Anti-Lipoteichoic Acid Antibodies Enhance Release of Cytokines by Monocytes Sensitized with Lipoteichoic Acid

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Lipoteichoic acid (LTA) from gram-positive bacteria can stimulate monocytes to produce cytokines. To ascertain whether aggregation of LTA receptors can contribute to this effect, human monocytes were sensitized with LTA from *Streptococcus pyogenes*, washed, and treated with anti-LTA antibodies. The addition of anti-LTA antibodies or F(ab')2 fragments markedly enhanced the aggregation of LTA receptors, as evidenced by indirect immunofluorescence and the release of tumor necrosis factor alpha and interleukin-1p. These findings suggest that aggregation of LTA receptors of monocytes is required for triggering marked cytokine responses.

Septic shock caused by gram-positive bacteria shares many clinical features with that caused by gram-negative bacteria (4). In some animal models, lethality and pathophysiological changes of both types of shock are mediated by tumor necrosis factor alpha (TNF-α) (7, 23) and interleukin-1 (IL-1) (1, 17). The role of lipopolysaccharide (LPS) of gram-negative bacteria in inducing phagocytes to release these cytokines is well established (15). Less is known, however, about gram-positive components responsible for similar activities.

Because many of the biological activities of LPS are shared by lipoteichoic acid (LTA) produced by gram-positive bacteria (reviewed in reference 25), several studies have focused on the ability of LTA to trigger the release of a number of products from phagocytes, including cytokines and oxygen radicals (11, 18), as well as to induce tumoricidal activity (9, 11). In vivo, it has been shown that streptococcal LTA induces a marked increase of serum TNF levels in mice previously sensitized with *Propionibacterium acnes* but not in unprimed mice (26). In vitro, incubation of monocytes with pneumococcal LTA caused the release of IL-1β (19), and staphylococcal LTA caused the release of TNF and IL-1α as well (13, 24).

Systematic studies on the efficacy of LTAs from several gram-positive bacterial species to induce the release of IL-1β, IL-6, and TNF-α by human monocytes (2) and murine bone marrow macrophages (11) have been recently undertaken. In these studies, it was noted that the magnitude of stimulation varied considerably with LTAs from different bacterial species. Intraspecies variability of LTA preparations was also noted (2, 11). Differences in stimulatory activity could not be related to structural features of the various LTAs such as chain length, alanine composition, or kind or sugar substitutions or fatty acid composition.

Aggregation and redistribution of receptors on the cell surface have important roles in transmembrane signalling (reviewed in reference 5). In one study, it was found that LTA alone was not able to induce an oxidative burst in polymorphonuclear leukocytes, but upon treatment of the cells with anti-LTA antibodies, a brisk production of oxygen radicals was observed (8). Because LTA alone bound to polymorphonuclear leukocytes but was not capable of inducing patching and redistribution of LTA receptors unless anti-LTA was present (6), it was postulated that cross-linking of LTA receptors is required for production of oxygen radicals. Indeed, in human monocytes, the stimulation of the oxidative burst induced by LTA alone was associated with some degree of cross-linking of LTA receptors, as evidenced by patching and capping (12).

To better understand the mechanisms whereby LTAs induce the production of cytokines, we investigated whether anti-LTA antibodies could modulate these effects in monocytes sensitized with LTA. We found that anti-LTA caused significant enhancement of secretion of IL-1β and TNF-α by LTA-treated monocytes, suggesting that cross-linking of LTA receptors results in marked stimulation of monocytes to release cytokines.

LTA from *Streptococcus pyogenes* M type 3 was extracted with hot phenol-water and purified by gel filtration chromatography as described previously (6, 20). Decayed LTA (d-LTA) was obtained by subjecting LTA to mild alkaline (0.2 M NaOH at 37°C) treatment for 60 min followed by neutralization with 0.1 M HCl (11). LPS from *Salmonella enteritidis*, used as a positive control, was purchased from Difco (Diagnostic International Distribution, Milan, Italy). Anti-LTA serum was prepared by immunizing rabbits with LTA-methylated albumin complexes (6, 22). Immunoglobulin G (IgG) was purified from anti-LTA serum by ion-exchange chromatography, using a quaternary aminooethyl Sephadex A-50 column equilibrated with 0.1 M Tris buffer, pH 6.5, as described previously (3). F(ab')2 fragments were obtained by pepsin digestion of anti-LTA IgG followed by protein A chromatography (10). These fragments were free of whole antibody molecules, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions (10). Anti-LTA serum and F(ab')2 fragments were tested for the presence of endotoxin by a *Limulus* amoebocyte lysate assay kit (E-Toxate; Sigma Chimica, Milan, Italy). Levels of endotoxin in these materials were <0.05 ng/ml.

Mononuclear cells were obtained from the peripheral blood of healthy volunteers by centrifugation on Ficoll-Paque (Pharmacia Biotech S.p.A., Cologno Monzese, Italy) and suspended to a density of 1.5 × 10⁷/ml in culture medium (RPMI 1640, 10% fetal calf serum, 50 μg of streptomycin per ml, 50 IU of benzylpenicillin per ml, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]; GIBCO; distributed by Mascia Brunelli, Milan, Italy) as described previously (12). One-milliliter aliquots were dispensed to plastic dishes and
incubated for 2 h at 37°C. After removal of nonadherent cells, monocytes were cultured with the stimuli in serum-free medium (1 ml per well). TNF was measured in monocyte supernatants by cytof ctotoxicity assay in L929 murine fibroblasts as previously described (21). In selected supernatants, virtually identical results were obtained with the cytotoxicity assay and an immunoassay specific for human TNF-α (Factor-Test human TNF-α enzyme-linked immunosorbent assay kit; Genzyme). IL-1β was measured with a commercial radioimmunoassay (Interleukin-1βII-125 assay; Amersham Italia S.r.l., Milan, Italy) according to the manufacturer’s instructions. Localization of LTA on the cell membrane was performed by indirect immunofluorescence using anti-LTA antibodies (6). The specificity of these antibodies was assessed by adding to LTA-sensitized monocytes anti-LTA serum containing d-LTA (100 μg/ml) for 30 min followed by fixation and labeling. Fluorescence of the cells was completely blocked (data not shown), suggesting that it is due to LTA-anti-LTA interaction. Polarization or capping of LTA receptors on the monocyte surface was expressed as the percentage of cells showing the fluorescence label on less than half of the surface.

