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Augmented Immunological Activities of Recombinant Lipopolysaccharide Possessing the Mannose Homopolymer as the O-Specific Polysaccharide

NORIKO PAENG,[1] NOBUO KIDO,[1] GÜNTER SCHMIDT,[2] TSUYOSHI SUGIYAMA,[3] YUTAKA KATO,[3] NAOKI KOIDE,[3] AND TAKASHI YOKOCHI[3]*

Department of Natural Science Informatics, School of Informatics and Sciences, Nagoya University, Nagoya, Aichi 464-01,[1] and Department of Microbiology and Immunology, Aichi Medical University, Nagakute, Aichi 480-11,[3] Japan, and Forschungsinstitut, Borstel, Germany[2]

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Recombinant lipopolysaccharide possessing the mannose homopolymer as the O-specific polysaccharide was manufactured genetically by transforming *Escherichia coli* K-12 with various *rfb* genes capable of synthesizing the mannose homopolymer. Recombinant lipopolysaccharide exhibited levels of anticomplement activity, adjuvant activity, and regional lymph node-enlarging activity much higher than those exhibited by the original rough-type lipopolysaccharide from *E. coli* K-12 or lipopolysaccharide possessing the heteropolysaccharide from *E. coli* O111. Immunological activities of recombinant lipopolysaccharide were as strong as those of wild-type lipopolysaccharide possessing the mannose homopolymer. Characteristic activities of wild-type lipopolysaccharide possessing the mannose homopolymer were exhibited by recombinant lipopolysaccharide. The abilities of lipopolysaccharide to activate B cells polyclonally and to produce cytokines did not seem to be related to the presence of the mannose homopolymer. Therefore, it was suggested that the mannose homopolymer in the O-specific polysaccharide might exclusively enhance anticomplement activity, adjuvant activity, and regional lymph node-enlarging activity among various lipid A activities.

Bacterial lipopolysaccharide (LPS) exhibits a variety of biologic activities, such as toxicity, adjuvanticity, anticomplement activity, polyclonal B-cell activation, and cytokine-inducing activities (12, 13). LPS is usually made up of the O-specific polysaccharide that consists of the heteropolysaccharide and the core oligosaccharide that is ketosidically bound to a lipid component called lipid A (18). The O-specific polysaccharide is devoid of typical endotoxic properties, whereas free lipid A, in a soluble form, expresses all the characteristic in vivo activities of LPS (18). Although lacking endotoxicity, the O-specific polysaccharide moiety may modulate the lipid A activity (14, 24). It has been reported that LPS possessing the mannose homopolymer as the O-specific polysaccharide, such as LPS from *Klebsiella* O3 (2), *Klebsiella* O5 (5), *Escherichia coli* O8 (5), and *E. coli* O9 (17), exhibits several characteristic immunological activities. In particular, LPS possessing the mannose homopolysaccharide is more than 100 times more potent in activating the complement system than LPS possessing the heteropolysaccharides (6, 24). LPS possessing the mannose homopolymer also exhibits an extraordinarily strong adjuvant action (7, 9, 16, 22, 24) and induces the production of autoimmune responses to various tissue extracts (21–23). Furthermore, subcutaneous injection of such LPS induces marked swelling of the regional lymph node (7, 26, 27). On the other hand, LPS possessing the mannose homopolymer does not modulate lethality and B-cell mitogenicity (15). In this study we constructed a series of *E. coli* strains expressing the mannose homopolymer as the O-specific polysaccharide as a consequence of genetic manipulation. Since these strains had the same genetic backgrounds except for the O-specific polysaccharide biosynthetic pathway, we examined the effect of the mannose homopolymer on various immunological activities of recombinant LPS prepared from these strains. Here we report that recombinant LPS expressing the mannose homopolymer selectively exhibits strong anticomplement, adjuvant, and regional lymph node-enlarging activity.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Aichi Medical University, Nagakute, Aichi 480-11, Japan. Phone: 81-561-62-3311, ext. 2269. Fax: 81-561-63-9187.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. JA221 is an *E. coli* K-12 strain used previously for the cloning of the *E. coli* O9 *his-rfb* region (8). Clone 31 was isolated from *E. coli* O8 strain F492 by G. Schmidt. The plasmid carries the *rfb* region which directs the synthesis of the O8 polysaccharide in *E. coli* K-12 strains. O8 polysaccharide synthesis was confirmed by a slide agglutination test using anti-*E. coli* O8 polyclonal rabbit serum and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Plasmid pNKB26 carries the whole *E. coli* O9 *rfb* region (10), and its derivative, del 21, carries the deleted *E. coli* O9 *rfb* region which directed the synthesis of the mannan O polysaccharide consisting of only 1,2 bonds (8). The recombinant strains were constructed by introducing the cloned *rfb* regions into JA221. Bacterial cells were cultivated in L broth. For the culture of bacteria carrying plasmids L broth was supplemented with chloramphenicol (30 μg/ml) for pACYC184-based clones of pNKB26 and del 21 and ampicillin (50 μg/ml) for a pHC79-based clone of clone 31.

