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Salmonella-Type Heptaacylated Lipid A Is Inactive and Acts as an Antagonist of Lipopolysaccharide Action on Human Line Cells

Ken-ichi Tanamoto2 and Satoko Azumi1

The stimulation of both THP-1 and U937 human-derived cells by Salmonella lipid A preparations from various strains, as assessed by TNF-α induction and NF-κB activation, was found to be very low (almost inactive) compared with Escherichia coli lipid A, but all of the lipid A exerted strong activity on mouse cells and on Limulus gelation activity. Experiments using chemically synthesized E. coli-type hexaacylated lipid A (506) and Salmonella-type heptaacylated lipid A (516) yielded clearer results. Both lipid A preparations strongly induced TNF-α release and activated NF-κB in mouse peritoneal macrophages and mouse macrophage-like cell line J774-1 and induced Limulus gelation activity, although the activity of the latter was slightly weaker than that of the former.

However, 516 was completely inactive on both THP-1 and U937 cells in terms of both induction of TNF-α and NF-κB activation, whereas 506 displayed strong activity on both cells, the same as natural E. coli LPS. In contrast to the action of the lipid A preparations, all the Salmonella LPSs also exhibited full activity on human cells. However, the polysaccharide portion of the LPS neither exhibited TNF-α induction activity on the cells when administered alone or together with lipid A nor inhibited the activity of the LPS. These results suggest that the mechanism of activation by LPS or the recognition of lipid A structure by human and mouse cells may differ. In addition, both 516 and lipid A from Salmonella were found to antagonize the 506 and E. coli LPS action that induced TNF-α release and NF-κB activation in THP-1 cells. The Journal of Immunology, 2000, 164: 3149–3156.

The biological activity of lipid A depends on its chemical structure, and extraordinary progress has been made on the relationship between chemical structure and biological activity of lipid A by using chemically synthesized lipid A analogues (1). Recently, several nontoxic lipid A that closely chemically resemble active lipid A but are completely devoid of endotoxic activity have been reported (2–4). Many of these preparations have also been found to act as antagonists (3, 5, 6), and simple chemical modifications have also been found to change biologically active lipid A to completely nontoxic derivatives (7, 8). These findings indicate that the biological activity of lipid A is controlled by fine structural variations that are still ill-defined. In addition, cells of different species have been discovered to respond in different ways to certain lipid A derivatives, such as lipid A precursor structure, which is an agonist in mouse cells but an antagonist in human cells (9–11), indicating that the cells (or receptor molecules) discriminate fine differences in the chemical structure of lipid A. These findings indicate that understanding the biological activity of lipid A is not a simple task and that the mechanism of action of lipid A is different in humans and mice.

Escherichia coli- and Salmonella-type lipid A have the most typical lipid A structures so far defined, and both have been synthesized chemically (12–14) and well characterized biologically by many investigators (15–20). The chemical structure of these lipid As consists of a hexa- and heptaacylated diglucosamine bearing six and seven fatty acids, respectively, as shown in Fig. 1. The only structural difference between them is the hexadecanoyl acid attached to the hydroxyl residue of 3-hydroxy tetradecanoic acid bound to the 2 position of reducing glucosamine.

Previous studies on structure-activity relationships performed by using chemically synthesized analogues have revealed that Salmonella-type lipid A is one of the active lipid As and exhibited activity in all of the test systems employed at that time, although its activity was a little less than that of E. coli-type synthetic lipid A, which has, therefore, been subsequently recognized as representative of the structure of active lipid A.

In view of the species-specific actions of endotoxin, it is necessary to clarify the actual action of lipid A molecules on human cells to better understand endotoxins, and thus the activity of endotoxin must be considered based on its actions on human cells.

In this study, we show that one of the typical lipid As from Salmonella is not an activator on human cells.