Monocytes exposed to LTA secreted low or undetectable amounts of TNF-α or IL-1β, respectively (Fig. 1). The addition of anti-LTA serum to LTA-sensitized monocytes caused a marked increase in secretion of both cytokines, reaching levels similar to those obtained in monolayers exposed to LPS. The effect of anti-LTA serum was specific because normal serum was not effective. Results similar to those reported in Fig. 1 were obtained when the endotoxin-neutralizing agent polymyxin B (20 μg/ml; Sigma) was present in the medium throughout the experiments (data not shown).

F(ab')2 anti-LTA (100 μg/ml) also triggered cytokine release, suggesting that the Fc portion of the antibodies is not required (Fig. 1). In control experiments with monocytes that were not exposed to LTA, F(ab')2 fragments did not induce any significant cytokine secretion (data not shown). The stimulatory effect of F(ab')2 fragments on monocytes sensitized with LTA was totally abrogated by heating the fragments to 100°C for 30 min (not shown). This and the previous finding that endotoxin levels of the F(ab')2 preparation were below the limit of detection (0.05 ng/ml) of a Limulus assay ruled out contamination with LPS as a possible cause of cytokine induction by the fragments.

d-LTA was not active either alone or with anti-LTA serum (Fig. 1). This result is consistent with those of other studies (2, 11), suggesting that LTA-associated activities are dependent on intact ester-linked lipids known to be required for cell binding (16).

The time course of TNF-α secretion induced by anti-LTA in LTA-sensitized monocytes was similar to that observed when monolayers were exposed to LTA or LPS alone for the entire culture period (Fig. 2). Maximal levels in the supernatants were attained at 8 h and remained unchanged thereafter for at least 24 h. Similar kinetics of IL-1β secretion were observed (data not shown).

Incubation with LTA alone for up to 30 min before fixation caused moderate capping of LTA receptors (Table 1). Addition of anti-LTA, but not normal, serum to the LTA-sensitized monocytes caused a marked increase in the percentage of capped cells.

LTA binds readily to specific receptors on a number of cell

**FIG. 1.** Effect of anti-LTA on the release of cytokines by human monocytes sensitized with LTA. Monocytes were incubated for 30 min at 37°C with increasing amounts of LTA, washed four times, and cultured with anti-LTA (▲), normal serum diluted to 1:100 with medium (●), or serum-free medium (■). Monocytes were also incubated with 100 μg of d-LTA per ml, washed, and treated with anti-LTA (○). To exclude Fc-mediated effects, cells were treated with 100 μg of LTA per ml followed by 100 μg of F(ab')2 anti-LTA per ml (△). As a positive control, monocytes were exposed to LPS (10 μg/ml) for the entire culture period (●). After culturing of the monocytes for 20 h, the supernatants were removed, centrifuged, and assayed for TNF-α (top) and IL-1β (bottom). Points and bars represent means ± standard deviations of three experiments. Duplicate samples were tested in each experiment. * significantly different (P < 0.05) from normal serum by one-way analysis of variance and Student-Newman-Keuls test.

**FIG. 2.** Time course of TNF-α release induced by anti-LTA in human monocytes sensitized with LTA. Monocytes were incubated for 30 min with 10 μg of LTA per ml, washed, and cultured with anti-LTA diluted 1:100 in medium (▲). Kinetics of TNF-α release were compared with those induced by 10 μg of LTA (●) or LPS (●) per ml continuously present throughout the culture period. Supernatants were collected at various times after the addition of the stimuli and assayed for TNF-α. Points and bars represent means ± standard deviations of three experiments. Duplicate samples were tested in each experiment.
types (6) but is consistently less potent than LPS in inducing cytokines (2, 11, 24). In the present study, some degree of capping and secretion of TNF-α was induced by LTA alone. However, the addition of anti-LTA to monocytes sensitized with LTA induced a rapid redistribution of LTA receptors and enhanced secretion of TNF-α and IL-1β. Our findings suggest that once fully aggregated, LTA receptors of monocytes are capable of triggering cytokine responses to levels similar to those obtained in monolayers exposed to LPS.

Bhakdi et al. noted that stimulation of monocytes by LTA occurred in the presence or absence of 10% autologous serum and concluded that naturally occurring anti-LTA serum antibodies were not inhibitory (2). Our data suggest that these antibodies may be actually stimulatory if present in sufficient concentrations. However, since Bhakdi et al. did not report the anti-LTA titers of the sera that they used, it is difficult to compare their data with ours.

We cannot speculate at this stage about the implications of our findings for events occurring during the natural course of streptococcal infections, especially those associated with septic shock. Anti-LTA antibodies are normally present in the majority of normal human sera (14). Because monocytes may be sensitized by LTA in the course of infections, it will be of interest to ascertain whether naturally occurring anti-LTA antibodies contribute to the release of cytokines and to the pathophysiology of gram-positive septic shock.

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