Construction of the recombinant strains. Recombinant strains of *E. coli* K-12 carrying the cloned *rfb* genes were constructed as reported previously (8, 10). In brief, competent cells of JA221 were prepared by the method described by Sambrook et al. (19). *E. coli rfb* clones were mixed with the competent cells, and recombinant strains carrying each plasmid were selected on agar plates containing antibiotics. Slide agglutination test-positive strains were used for the extraction of LPS.

LPS. LPS preparations were extracted from recombinant strains JA221(pNKB 26), JA221(del 21), and JA221(clone 31) by the phenol-water method as described previously (20, 25). The O-specific antigenic structure of recombinant LPS was also confirmed by immunoblotting with antisera. Wild-type LPS was also prepared from *E. coli* O8 strain F492, *E. coli* O9 strain B993, and *Klebsiella* O3 strain LEN-1. LPS extracted by the phenol-water method from *E. coli* O111 was obtained from Difco Laboratories (Detroit, Mich.). The structures of the

TABLE 1. Bacterial strains used in this study

Strain	O antigen (reference or source)
Wild type	
E. coli O8 F492	O8
E. coli O9 B993	O9
Klebsiella O3 strain LEN-1	O3
E. coli K-12 JA221	None[a]
Recombinant	
JA221(clone 31)	O8 (G. Schmidt)
JA221(pNKB26)	O9 (10)
JA221(del 21)	O9 derivative (8)

[a] Rough-type LPS.

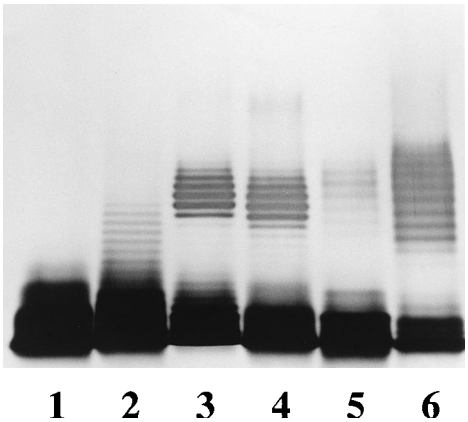

FIG. 1. Silver staining of LPS. LPS was separated on a 5 to 20% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate and detected by silver staining. Note the rough-type LPS from *E. coli* K-12 JA221. Lane 1, *E. coli* K-12 JA221 LPS; lane 2, recombinant del 21 LPS; lane 3, wild-type *E. coli* O8 LPS; lane 4, recombinant *E. coli* O8 LPS; lane 5, wild-type *E. coli* O9 LPS; lane 6, recombinant *E. coli* O9 LPS.

repeating units in the O-specific polysaccharides of the LPSs used in this study are shown in Table 2.

Analysis of LPS. LPS was separated on a 5 to 20% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate under reducing conditions, and LPS bands were detected by the silver staining method of Hitchcock and Brown (3). The staining pattern of the LPS tested is shown in Fig. 1.

