The Preparation of a Lipidic Endotoxin Affects Its Biological Activities

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Bacterial membrane constituents, such as Ornithine-containing lipid (OL) and the lipid A portion of lipopolysaccharide, trigger various immune responses through recognition by Toll-like receptor (TLR) 4. Usually, these lipids are dissolved in a small amount of aqueous or organic solvent before being added to the culture medium for examination of their biological activities. Macrophages stimulated with OL or lipid A sonically dissolved in saline released both interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). In contrast, macrophages stimulated with OL or lipid A sonically dissolved in ethanol or dimethyl sulfoxide (DMSO) secreted much TNF-α, but very little IL-1β. These results, taken together, indicate that how an endotoxin is prepared affects its biological activities. In addition, electronmicroscopic analysis revealed that sonication of air-dried OL or lipid A in DMSO produced larger particles than those produced in saline, suggesting that the process of preparing lipidic TLR4-ligands affects their physical state including particle size, and that the physical state might be an important determinant of biological activity.

Key words endotoxin; lipid; lipopolysaccharide; Toll-like receptor 4

Lipopolysaccharide (LPS) is involved in bacterial pathogenesis and elicits a variety of biological responses including endotoxin shock (reviewed in1). LPS consists of a hydrophobic domain known as lipid A, which comprises the outer leaflet of the bacterial outer membrane, and a non-repeating core oligosaccharide coupled to a distal polysaccharide which extends from the bacterial surface.2 Lipid A, which is known as an endotoxin, is the bioactive component of LPS involved in bacterial virulence through its recognition by Toll-like receptor (TLR) 4.3,4 The chemical structure of lipid A, such as numbers of acyl chains and existence of phosphate residues, is very important for recognition by TLR4.5–7 TLR4 is involved in the precise discrimination of structures of lipid A.5,7 Ornithine-containing lipid (OL), a lipidic component of outer and inner membranes of various pathogenic Gram-negative bacteria such as Achromobacter xylosoxidans and Pseudomonas aeruginosa,8 has similar biological activity to LPS, inducing the release of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) from mouse macrophage-like cell lines.9 OL-induced cellular signaling is mediated by TLR4, and the structural similarity of OL with LPS may explain their LPS-mimetic biological activities.10

Since TLR4-mediated signaling enhances immune responses, such as the release of cytokines, LPS is thought to be a potent adjuvant; however, it is not useful because of its high toxicity. A lipid A derivative, monophosphoryl lipid A, exhibits potent adjuvant activity, but is 100- to 1000-fold less toxic than LPS.11,12 Our previous observations suggested that the lack of ability of MPL to induce secretion of IL-1β from macrophages is involved in the low toxicity.5 Analyses of chemical and physical factors which influence the ability of TLR4-ligands to induce secretion of cytokines, including IL-1β and TNF-α, are important for the development of useful adjuvant.

In this paper, we showed that the abilities of OL and lipid A to induce the release of IL-1β from mouse macrophages were influenced by how the OL or lipid A suspension was prepared, although the molecular machinery underlying the difference remains to be elucidated. The preparation of TLR4 ligands is an important factor to be considered in the development of useful adjuvants.

MATERIALS AND METHODS

Animals C3H/HeN (male, 6 weeks old) mice were obtained from Clea Japan, Inc. (Tokyo, Japan). The mice were housed in a specific pathogen-free environment with a 12 h light–dark cycle in the animal house of the National Institute of Infectious Diseases.

Reagents The OL of A. xylosidans was purified as described previously.9 Synthetic OL was produced previously.10 Lipid A from the Escherichia coli F583 Rd mutant (E. coli lipid A) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Chemically synthesized E. coli-type lipid A (compound 506; Synthetic lipid A), was generously provided by Drs. S. Kusumoto and K. Fukase (Department of Chemistry, Graduate School of Science, Osaka University).13

Preparation of OL and Lipid A Suspensions OL or lipid A was dissolved in chloroform:methanol (2:1, v/v). The solvent was dried in a glass tube under a stream of nitrogen. The glass tubes were supplemented with phosphate-buffered saline (PBS), and subjected to sonication for 5 min at room temperature (20—25 °C) in a bath-type sonicator (Branson Type 2, Emerson Japan Ltd.) for preparation of the OL or lipid A suspension (100 μg/ml). Alternatively, the glass tubes were supplemented with a small amount of dimethyl sulfoxide (DMSO) or methanol, and subjected to sonication for 5 min at room temperature (20—25 °C) to prepare the suspensions (10 mg/ml). Finally, the suspensions were diluted with phosphate-buffered saline (100 μg/ml). The final concentration of DMSO or methanol in the culture medium was less than 0.3% (v/v).