Materials and Methods

Materials

Recombinant TNF-α standards and rabbit polyclonal antisera against murine TNF-α were obtained from Asahi Kasei Kogyo (Tokyo, Japan). Rabbit IgG was obtained from Zymed Laboratories (South San Francisco, CA). Anti-human and -mouse IgG-α antisera (1309 and 751, respectively) were gifts from Dr. Nancy Rice (Advanced BioScience Laboratories-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center). Synthetic lipid A analogues 506 and 516 were a gift from Daiichi Kagaku (Tokyo, Japan). RPMI 1640 medium with glutamine and IMDM were the products of Life Technologies (Grand Island, NY). Quantitative Limulus assay reagent (Endospecy) was obtained from Seikagaku Kogyo (Tokyo, Japan). Pyrogen-free water was a product of Otsuka Seiyaku (Tokyo, Japan). LPS was extracted from E. coli 03K2a2b:H2, Salmonella minnesota (S type), Salmonella typhimurium LT2, and Salmonella abortus equi by the aqueous phenol method (21). Lipid A was obtained as an insoluble substance after 1% acetic acid treatment of LPS at 100°C for 90 min (22). The polysaccharide portion was obtained from the supernatant of the reaction mixture by evaporating to dryness and washing with chloroform three times. No residual lipid A portion was detected by

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either fatty acid analysis by Gas chromatography or by Limulus gelation assay.

Gas-liquid chromatography conditions
Gas-liquid chromatography analysis was performed on a model GC-14A (Shimadzu, Kyoto, Japan) with a HiCap-CB5 fused silica capillary column (25 m x 0.25 mm; GL Science, Toyko, Japan) and temperature programs 120°C for 3 min to 250°C at 3°C/min. Nitrogen was used as carrier gas.

Mass spectrometry
Liquid secondary-ion mass spectrometry was performed on a VG ZAB-2SEQ spectrometer (VG Analytical, Manchester, U.K.) operated at 8 kV in a negative mode. The cesium gun was operated at 30 kV. Current-controlled scans were acquired at a rate of 30 s per decade. A mixture of ethanolamine and m-nitrobenzyl alcohol (1:1) was used as the matrix.

Induction of TNF-α release from mouse peritoneal macrophages, J774-1, THP-1, and U937 cells
Mouse peritoneal macrophages were obtained by washing the peritoneal cavity of female BALB/c mice (6–10 wk old, Japan SLC, Hamamatsu, Japan) with 5 ml of serum-free IMDM (23). The cell number was adjusted to 2 x 10^6 cells/ml. After adhesion, the cells were incubated with the stimulant for 6 h. J774-1, THP-1, and U937 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 50 μg/ml 1,25-dihydroxy vitamin D3 (Sigma, St. Louis, MO) with 5 ml of actinomycin D (4 μg/ml) and 2-ME, 5 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO2 atmosphere at 37°C. J774-1 cells were harvested by scraping with cell scraper (Costar, Cambridge, MA) and suspended in a fresh medium. The cells (1 x 10^6 cells/ml of 24-well dishes) were allowed to adhere to plastic for 3 h at 37°C, washed twice with medium, and incubated an additional 4 h for TNF-α induction with the stimulant. THP-1 cells (2 x 10^5 cells/ml of 24-well dishes) were prepared for the experiments by adding 100 ng/ml of PMA (Sigma, St. Louis, MO) and 0.1 μM 1,25-dihydroxy vitamin D3 (Wako Pure Chemical Industries, Tokyo, Japan) to 2 ml of lysis buffer (10 mM HEPES-KOH, 5 mM EDTA, and 10 mM KCl, pH 7.9) containing 0.5% (for J774-1) or 0.1% (for THP-1) Nonidet P-40, 1 mM DTT, and a protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany). The lysate was centrifuged for 5 min at 15,000 rpm at 4°C, and the resultant supernatant was used as the crude cytosol fraction.