Mice. BALB/c mice, 6 to 8 weeks of age, were purchased from Japan SLC (Hamamatsu, Japan).

Assay for anticomplement activity of LPS. Various concentrations of LPS in 0.01 M phosphate-buffered saline (PBS; pH 7.2; 12.5 μl) were incubated with human serum (50 μl) for 30 min at 37°C. Determination of anticomplement activity was performed according to Mayer's technique 1/2.5 (Denka Seiken Co., Tokyo, Japan) (24). Briefly, serum treated with LPS was adjusted to a total volume of 2 ml with Veronal buffer containing 0.06 M $CaCl_2$ and 0.1 M $MgCl_2$, pH 7.5. The diluted serum was mixed with Veronal buffer (1.1 ml), and then hemolysin-sensitized sheep erythrocytes (SRBC) (0.4 ml) were added. The mixture (3 ml) was incubated without mixing at 37°C for 60 min. The reaction was stopped by incubating the mixture at 4°C for 5 min. The suspension of SRBC was centrifuged at 1,000 × *g* for 10 min. The optical density (OD) of the supernatant was determined spectrophotometrically at 541 nm (Shimadzu Corp., Kyoto, Japan). Spontaneous hemolysis and 100% hemolysis were estimated by adding 2.6 ml of Veronal buffer and 2.6 ml of distilled water to 0.4 ml of sensitized SRBC suspension, respectively. The percent hemolysis was calculated as follows: % hemolysis = (experimental OD − spontaneous OD)/(100% OD − spontaneous OD).

Adjuvant activity of LPS on antibody response to deaggregated BSA. Bovine serum albumin (BSA) was purchased from Miles Laboratories, Inc. (Kankakee, Ill.), and was suspended at a concentration of 1 mg/ml in PBS. Deaggregated soluble BSA was prepared by ultracentrifugation at 100,000 × *g* for 1 h. Three mice per experimental group were injected subcutaneously with deaggregated BSA (1 ml) and LPS (100 μg) together or with BSA alone. Blood was taken from the retro-orbital venous plexus of mice at 14 days after the injection. Anti-BSA antibody titers in the sera were measured by an enzyme-linked immunosorbent assay (ELISA) (24). Briefly, microplates were coated with BSA at a concentration of 2 μg/ml overnight at room temperature. Each serum sample was subjected to twofold dilution from 1/50 to 1/12,800 with PBS and dispensed down a column of a microplate at 100 μl per well. Plates were incubated at 37°C for 1 h and then washed four times with PBS containing 0.05% Tween 20. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako Japan Co., Kyoto, Japan) was diluted to 1/1,000 with PBS containing Tween 20 and added at 100 μl per well, and the plates were incubated for a further 60 min at 37°C. The plates were washed four times with PBS containing Tween 20, and peroxidase substrate solution (200 μl) containing 0.01% *o*-phenylenediamine and 0.03% H_2O_2 in PBS was added. Reactions were stopped about 15 min later with 1 N H_2SO_4. The OD of wells was read relative to a substrate blank at 492 nm on an automatic microplate reader 450 (Bio-Rad, Hercules, Calif.). The mean OD with standard deviation at various dilutions of sera for each experimental group (four mice) was calculated, and the serum dilution showing an OD of 0.500 was estimated.

In vivo and in vitro polyclonal B-cell activation. Mice were injected intraperitoneally (i.p.) with 2×10^8 SRBC, and 2 weeks later LPSs (100 μg) were injected i.p. Two days after injection of LPS spleens were removed and dissected to prepare a single-cell suspension in RPMI 1640. The spleen cell suspensions were used for an anti-SRBC plaque-forming cell (PFC) assay. The number of anti-SRBC PFCs was determined by Cunningham's technique (1). In brief, a mixture of an appropriate diluted cell suspension in RPMI 1640 (50 μl), normal guinea pig serum (7 μl), and 50% SRBC (7 μl) was incubated at 37°C for 60 min in a Cunningham chamber for development of PFCs. The spleen cell suspension from mice which were injected with SRBC alone was cultured in vitro in RPMI 1640 culture medium supplemented with 5% decomplemented fetal calf serum. Spleen cell suspension (2×10^6 cells per ml) was seeded into a 24-well plate and incubated at 37°C in 5% CO_2 for 2 days. Various LPSs were added at a final concentration of 10 μg/ml at the start of incubation. In vitro-cultured spleen cells were also used for an anti-SRBC PFC assay.

