Succinylated lipid A is a potent and specific inhibitor of endotoxin mitogenicity

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Chemically modified lipopolysaccharides of *Salmonella abortus-equi* were tested for mitogenicity on mouse spleen cells as well as antagonism of the mitogenicity of intact lipopolysaccharide (LPS). All the lipopolysaccharide preparations deacylated by different alkaline treatments suffered a drastic loss of mitogenicity. The mitogenic activity of lipid A was also lost when succinic residues were introduced on hydroxyl groups. Partially deacylated alkaline-treated preparations (but not completely deacylated preparations) inhibited the activation of splenic B-cells by LPS. They were found to be toxic to spleen cells, however, and to suppress not only the mitogenicity of LPS but that of concanavalin A as well. This inhibitory action was not exhibited when all of the fatty acid was eliminated. Succinylated lipid A, on the other hand, was not toxic to the cells and inhibited the B-cell mitogenicity of lipopolysaccharide (but not the T-cell mitogenicity of concanavalin A). Chemical analysis revealed that about 4–6 mol of succinic acid had been introduced into lipid A by succinylation, and that the fatty acid and phosphate composition was unchanged by this treatment. Macrophages do not seem to participate in this inhibition. Inhibition was observed when succinylated lipid A was added either at the same time or after lipid A mitogen, but optimal inhibition was expressed when it was added to the culture 3 h before LPS. Inhibition was not affected by washing the cells before adding LPS. Inhibition increased as the ratio of suppressor to mitogen increased, suggesting that the succinylated lipid A competes with intact LPS.

Introduction

The lipid A moiety of lipopolysaccharides (LPS) is known to be the active centre of the endotoxin which exerts numerous biological activities both *in vivo* and *in vitro* (Lüderitz *et al.*, 1982). The chemical structure of lipid A from *Escherichia coli* was determined recently, and the lipid A synthesized chemically based on this structure proved to be identical to natural lipid A in many of its biological activities (Galanos *et al.*, 1985; Homma *et al.*, 1985; Kotani *et al.*, 1985). Chemically synthesized lipid A analogues have also contributed to the study of the relationship between chemical structure and biological activities of endotoxin (Galanos *et al.*, 1984, 1985, 1986; Homma *et al.*, 1985; Kanegasaki *et al.*, 1984, 1986; Kotani *et al.*, 1985, 1986; Tanamoto *et al.*, 1984a, b, 1990). During the course of such studies, we found that some of the chemically synthesized lipid A analogues, which were structurally distinct from native lipid A regarding the position of fatty acid substitution and were hence endotoxically inactive in many biological

tests (Tanamoto *et al.*, 1984b), antagonize the mitogenicity of LPS (Tanamoto *et al.*, 1984a). Recently the antagonism of endotoxic activity by nontoxic LPS (or lipid A) and its analogues has been studied intensively. Lipid X, which is known to be a precursor of lipid A, was found to inhibit LPS activation of 70Z/3 cells *in vitro* (Sibley *et al.*, 1988), and to prevent expression of the toxicity of LPS *in vivo* (Proctor *et al.*, 1986). It was also found that lipid X and its analogue 3-aza-lipid X inhibit neutrophil priming induced by LPS *in vitro* (Danner *et al.*, 1987). Another lipid A precursor, which lacks secondary fatty acids and did not stimulate human mononuclear cells to synthesize interleukin-1, inhibited LPS activity (Loppnow *et al.*, 1989). A nontoxic natural lipid A was also obtained from *Rhodopseudomonas sphaeroides* (Qureshi *et al.*, 1988; Strittmatter *et al.*, 1983) and found to antagonize LPS (Qureshi *et al.*, 1991) and to inhibit the activation of B cell line 702 B (Kirkland *et al.*, 1991) as well as tumor necrosis factor induction in the RAW 264-7 murine macrophage cell line (Takayama *et al.*, 1989). Detection of such an endotoxin inhibitor or antagonist is of great significance because of its possible application to the treatment of endotoxic diseases in

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Gram-negative bacterial infection. In this study, we attempted to modify native LPS and lipid A chemically in such a way as to render them endotoxically inactive and tested their inhibitory activity on the mitogenicity of LPS.

Methods

Materials. LPS was obtained from *Salmonella abortus-equi* (*S. choleraesuis* subsp. *choleraesuis* pathovar *abortus-equi*) by the aqueous phenol method (Westphal *et al.*, 1952). This strain was kindly provided by Dr O. Lüderitz, Max-Planck-Institut, Freiburg, Germany. Lipid A was obtained as an insoluble substance after treatment with 1% (v/v) acetic acid at 100 °C for 30 min (Galanos *et al.*, 1971).