The crude cytosol fractions (50 μg of protein) were subjected to 10% SDS-PAGE, and IκB-α was detected as follows. Protein was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) with a semidy blotting apparatus. The membrane was then probed with human (or mouse) IκB-α antisera (1:1000 dilution) followed by a peroxide-labeled goat anti-rabbit IgG Ab (Boehringer Mannheim; diluted 1:10000). Proteins were detected by using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Limulus amoebocyte gelation activity
Activation of the proclotting enzyme of the horseshoe crab was tested by a quantitative Limulus assay reagent (Endospecy). Pyrogen-free water was used to dilute the test samples. A 50-μl amount of each sample was incubated with the same volume of lysate containing chromogenic substrate in 96-well flat plates (Costar) for 30 min at 37°C. The reaction was stopped by adding 200 μl of 0.6 M acetic acid. The chromogen (p-nitroaniline) was measured at 405 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

Inhibition assay of endotoxin-mediated TNF-α induction and NF-κB activation in THP-1 cells
Inhibition of endotoxin-mediated TNF-α induction and NF-κB production in THP-1 cells was performed by adding the indicated concentration of the inhibitor to the assay system followed immediately by the addition of agonist, and TNF-α production or the degradation of IκB-α was compared with a control containing agonist alone. Inhibition of TNF-α induction is expressed as percent TNF-α production with production in the presence of agonist alone equal to 100%.

![Structure of E. coli-type lipid A](image)
Results

Fatty acid composition and liquid secondary-ion mass spectrometry analysis of lipid A from Salmonella and E. coli LPS

To examine the fatty acid composition of LPS and lipid A from the Salmonella species used in the present study, i.e., S. abortus equi, S. minnesota (S type), and S. typhimurium LT2 lipid A were hydrolyzed, and the methyl-fatty acids liberated were analyzed by gas-liquid chromatography. The results are shown in Table I, where the molar ratios of the fatty acids are expressed by assuming the number of 3-hydroxytetradecanoic acid molecules in each LPS to be 4.

Table I. Fatty acid composition of lipid A from various Salmonella and E. coli LPS

<table>
<thead>
<tr>
<th>Lipid A From</th>
<th>Amount of Fatty Acid (mol/4 mol β-OH C14:0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C12:0</td>
</tr>
<tr>
<td>S. minnesota</td>
<td>1.32</td>
</tr>
<tr>
<td>S. abortus equi</td>
<td>1.18</td>
</tr>
<tr>
<td>S. typhimurium LT2</td>
<td>0.89</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.86</td>
</tr>
</tbody>
</table>

*a Fatty acid composition was analyzed by gas-liquid chromatography. Molar ratios of the fatty acids are expressed by assuming the number of 3-hydroxytetradecanoic acid molecules in each LPS to be 4.

FIGURE 2. Salmonella LPS and lipid A induction of TNF-α release by mouse cells. A. Murine peritoneal macrophages. B. J774-1 cells. Cells (A. 2 × 10⁶ cells/ml/well of 24-well dishes in IMDM; B. 1 × 10⁶ cells/ml/well in RPMI 1640 medium supplemented with 10% (v/v) FCS) were cultured with test sample. After 6 h (A) or 4 h (B) of incubation at 37°C, the supernatants were examined for TNF-α. The results are expressed as means ± SD for triplicate wells from one of three experiments with similar results.

FIGURE 3. Salmonella LPS and lipid A induction of TNF-α release by human line cells. A. Human THP-1 cells. Cells (2 × 10⁶ cells/ml/well) were incubated with 100 ng/ml of PMA and 0.1 μM 1,25-dihydroxyvitamin D₃ for 3 days in RPMI 1640 medium containing 10% FCS at 37°C. B. Human U937 cells. Cells (1 × 10⁶ cells/ml/well) were incubated with 100 ng/ml of PMA for 2 days in RPMI 1640 medium containing 10% FCS at 37°C. After an additional 24 h (A, THP-1) or 8 h (B, U937) of incubation in medium containing 10% FCS with 10 μl of test sample, the supernatants were assayed for TNF-α. Values represent the mean concentration of TNF-α SD for triplicate wells from one of three experiments with similar results.
Salmonella LPS and lipid A induction of TNF-α release by mouse peritoneal macrophages and mouse macrophage-like J774-1 cells

