes* (○) and *S. faecalis* (●) LTA. Bars represent the standard error of the mean.

arations of either monocytes or PMN were incubated for various times with 30 μg of LTA per ml and fixed with 1% paraformaldehyde. Binding of LTA was visualized by incubating the fixed monolayers first with a 1:50 dilution of anti-LTA antibodies for 30 min at 37°C and then with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies. The binding was examined by fluorescence microscopy.

Superoxide anion generation was assayed by the superoxide dismutase-inhibitable reduction of cytochrome *c* as described by Johnston et al. (12). Monocytes (10^6) in HBSS containing 80 μM ferricytochrome *c* (Sigma) were placed in plastic tissue culture dishes (35 by 10 mm; Nunc, Roskilde, Denmark). The reaction was initiated with the addition of LTA, and the cells were incubated in a humidified incubator with 5% CO_2 in air at 37°C. The reaction mixture was removed, placed in chilled microcentrifuge tubes, and centrifuged. The optical density of the supernatants was measured spectrophotometrically at 550 nm, and the concentration of reduced cytochrome *c* was determined. Results are expressed as nanomoles of O_2 per hour per 10^6 cells with an extinction coefficient of 21×10^{-3}/M per cm. Cultures containing 50 μg of superoxide dismutase per ml served as blanks. The differences in means were analyzed by Student's *t* test. The plots were drawn as least-squares regression lines and tested by analysis of variance.

LTA purified from group A streptococci (*S. pyogenes*) and from group D streptococci (*S. faecalis*) stimulated superoxide generation by monocytes in a similar fashion (Fig. 1). In contrast, d-LTA had no detectable activity (data not shown). Thus, it appears that as in the case of other biological activities of LTA, the stimulation of the oxidative burst in monocytes requires ester-linked fatty acids. LTA had a biphasic effect on the maximal cytochrome *c* reduction in monocytes (range, 1 to 1,000 ng/ml). The most effective concentration of LTA from either *S. faecalis* or *S. pyogenes* was 50 ng/ml, which stimulated cytochrome *c* reduction to 4.52 ± 0.2 and 4.1 ± 0.4 nmol, respectively (mean ± standard error of the mean). The biphasic behavior induced by LTA is not surprising, since a similar effect was also shown for other stimuli such as formylmethionylleucylphenylalanine, lipopolysaccharide, and tuftsin (13, 18). However, unlike lipopolysaccharide, LTA does not stimulate PMN directly (9, 25; our unpublished results). This finding, together with the lack of activity in the d-LTA preparations, rules out the possibility that the observed effects are mediated by trace contaminants of lipopolysaccharide in the buffers.

The time course effect of LTA (50 ng/ml) on cytochrome *c* reduction was also studied (Fig. 2). LTA caused an immediate increase in the reduction of cytochrome *c*. Stimulation of the oxidative burst reached a plateau after 60 min.

Binding of *S. pyogenes* LTA to monocytes was confirmed by immunofluorescence techniques (Fig. 3). Unlike that in PMN, the immunofluorescence label in monocytes was polar, suggesting that LTA receptors in these cells can be capped directly by LTA. Previous results by Courtney et al.