Alkaline treatment of LPS. (a) Hydroxyaminolysis (Tanamoto *et al.*, 1979): LPS from *S. abortus-equi* was suspended in 2% (w/v) NH_2OH /ethanol solution containing 4% (w/v) NaOH and heated at 63 °C for 3 min. The liberated fatty acids and hydroxamates were removed by extraction with chloroform three times after acidification. The salt produced by neutralization was removed by dialysis against water. (b) Hydrazinolysis (Tanamoto *et al.*, 1979): *S. abortus-equi* LPS was treated with anhydrous hydrazine (1 ml) in a sealed tube for 40 h at 100 °C. After the reaction, excess hydrazine was diluted with water. The mixture was neutralized with HCl and the fatty hydrazides liberated were removed by extraction with chloroform. Deacylated LPS was obtained after eliminating the salt by gel filtration (Pharmacia, G-10). (c) Alkaline hydrolysis: LPS from *S. abortus-equi* was suspended in an alkaline solution [0.2 M-NaOH in 99% (v/v) ethanol] and heated at 50 °C for 18 h. The hydrolysate was neutralized, dialysed against water to remove the salt, and lyophilized.

Succinylation of lipid A. Succinylation was performed according a standard method (Rietschel *et al.*, 1971; Schenck *et al.*, 1969; Tanamoto *et al.*, 1984). Briefly, the suspension of 5 mg LPS (dried over P_2O_5 in a desiccator), 100 mg succinic anhydride, and 90 μl pyridine (freshly distilled over KOH), was heated in a sealed tube at 60 °C for 3 h. The mixture was poured into water (2 ml, 4 °C), dialysed and lyophilized.

Chemical analysis of lipid A preparations. Lipid A fatty acids were determined by gas chromatography using heptadecanoic acid as the internal standard (Tanamoto *et al.*, 1978).

Amino sugar and phosphate were determined in accordance with the methods described by Strominger *et al.* (1959) and Lowry *et al.* (1954), respectively. Succinic acid was determined as described below, using glutaric acid as the internal standard. Test samples were dissolved in 4 M-HCl and hydrolysed for 3 h at 100 °C. The reaction mixture was then evaporated by blowing with nitrogen gas and dissolved in 1 ml distilled water. A 100 μl sample of the solution was injected into a high-performance liquid chromatograph (HPLC; Hitachi model L-6200) equipped with an ODS reverse-phase column (C18) (5 μm , 4 $\times$ 250 mm) containing Lichrosorb. Elution was performed with distilled water containing 0.1% phosphoric acid at a flow rate of 1 ml min^{-1} . Free succinic acid was detected by absorption at 210 nm.

Preparation of cells. Spleen cells from female BALB/C mice (6 to 10 weeks old) were pressed through a wire grid and suspended in Iscove modified Dulbecco medium containing L-glutamine and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer (Gibco) supplemented with penicillin (100 $\mu\text{g ml}^{-1}$) and streptomycin (100 $\mu\text{g ml}^{-1}$). The cell suspension was washed three times with the medium. The spleen cells obtained were approx. 45% B cells, 45% T cells and 10% macrophages. Macrophages in spleen cells were allowed

to adhere to a plastic Petri dish (Eiken Kizai, Japan) for 3 h at 37 °C in the presence of 5% (v/v) CO_2 and the nonadherent cells in the medium were collected and used as macrophage-free cells.

Cell culture and assay of mitogenicity. Cells were cultured in Iscove medium as described above on microplates (Corning, No. 25850-96) at 37 °C with 5% (v/v) CO_2 . Each well contained 8×10^5 cells in 200 μl and the mitogen. After 48 h of incubation, [^3H]thymidine [0.2 μCi (7.4 kBq) per culture; Amersham] was added. After a further 24 h incubation, cultures were harvested, collected on glass fibre filters, and washed with distilled water. The filters were dried and transferred to scintillation vials. Xylol-based scintillation fluid (5 ml) was added, and radioactivity was measured in a scintillation counter. All experiments were done in triplicate. Standard errors were lower than 10% in the mitogenicity assay and are not indicated in the figures.