TNF-α release into the medium was estimated by cytotoxicity against actinomycin-D-sensitized L929 murine fibroblasts. Murine peritoneal macrophages were found to react to stimulation by all Salmonella LPSs from the three different species, S. abortus equi, S. minnesota, and S. typhimurium. As shown in Fig. 2A, the cells started to secrete TNF-α at a LPS concentration of 0.1 ng/ml, and TNF-α production by the macrophages increased dose-dependently. Although the activity of the lipid A preparations was less than that of their respective parent LPS, they stimulated cells at a concentration of 1–10 ng/ml.

A similar response to Salmonella LPS and lipid A was observed in mouse macrophage-like J774-1 cells. Although the activity of each of the lipid A was about 10 times less than that of its parent LPS, they stimulated cells at a concentration of 1–10 ng/ml.

Salmonella LPS and lipid A induction of TNF-α release by THP-1 and U937 line cells

Human THP-1 and U937 line cells also reacted to stimulation with all three Salmonella LPSs, and both lines started to secrete TNF-α at a concentration of 10 ng/ml. The LPS-induced production of TNF-α increased dose-dependently (Fig. 3). However, the cells did not produce TNF-α when exposed to any of the lipid A preparations, and significant amounts of TNF-α were first detected at a concentration of 10 μg/ml in THP-1 cells, showing that the activity of each lipid A was 10^3 times less or more than that of their parent LPS.

E. coli- and Salmonella-type synthetic lipid A induction of TNF-α release by mouse peritoneal macrophages and mouse macrophage-like J774-1 cells

Lipid A preparations obtained from natural LPS are usually heterogeneous, and all of the lipid A preparations from Salmonella LPS used in the present study partially included a significant amount of E. coli-type lipid A and may also have included contaminating parent LPS, both of which stimulate human cells. Because the data obtained by using these preparations are, therefore, not reliable, the same experiments described above were performed with chemically synthesized Salmonella-type lipid A (516; Fig. 1) using E. coli-type synthetic lipid A (506; Fig. 1) as a control. The results in mouse cells are shown in Fig. 4, A and B. Murine peritoneal macrophages reacted to stimulation with 516 in a dose-dependent manner and started to secrete TNF-α at a concentration of 10 ng/ml (Fig. 4A). A similar response to 516 was also observed in mouse macrophage-like J774-1 cells (Fig. 4B). The activity of 506 in inducing TNF-α was greater than that of 516, and the activity was expressed even at 1 ng/ml in both mouse peritoneal and J774-1 line cells, showing that its activity is about ten times more powerful than that of 516.

Synthetic lipid A, 506 and 516, induction of TNF-α release by THP-1 and U937 line cells

Human THP-1 and U937 line cells also reacted to stimulation with E. coli-type hexaacylated lipid A and started to secrete significant amounts of TNF-α at a concentration of 100 ng/ml in both THP-1 and U937 cells, showing that the activity of E. coli-type lipid A is about 10^3 times more powerful than that of Salmonella-type lipid A.
To examine endotoxin-mediated NF-κB activation, J774-1 cells were treated with LPS and lipid A from S. abortus equi, and cell extracts were analyzed for degradation of IκB-α protein by Western blotting (50 μg protein/lane). 

Activation of NF-κB in mouse macrophage-like J774-1 cells by Salmonella LPS and lipid A, and synthetic lipid As 

To examine endotoxin-mediated NF-κB activation, J774-1 cells were treated with LPS and lipid A from S. abortus equi, and cell extracts were analyzed for degradation of IκB-α protein by Western blotting. The results are shown in Fig. 5A. Stimulation of the cells with both S. abortus equi LPS and lipid A resulted in a rapid decrease and disappearance of IκB-α protein at 1 ng/ml, and 10 ng/ml, respectively. Both synthetic lipid A preparations 506 and 516 also expressed strong IκB-α degrading activity in J774-1 cells (Fig. 5B). However, the activity of 506 was about ten times stronger than that of 516, consistent with the results of TNF-α induction.