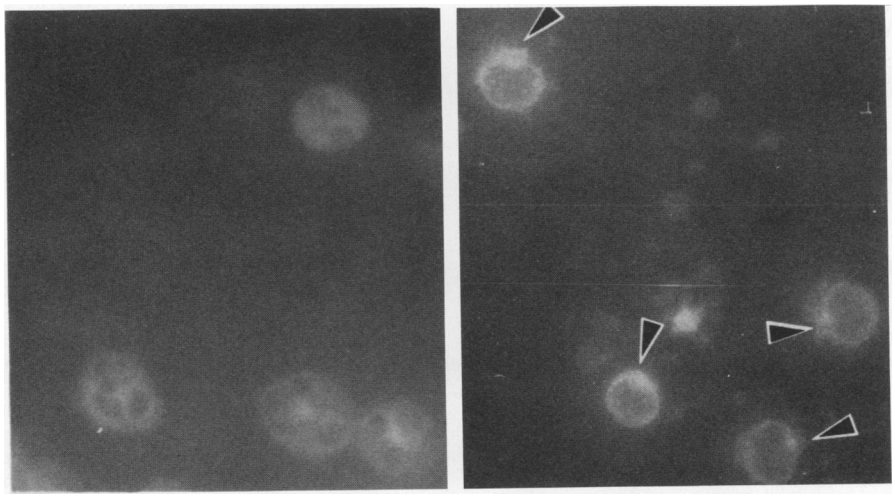

FIG. 3. Binding of LTA to human monocytes and PMN. Monolayers of monocytes (right) and PMN (left) were exposed to LTA (30 μg/ml) for 30 min at 37°C. The monolayers were fixed, washed, and reacted with anti-LTA antibody followed by fluorescein isothiocyanate goat anti-rabbit antibody as described in the text. Note that LTA caused capping of receptors in monocytes (arrowheads), while binding of LTA to PMN was uniformly distributed along the cell membrane. No fluorescence was seen in either monolayer when LTA was omitted (data not shown).

(7) demonstrated that LTA binds to PMN, but capping occurred only in the presence of anti-LTA antibodies. Recently, Ginsburg et al. (9) reported that LTA can stimulate an oxidative burst in PMN only in the presence of anti-LTA antibodies. In contrast, Ohshima et al. (25) observed direct stimulation of chemiluminescence in human monocytes by staphylococcal LTA. In our studies, we found that the magnitude of cytochrome *c* reduction in monocytes that were first incubated in the cold (4°C) with 50 ng of LTA per ml, washed, and then suspended in HBSS was the same as that in monocytes suspended in a 1:50 dilution of anti-LTA antibodies in HBSS (data not shown). Taken together, these observations are in line with reports that ligand-induced receptor cross-linking is required for certain membrane-initiated intracellular events (6).

In conclusion, it appears that compared with those on PMN, LTA receptors on monocytes are either distinct or presented in a way that allows LTA to stimulate oxidative burst directly in these cells, probably by binding and cross-linking the receptors.

It has been suggested that stimulation of LTA-sensitized PMN or monocytes by anti-LTA antibodies, which are present in most human sera, may occur during inflammation caused by gram-positive bacteria in deep tissues (9). Our findings suggest that direct stimulation of mononuclear phagocytes by LTA may be of significance in gram-positive infections at serum-poor sites such as the lung or renal medulla (24).

This study was supported by research funds from the U.S. Veterans Administration and by Public Health Service research grants GM-38530, AI-10085, and AI-13550 to M. Kotb and E. H. Beachey from the National Institutes of Health.

We are grateful to H. Courtney for preparing *S. pyogenes* LTA.