Inhibition of mitogenicity by chemically modified LPS. The chemically modified preparations were added to the assay system before, together with, or after mitogen, which was used in a dose leading to optimal stimulation [33 $\mu\text{g ml}^{-1}$ LPS and 2 $\mu\text{g ml}^{-1}$ concanavalin A (Con A)]. The reduction in [^3H]thymidine incorporation was tested and compared with a control containing mitogen only.

Cell toxicity of preparations for spleen lymphocytes. Mouse spleen cells (4×10^6 cells ml^{-1}) and test samples were incubated for different periods of time in Falcon plastic tubes at 37 °C in the presence of 5% (v/v) CO_2 . After the incubation, the cells were stained with erythrocyan and the viable cells were enumerated.

Results

Chemical composition of LPS after chemical modification

The fatty acid composition of the alkali-treated LPS preparations is shown in Table 1. Ester-bound fatty acids were partially eliminated from LPS by treatment with 0.2 M-NaOH, while most of the ester-linked fatty acids were removed by hydroxyaminolysis. On the other hand, almost all fatty acids were removed by hydrazinolysis. The chemical composition of lipid A before and after succinylation was compared. The results are shown in Table 2. Calculated on the basis of the glucosamine molecule being equivalent to 2 mol, 4.57 mol of succinic acid was found to have been introduced into lipid A by succinylation. The other components (phosphorus, fatty acids, glucosamine) were unaffected by the treatment.

Mitogenic activity of chemically modified LPS

Chemically modified LPS or lipid A preparations were first tested for mitogenic activity on mouse spleen cells. Each experiment was done in triplicate. Activity of all of the preparations was markedly less than that of the original LPS (Fig. 1). The 0.2 M-NaOH-treated LPS, whose ester-bound fatty acids were partially (about 50%) removed, retained moderate activity, but it amounted to only about 30% of the activity of LPS when compared with optimal stimulation.

Fig. 1. Mitogenic response of murine spleen cells to lipopolysaccharides deacylated with Δ , 0.2 M-NaOH; $\blacktriangle$, NH_2OH ; $\times$, hydrazine; and to succinylated lipid A $\circ$ in comparison with the original lipopolysaccharide ($\square$) and lipid A ($\bullet$) from *S. abortus-equi*.

Table 1. Fatty acid composition of alkaline-treated LPS

Figures are mol fatty acid per 2 mol glucosamine.

Fatty acid	LPS	LPS treated with		
		N_2H_4	NH_2OH	NaOH
12:0	0.85	0.08	0.04	0.57
14:0	0.61	0.04	0.03	0.27
16:0	0.59	0.03	0.00	0.27
3-OH-14:0	3.75	0.14	1.37	2.10

Table 2. Chemical composition of lipid A before and after succinylation

Figures are mol per 2 mol glucosamine.

	GlcN	Phosphate	Fatty acid				Succinic acid
			12:0	14:0	16:0	3-OH-14:0	
Lipid A	2.00	1.45	0.85	0.61	0.59	3.75	—
Succinylated lipid A	2.00	1.34	0.79	0.56	0.58	3.95	4.57

Inhibition of the mitogenic activity of LPS and Con A by chemically modified LPS

Since most of the chemically modified LPS preparations failed to activate spleen cells, this inhibitory effect on the mitogenicity of active LPS was tested by adding them to spleen cell cultures together with the mitogen. Unless otherwise specified, the concentration of LPS used for the stimulation was $33 \mu\text{g ml}^{-1}$. The results are shown in

Fig. 2. Inhibition of splenocyte mitogenic activity of LPS and Con A by deacylated LPS. Inhibition by (a) 0.2 M-NaOH-treated LPS, (b) NH_2OH -treated LPS, and (c) hydrazine-treated LPS. Mitogen: $\circ$, $33 \mu\text{g LPS ml}^{-1}$; $\bullet$, $2 \mu\text{g Con A ml}^{-1}$; $\square$, $1.2 \mu\text{g LPS ml}^{-1}$.

Fig. 3. Inhibitory activity of succinylated lipid A on the activation of whole spleen cells (—) and macrophage depleted spleen cells (---) by LPS ($\circ$) from *S. abortus-equi* and ConA ($\bullet$).