Cell Culture Thioglycolate-elicited peritoneal macrophages from C3H/HeN were cultivated in RPMI 1640 medium (Invitrogen Co., Carlsbad, CA, U.S.A.) containing

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10% heat-inactivated FBS (Equitech-Bio, Inc., Kerrville, TX, U.S.A.). Before the stimulation with lipid A or OL, 1 ml of cell suspension (5×10^5 cells/ml) per well was added to 24-well culture plates.

**Measurement of the IL-1β mRNA Levels by RT-PCR**

Peritoneal macrophages in 24-well culture plates were allowed to adhere for 2 h, and *A. xylosoxidans* OL, synthetic OL, *E. coli* lipid A, or synthetic lipid A was added to the wells at an appropriate concentration. After 3 h of cultivation, total cellular RNA was extracted from the macrophages using an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). First strand cDNA synthesis was performed using a SuperScript First Strand Synthesis System (Invitrogen). Polymerase chain reaction (PCR) was performed with 0.3 μg of cDNA and primers specific for IL-1β (5′-TACAGGCTCCGAGATGAACAACAA-3′, 5′-TGGGGAAGGCATTAGAATCC-3′) using Taq polymerase (Takara Shuzo, Shiga, Japan).

**Electron Microscopy**

The OL or lipid A suspension (5 μg/ml PBS) was deposited onto thin amorphous carbon films supported by electron microscope specimen screens for about 30 s, and a drop was then stained with uranyl acetate. The negatively stained samples were dried and examined in a HITACH H-7000 high voltage electron microscope. Micrographs were recorded at a magnification of 30000.

**RESULTS**

**Macrophages Stimulated with OL or Lipid A Sonically Dissolved in Methanol or DMSO Secrete TNF-α Well, But Secrete IL-1β very Weakly**

OL as well as lipid A is a potent activator of macrophages. They both induce the release of inflammatory cytokines, such as IL-1β and TNF-α. Consistent with previous observations, the OL or lipid A suspension prepared by sonication of dried OL or lipid A in PBS (aq-OL and aq-lipid A, respectively) induced the secretion of both IL-1β and TNF-α from mouse peritoneal macrophages in a dose-dependent manner (Fig. 1). OL and lipid A sonically dissolved in methanol or DMSO (or-OL and or-lipid A, respectively) also induced the release of TNF-α from mouse peritoneal macrophages at levels similar to those induced by aq-OL or aq-lipid A (Figs. 1B, D). On the other hand, or-OL and or-lipid A showed weaker activity to induce secretion of IL-1β from mouse peritoneal macrophages than did aq-OL and aq-lipid A (Figs. 1A, C). These results suggest that or-OL and or-lipid A specifically lost the ability to induce the release of IL-1β from mouse peritoneal macrophages.

IL-1β gene expression was measured in a RT-PCR-based examination of IL-1β mRNA. The levels of IL-1β mRNA expression induced by aq-OL and aq-lipid A were significantly higher than those induced by or-OL and or-lipid A, respectively (Fig. 1E). A similar difference was observed in the chemically synthesized OL and lipid A preparations (Fig. 1E), ruling out the possibility that the observations described above were not due to possible contaminants in the natural OL or lipid A preparation. These results, taken together, indicate that the ability of OL and lipid A to induce the expression of the IL-1β gene in macrophages is affected by the procedures used to dissolve OL and lipid A before addition to the cell culture medium.