LITERATURE CITED

1. **Alkan, M. L., and E. H. Beachey.** 1978. Excretion of lipoteichoic acid by group A streptococci. Influence of penicillin on excretion and loss of ability to adhere to human oral mucosal cells. J. Clin. Invest. **61**:671–677.
2. **Beachey, E. H.** 1981. Bacterial adherence: adhesion receptor interaction mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:990–1003.
3. **Beachey, E. H., and I. Ofek.** 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid in fimbriae denuded of M protein. J. Exp. Med. **143**:759–771.
4. **Birdsell, D. C., R. J. Doyle, and M. Morgenstern.** 1975. Organization of teichoic acid in the cell wall of *Bacillus subtilis*. J. Bacteriol. **121**:726–734.
5. **Boyuam, A. C.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):77–89.
6. **Cohen, P., and M. D. Houslay.** 1985. Molecular mechanisms of transmembrane signalling, p. 1–487. Elsevier Biomedical Press, Amsterdam.
7. **Courtney, H., I. Ofek, W. A. Simpson, and E. H. Beachey.** 1981. Characterization of lipoteichoic acid binding to polymorphonuclear leukocytes of human blood. Infect. Immun. **32**:625–631.
8. **Fischer, W., H. U. Koch, and R. Haas.** 1983. Improved preparation of lipoteichoic acids. Eur. J. Biochem. **133**:523–530.
9. **Ginsburg, I., S. E. Fligiel, P. A. Ward, and J. Varani.** 1988. Lipoteichoic acid-antilipoteichoic acid complexes induce superoxide generation by human neutrophils. Inflammation **12**:525–548.
10. **Gordon, S.** 1980. Lysozyme and plasminogen activator: constitutive and induced secretory products of mononuclear phagocytes, p. 1273–1298. *In* R. van Furth, (ed.), Mononuclear phagocytes. Part II. Functional aspects. Martinus Nijhoff Netherlands, The Hague.
11. **Hamada, S., T. Yamamoto, T. Koga, J. R. McGhee, S. M. Michalek, and S. Yamamoto.** 1985. Chemical properties and immunological activities of streptococcal lipoteichoic acids. Zentralbl. Bakteriol. Parasitenkd. Infebktionskr. Hyg. Abt. 1 Orig. Reihe A **259**:228–243.
12. **Johnston, R. B., C. A. Godzik, and Z. A. Cohn.** 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J. Exp. Med. **148**:115–127.
13. **Kain, Z., M. Alkan, C. Chaimovitz, S. Segal, M. Fridkin, and R. Levy.** 1989. Human peritoneal macrophage activity is increased by tuftsin. Immunol. Lett. **21**:257–261.
14. **Klebanoff, S. J.** 1980. Oxygen intermediates and the microbicidal event, p. 1105–1137. *In* R. van Furth, (ed.), Mononuclear phagocytes. Part II. Functional aspects. Martinus Nijhoff Netherlands, The Hague.
15. **Koch, H. V., and W. Fischer.** 1978. Acyldiglucoxyldiacylglycerol-containing lipoteichoic acid with a poly (3-0-galabioxyl-2-0-galactosylsn-glycerol-1-phosphate) chain from *Streptococcus lactis* kiel- 42172. Biochemistry **17**:5275–5281.
16. **Lambert, P. A., I. C. Hancock, and J. Baddiley.** 1977. Occurrence and function of membrane teichoic acids. Biochim. Biophys. Acta **472**:1–12.
17. **Lehrer, R. I., D. Szklarek, M. E. Selsted, and J. Fleischmann.** 1981. Increased content of microbicidal cationic peptides in rabbit alveolar macrophages elicited by complete Freund adjuvant. Infect. Immun. **33**:775–778.
18. **McPhail, L. C., C. C. Clayton, and R. Snyderman.** 1984. The NADPH-oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals. J. Biol. Chem. **259**: 5768–5775.
19. **Nathan, C. F.** 1982. Secretion of oxygen intermediates: role in effector function of activated macrophages. Fed. Proc. **41**: 2206–2211.
20. **Nathan, C. F., and A. Nakagaware.** 1982. Role of reactive oxygen intermediates in macrophage killing of intracellular pathogens: a review, p. 279–294. *In* D. Mizuno, Z. A. Cohen, K. Kakeya, and N. Ishida (ed.), Self-defense mechanisms: role of macrophages. University of Tokyo Press, Tokyo, and Elsevier Biomedical Press, Amsterdam.
21. **Neeman, N., and I. Ginsburg.** 1979. Red cell sensitizing antigen of group A streptococci. II. Immunological and immunopathological properties. Isr. J. Med. Sci. **8**:1807–1816.
22. **Ofek, I., E. H. Beachey, and A. L. Bisno.** 1973. Resistance of Neisseria gonorrhoeae to phagocytosis: relationship to colonial morphology and surface pili. J. Infect. Dis. **129**:310–316.
23. **Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell.** 1975. Cell membrane binding properties of group A streptococcal lipoteichoic acid. J. Exp. Med. **141**:990–1003.
24. **Ofek, I., and N. Sharon.** 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect. Immun. **56**: 539–547.
25. **Ohshima, Y., J. Beuth, A. Yassin, H. L. Ko, and G. Pulverer.** 1988. Stimulation of human monocyte chemiluminescence by staphylococcal lipoteichoic acid. Med. Microbiol. Immunol. **177**:115–121.
26. **Patterson-Delafield, J., R. J. Martinez, and R. I. Lehrer.** 1980. Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host defense mechanism. Infect. Immun. **30**:180–192.
27. **Pertoft, H., K. Rubin, L. Kjellen, T. C. Laurent, and B. Klingeborn.** 1977. The viability of cells grown or centrifuged in a new density gradient medium, Percoll™. Exp. Cell. Res. **110**:449–457.
28. **Sen, V., D. P. Nerkar, D. S. Joshi, A. S. Kamat, and N. F. Lewis.** 1986. *In vivo* effect of lipoteichoic acid on biochemical function of peritoneal macrophages. Indian J. Exp. Biol. **24**:159–162.
29. **Wicken, A. J., and K. W. Knox.** 1975. Lipoteichoic acids: a new class of bacterial antigen. Science **187**:1161–1167.
30. **Wicken, A. J., and K. W. Knox.** 1980. Bacterial cell surface amphiphiles. Biochim. Biophys. Acta **604**:1–26.