Fig. 2. Both NaOH- and NH_2OH -treated partially-deacylated LPS preparations inhibited the activation of B cells by LPS at concentrations higher than 33 and $100 \mu\text{g ml}^{-1}$, respectively. These samples, however, also inhibited the mitogenicity of Con A ($2 \mu\text{g ml}^{-1}$) in a similar dose-dependent manner. More pronounced inhibition by NH_2OH -treated LPS was observed when $1.2 \mu\text{g ml}^{-1}$ LPS was used as the spleen cell stimulant (Fig. 2b). In this case, inhibition occurred at a lower dose, and cell activation was completely inhibited at $100 \mu\text{g ml}^{-1}$ concentration of the de-O-acylated LPS. No inhibition by hydrazine-deacylated preparation of the thymidine incorporation induced by either LPS or Con A was observed. The polysaccharide portion of LPS from *S. abortus-equi* did not affect the activation of spleen cells by LPS at any concentration tested (up to 1 mg ml^{-1}).

Succinylated lipid A strongly inhibited the mitogenicity of LPS (Fig. 3). Inhibition was observed at concentrations as low as $11 \mu\text{g ml}^{-1}$, and more than 90% inhibition was observed at $100 \mu\text{g ml}^{-1}$. On the other hand, it did not inhibit the mitogenicity of Con A even at $100 \mu\text{g ml}^{-1}$, as shown in Fig. 3.

Fig. 4. Viability of spleen cells after the incubation with △, 0.2 M-NaOH-treated LPS, ▲, NH₂OH-treated LPS or ○, succinylated lipid A. □, Control (water used to dissolve the preparations).

Cytotoxicity of chemically modified LPS toward spleen cells

Since both NH₂OH- and NaOH-treated preparations antagonized spleen-cell-induced mitosis by both Con A and LPS, the spleen cell cytotoxicity of the chemically modified LPS preparations was tested using the erythrocin staining assay. The results are shown in Fig. 4. Preparations partially deacylated by both NH₂OH and NaOH were strongly toxic to the cells and more than 50% of the cells died after 48 h of incubation at 100 µg ml⁻¹ concentrations of both samples. No toxicity, on the other hand, was observed for LPS, hydrazine-treated LPS (data not shown) or succinylated lipid A.

Inhibitory action of succinylated lipid A on splenic B cells

Since succinylated lipid A was found to specifically inhibit the mitogenicity of LPS, the mode of the inhibitory action of the succinylated lipid A was investigated. To determine whether macrophages participated in this inhibition, macrophages were eliminated from the spleen cells by allowing them to adhere to plastic, and the non-adherent cells in the medium were collected and used as macrophage-free cells. The results of mitogenicity inhibition testing of succinylated lipid A on these cells are shown in Fig. 3. The same phenomenon was seen using these cells as with whole spleen cells in both dose-response and inhibition of a B cell-specific mitogen.

The effect of varying the timing of addition of the inhibitor relative to the addition of mitogen to the culture was investigated. LPS (33 µg ml⁻¹) was added to the culture at time zero, and succinylated lipid A (33 µg ml⁻¹) was added at -3, 0, 3, 24, and 48 h. Although inhibition was observed regardless of the timing of the addition, prior addition was most effective in inhibiting mitogenicity (-3 h, 95% inhibition; 0 h, 72%; 3 h, 43%; 24 h, 24%; 48 h, 8%). In a separate experiment, succinylated lipid A (100 µg ml⁻¹) was

Fig. 5. Dose-response of succinylated lipid A inhibition of the activation of spleen cells by different doses of mitogen LPS. □, 100 µg LPS ml⁻¹; ○, 33 µg ml⁻¹; △, 3.7 µg ml⁻¹; and ▲, 1.2 µg ml⁻¹.

preincubated with spleen cells for 3 h to assess the effect of preincubation with the cells on inhibition. The cells were then washed prior to the addition of mitogen. Strong inhibition (74%) was observed.

To investigate the relationship between the dose of succinylated lipid A and the mitogen used for inhibition, different dose ratios were tested. The results are shown in Fig. 5. Inhibition increased in proportion to the ratio of the inhibitor relative to the mitogen. About half of the mitogenicity caused by 100 µg LPS ml⁻¹ was inhibited by 100 µg succinylated lipid A ml⁻¹. The same inhibition occurred with 3.7 µg succinylated lipid A ml⁻¹ when the mitogen was used at a concentration of 3.7 µg ml⁻¹.