**Aq-OL and Aq-Lipid A Particles Were Smaller Than Or-OL and Or-Lipid A Particles, Respectively**

We speculated that the procedures used to make the OL or lipid A

**Fig. 1.** Expression and Secretion of IL-1β on Stimulation with Aq-OL, Or-OL, Aq-Lipid A and Or-Lipid A in Mouse Macrophages

(A, B, C, D) Peritoneal macrophages from C3H/HeN (5×10^5 cells/ml) cultivated in 24-well dishes were allowed to adhere for 2 h. Thereafter, *A. xylosoxidans* OL (A, B) and *E. coli* lipid A (C, D) were added. After 20 h, the amounts of IL-1β and TNF-α were determined by ELISA. The closed circles (aq) indicate OL or lipid A sonically prepared in saline, the open circles (or (Methanol)) indicate OL or lipid A sonically prepared in methanol, and the open triangles (or (DMSO)) indicate lipid A or OL sonically prepared in DMSO. The results shown are averages for triplicate wells with the standard deviations. (E) Macrophages were stimulated with 5 μg/ml of OL (from *A. xylosoxidans*), synthetic OL, lipid A (from *E. coli*), and synthetic lipid A for 3 h, and then IL-1β mRNA expression was examined by RT-PCR. The PCR products obtained at 20 cycles were subjected to agarose gel electrophoresis, and then visualized by ethidium bromide staining. The results shown are representative of three independent experiments.
suspension affect particle shape or size, which are implicated in biological activities of endotoxin.\textsuperscript{15)} The difference between aq-OL and or-OL or between aq-lipid A and or-lipid A may affect their ability to induce secretion of IL-1\textbeta{} from mouse peritoneal macrophages. An electromicroscopic analysis of OL and lipid A was performed to examine differences in shape and size. The aq-OL particles are approximately 10—40 nm in diameter (Fig. 2A), while the or-OL particles are approximately 50—100 nm (Fig. 2B), indicating that the procedures followed to prepare OL aggregates affect their size. The aq-lipid A aggregates had much smaller particles (less than 50 nm in diameter, Fig. 2C) than or-lipid A (more than 100 nm in diameter, Fig. 2D). In addition to the difference in size, or-lipid A displayed a multi-lamellar shape, which was apparently different from that of aq-lipid A (Figs. 2C, D). These results indicated that how OL and lipid A suspensions are prepared greatly affects aggregate size and shape, which may affect their ability to induce IL-1\textbeta{} secretion from mouse peritoneal macrophages.

**DISCUSSION**

In this paper we demonstrated that macrophages stimulated with OL or lipid A sonically dissolved in methanol or DMSO secrete much TNF-\alpha{}, but little IL-1\beta{}. On the other hand, macrophages stimulated with OL or lipid A sonically dissolved in saline secrete both IL-1\beta{} and TNF-\alpha{}. These results indicate that the procedures used to prepare endotoxins affect their biological activities. Differences of particle size and shape were observed between or-lipid A and aq-lipid A and between or-OL and aq-OL and aq-lipid A on electron microscopic analysis, indicating that the preparation of OL and lipid A affects aggregate size and structure.

The difference in aggregate structure of endotoxins might affect their biological activity to induce the release of IL-1\beta{}. Previously, Mueller et al. demonstrated that the aggregate is an active unit of endotoxin; Endotoxin aggregates induced the secretion of TNF-\alpha{} from human mononuclear cells, but the monomers did not.\textsuperscript{15)} Their observations suggest that the formation of aggregates is essential for recognition by endotoxin receptors, including TLR4. Our observations were consistent with theirs in that endotoxin aggregates, such as aq-OL, aq-lipid A, or-OL and or-lipid A, induced the secretion of TNF-\alpha{} from mouse macrophages; however, the reduced ability of or-OL and or-lipid A to induce the secretion of IL-1\beta{} from mouse macrophages could not be explained by their observations. It is possible that the physical state of an endotoxin, including particle shape as well as size, is an important determinant of its biological activities, and the identification of other determinants remains to be carried out.

TLRs are thought to be very important targets for medicines that modulate immune responses, because TLR-mediated immune responses are essential for host defense against bacterial and viral infections (reviewed in\textsuperscript{15}). Many bacterial membrane components, including LPS, OL, lipoteichoic acid, and lipoarabinomannan, which have a lipid portion, have been demonstrated to be TLR ligands. Their structural mimics are also thought to be potential candidates for drugs. Our results suggest that the procedures used to prepare such lipidic medicines, which are designed to activate or repress TLRs, to be important determinants of their effects.

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**REFERENCES**