Activation of NF-κB in THP-1 line cells by Salmonella LPS and lipid A, and synthetic lipid As 

Human THP-1 line cells also reacted to stimulation with Salmonella LPS and E. coli-type hexaacylated lipid A, and IκB-α protein degradation started at a concentration of 1 ng/ml (Fig. 6, A and B). In contrast, Salmonella lipid A exhibited much less activity than the parent LPS (104 times less), and Salmonella type synthetic lipid A did not induce degradation of IκB-α at all, even at a concentration of 1 μg/ml (Fig. 6).

Limulus amoebocyte gelation activity of Salmonella LPS and lipid A, and synthetic lipid A 

Activation of the clotting system cascade of the horseshoe crab was used to assess differences in the activity of Salmonella LPS and lipid A, 506 and 516. The results are shown in Fig. 7. Although the activity of 516 was slightly lower than that of 506, all of the preparations exhibited strong Limulus gelation activity.

Inhibition of the TNF-α induction activity of E. coli LPS by Salmonella lipid A and 506 

Because the lipid A from Salmonella and synthetic Salmonella-type lipid A failed to stimulate TNF-α production by human THP-1 cells, their antagonistic activity on the response of cells to active LPS was tested by adding them to the cell cultures together with the agonist. As shown in Fig. 8, both Salmonella lipid A and synthetic Salmonella-type lipid A displayed inhibitory activity on TNF-α production stimulated by E. coli LPS. Suppression by synthetic Salmonella-type lipid A was observed in a dose-dependent manner without regard to the stimulatory dose of the agonist (10 and 100 ng/ml). Significant inhibitory effects were observed at an agonist to antagonist ratio of 1:10 (w/w), and almost complete inhibition was possible at a 100-fold excess of both antagonists to stimulant.
In the present study, we found that all lipid A preparations isolated from several Salmonella strains exhibited far lower activity on human cells in inducing TNF-α and NF-κB activation than E. coli lipid A. According to the fatty acid analysis by gas-liquid chromatography and mass spectrometry (data not shown), the major lipid A structure of all of the lipid A preparations from the Salmonella strains used in the present study was heptaacylated diglucosamine, which is referred to as Salmonella-type lipid A, because it is the typical structure present in Salmonella, as reported previously (30–32). In addition to the Salmonella-type lipid A, they also contained a significant amount of E. coli-type hexaacylated diglucosamine lipid A structure.

Because natural lipid A usually consists of a mixture of various structures originated from the biosynthesis and extraction procedure, as was also the case in the present study, and its biological activity depends on the chemical structure, experiments using natural lipid A may give rise to misunderstandings regarding structure-activity relationships.

To confirm the phenomenon observed when natural Salmonella lipid A was used, and to obtain more definitive results, experiments have been performed with synthetic E. coli- and Salmonellatype lipid A (506 and 516, respectively). The results showed that Salmonella-type lipid A is completely inactive on human cells, but is a strong agonist in mice and active in inducing Limulus gelation activity as well. In contrast, E. coli-type lipid A exhibited full activity in human cells as well.

Both of these lipid As, the Salmonella-type heptaacylated and E. coli-type hexaacylated forms, had been successfully synthesized chemically in the past (12–14), and their biological properties had been carefully tested by many investigators (15–20). The Salmonella-type lipid A had been demonstrated to be very active in mice and rabbits and subsequently had been thought to be a typical lipid A (18–20) along with that from E. coli. However, Salmonella-type lipid A (516) was not tested for activity on human systems, and nobody at that time expected that it would be discriminated by human and mouse cells.