Discussion

After the chemical structure of lipid A responsible for the endotoxic activity was determined and synthetic lipid A of the *E. coli* type proved to be biologically identical to natural lipid A in many test systems, efforts were directed toward resolving the question of whether the variety of endotoxic activities could be separated structurally. Thus far, however, it has been difficult to obtain substances which retain full activity but completely lack toxicity. Another strategy is directed toward finding LPS analogues which are nontoxic but inhibit LPS activity. Such analogues may become candidates for treatment of endotoxicoes and at the same time be useful in biological studies of endotoxin, including the receptor issue. Some candidates have been reported by us and others (Danner *et al.*, 1987; Erwin *et al.*, 1991; Kirkland *et al.*, 1991; Proctor *et al.*, 1986; Qureshi *et al.*, 1991; Takayama *et al.*, 1989; Tanamoto *et al.*, 1984b). With this in mind, we tried several chemical modifications of LPS in order to produce inactive forms of LPS

and tested both their mitogenicity and inhibitory activity on the mitogenicity of LPS. Since the importance of fatty acids to the biological activity of endotoxin has been emphasized in previous studies (Kanegasaki *et al.*, 1986; Tanamoto *et al.*, 1979, 1984a), LPS was first exposed to several alkaline conditions to eliminate fatty acids from LPS. The mitogenicity of LPS was drastically reduced by all treatments regardless of the degree of fatty acid elimination, again corroborating the importance of fatty acids to biological activity. Partially deacylated LPS, but not completely deacylated preparation, inhibited the mitogenicity of LPS. This inhibition, however, was not specific for B-cell mitogen but also suppressed spleen cell activation by Con A. They were also found to be toxic for spleen cells. The inhibition of B-cell mitogen LPS by de-*O*-acylated LPS was more pronounced when a lower concentration of LPS was used as the stimulant. The inhibitory activity of these compounds, therefore, may be the result of a complex phenomenon involving both competitive inhibition of LPS mitogen and cytotoxicity. When all of the fatty acids had been eliminated, LPS exerted neither mitogenicity nor inhibition of LPS mitogenicity, suggesting that some fatty acids, if not all, are indispensable not only to mitogenic activity but to inhibition of LPS mitogenicity. A similar phenomenon was recently reported by Erwin *et al.* (1991), i.e. that enzymically deacylated *Neisseria* LPS inhibits murine splenocyte mitogenesis induced by LPS. They did mention, however, that the inhibition was specific for the B-cell mitogen LPS but not the T-cell mitogen Con A. Pohlman *et al.* (1987) showed that selective deacylation of the nonhydroxylated fatty acids of LPS renders it less toxic and effective in inhibiting neutrophil adhesion to endothelial cells specifically caused by LPS. The discrepancy between the inhibition specificity in their study and our own may be explained by the difference in degree of deacylation. The most effective inhibition in our experiments was manifested by succinylated lipid A. Based on the results of the chemical analysis, most of the hydroxyl groups in lipid A were replaced by succinic acid during succinylation, but the other components of lipid A were unaffected. Lipid A lost its mitogenicity on succinylation, and this is consistent with the results obtained from tests using chemically synthesized lipid A analogues, in which overacylated lipid A reduced endotoxic activity (Galanos *et al.*, 1986; Kanegasaki *et al.*, 1986; Kotani *et al.*, 1986). Native lipid A itself, but not LPS, exhibited cell toxicity at higher concentrations and hence reduced the mitogenicity of LPS, as well as that of Con A, non-specifically. It may be the reason why lipid A can not cause mitosis on B cells at higher concentration (Fig. 1). On the other hand, succinylated lipid A inhibited the mitogenic activity of LPS specifically but did not affect the mitogenicity of Con A, even at

higher concentrations, and is not toxic to spleen cells. Some aspects of the inhibitory action of succinylated lipid A were investigated. Macrophages did not seem to play a decisive role in the inhibition, indicating that the various mediators secreted by macrophages in response to stimulation by LPS do not play a role in inhibition. Regarding the timing of addition of this inhibitor, optimal inhibition was expressed when it was added to the culture before the mitogen. Preincubation of inhibitor with the cells was adequate for the inhibition to be expressed, and inhibition was not affected by washing the cells before exposure to the mitogen. Inhibition became even more pronounced as the ratio of inhibitor to mitogen increased. Taking all of these findings into consideration, succinylated lipid A acts competitively with LPS and specifically suppresses the mitogenic activity of LPS.

These data demonstrate that succinylated lipid A is a much more potent and specific inhibitor of LPS mitogen than deacylated LPS in our test system. Many other interesting questions concerning succinylated lipid A remain to be resolved, e.g. whether it acts as an inhibitor of other endotoxic activities *in vivo* or *in vitro*, and which structural component of succinylated lipid A is the most potent inhibitor of endotoxic activities.

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