Although natural Salmonella lipid A contains a significant amount of the E. coli-type hexaacylated diglucosamine structure, as recognized by mass spectrometry, its activity on human cells was much lower than expected. In the present study, biologically
inactive *Salmonella* lipid A was found to antagonize the agonist (*E. coli* LPS and 506) action of inducing TNF-α and NF-κB activation in THP-1 cells. This may explain the low activity of natural lipid A from *Salmonella*. The apparent activity of *Salmonella* lipid A must be the result of the overall reaction of active structures, including *E. coli*-type lipid A, and the antagonistic activity of the *Salmonella*-type structure.

In contrast to the inactivity of *Salmonella* lipid A on human cells, all of the parent LPS preparations stimulated human cells to the same extent as *E. coli* LPS and induced both TNF-α release and NF-κB activation.

The results obtained in the present study are very interesting in two senses. First, the lipid A from *Salmonella* LPS is specifically inactive on human cells, showing that recognition of the lipid A structure by human cells is different from that of mice or rabbits. The same species-specific phenomenon had been observed with tetraacylated lipid A precursor (lipid IVα), which is also active in mice and rabbits, but inactive on human cells (9, 33). However, the meaning of these two lipid A is completely different in view of the fact that the precursor structure is an intermediate in the biosynthesis of LPS, whose structure is not found as a component of any LPS. In contrast, the *Salmonella*-type tetraacylated lipid A actually exists as the major component of *Salmonella* lipid A, along with the hexaacylated lipid A, e.g., *E. coli*-type lipid A.

The second point is that *Salmonella* lipid A is inactive, but that the LPS exhibits full activity in terms of both the induction of TNF-α release and NF-κB activation in human macrophage-like cells, while both preparations are equally active in the other assay systems employed in the present study, including *Limonula* gelation activity and the activation of mouse cells, both peritoneal macrophages and the J774-1 line, as shown by many previous studies performed both by us and other investigators. These facts suggest that the polysaccharide portion plays an important role in the manifestation of activity in human cells. However, the polysaccharide portion alone did not activate the cells at all, and no LPS activity was recovered when lipid A and polysaccharide of the same LPS origin were added to the cells simultaneously. Furthermore, the activity of LPS in human cells was not inhibited even by adding a 100 times excess amount of the polysaccharide portion.

The reason why *Salmonella* lipid A, but not LPS, fails to stimulate human cells, but not mouse cells, is unclear. Until now, the active center of the endotoxic activity of LPS has been thought to reside in the lipid A portion. There have been no reports claiming that the polysaccharide portion plays a role in the activation of macrophages as well as the activation of other endotoxic activities. Although the role of polysaccharide in human cell activation is still unknown, these results suggest that the activation mechanism of LPSs may differ. Recently, Shimazu et al. have reported that MD-2, a molecule that confers LPS responsiveness on Toll-like receptor 4 (TLR4), has broader specificity in the form of the TLR4-MD2 complex than recently described for TLR2 (34), and they suggested that the polysaccharide portion selectively affects recognition by TLR2 (35). All considered, further study concerning the role of the polysaccharide portion in LPS signaling in human cells is needed and is currently under way.

The findings in the present study also suggest that the activity of *Salmonella* LPS introduced into the body may be altered by circumstances or by host defense mechanisms: *Salmonella* LPS is easily detoxified because the ketodic linkage that links lipid A, and the polysaccharide portion is quite susceptible to acidic conditions (36), whereas inactive *Salmonella* lipid A may change to the active form by being split off the acyloxyacylated fatty acids by the enzyme in macrophages (37, 38). These changes may make understanding the reaction of endotoxin in sepsis complex.

Based on the results of the present and previous studies, it seems that higher animals have higher specificity for the recognition of lipid A structures as observed in the case of lipid A precursor and chemically modified lipid A analogues (39). This seems to suggest that the active endotoxin is effectively detoxified in higher animals.

Experiments concerning the structure-activity relationships of lipid A as have mainly been performed in animal systems thus far, and thus an important finding in the present study had been overlooked. Accordingly, the study should be performed again from the viewpoint of human endotoxinsis by using a human system and chemically pure materials.

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**References**