Weight of the regional lymph node. Previously we showed that the increase in weight of the regional lymph node reached a peak 10 to 14 days after the injection of LPS (7, 26, 27). LPS (100 μg) was injected subcutaneously into the right inguinal region of mice, and the wet right inguinal lymph nodes were weighed 14 days after the injection of mice with LPS. The right inguinal lymph nodes from untreated control mice were used for the negative control.

Estimation of TNF-α and IFN-γ levels in the serum. Levels of circulating tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) in peripheral blood were estimated by ELISA (Genzyme Corp., Cambridge, Mass.). Three mice per experimental group were injected i.p. with LPS (100 μg). On the basis of the maximum release of TNF-α and IFN-γ, blood was taken from the retro-orbital venous plexus at 1 and 6 h after the injection. The sera were then analyzed with respect to concentrations by performing an ELISA according to the manufacturer's instructions.

TABLE 2. Structures of repeating units of LPSs used in the present study

O antigen	Structure of the repeating unit
E. coli O8	α-Man3Me-(1┼→3)-β-Man-(1→2)-α-Man-(1→2)-α-Man-(1┼→$_n$
E. coli O9	┼→3)-α-Man-(1→3)-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1┼→$_n$
Klebsiella O3	┼→3)-α-Man-(1→3)-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1┼→$_n$
E. coli O9 derivative	┼→2)-α-Man-(1→2)-α-Man(1→2)α-Man-(1┼→$_n$ Col
E. coli O111	α ↓ 1.3 ┼→4)-α-Glc-(1→4)-α-Gal(1→3)-β-GlcNAc-(1 ┼→$_n$ α ↑ 1.6 Col

TABLE 3. Activation of complement by various LPSs

LPS	Inhibition of hemolysis (%) with the following concn of LPS in the serum[a]:				
	2,500	250	25	2.5	ED_{50}
Recombinant *E. coli* O8	96	93	30	5	54[b]
Recombinant *E. coli* O9	97	98	39	14	35[b]
Recombinant *E. coli* O9 derivative	98	98	19	0	65[b]
E. coli O8	95	93	36	13	42[b]
E. coli O9	96	93	43	9	39[b]
Klebsiella O3	91	98	38	10	43[b]
E. coli O111	49	7	0	0	>2,500
E. coli K-12	94	54	2	0	173

[a] Concentrations are in micrograms per milliliter except for the 50% effective dose (ED_{50}).
[b] $P < 0.01$ versus *E. coli* O111 by Student's *t* test.

RESULTS

Anticomplement activity of recombinant LPS. The ability of recombinant LPS to activate serum complement was examined (Table 3). Recombinant LPS containing the mannose homopolymer inhibited hemolysis (i.e., consumed complement) completely at a concentration of 250 μg/ml and partially at a concentration of 25 μg/ml. The intensity of anticomplement activity was as high as that of wild-type LPS possessing the mannose homopolymer. On the other hand, the level of activity of LPS from *E. coli* O111, which does not contain the mannose homopolymer, was very low. This suggests that the presence of the mannose homopolymer as the O-specific polysaccharide can enhance anticomplement activity of LPS. Rough-type LPS from *E. coli* K-12 exhibited levels of activity higher than those exhibited by LPS from *E. coli* O111 but lower than those exhibited by recombinant LPS. The high-level anticomplement activity of rough-type LPS could be explained by the binding of Ra factor to rough-type LPS (4).

Adjuvant action of recombinant LPS on antibody response to deaggregated BSA. The antibody titers to BSA in sera from mice immunized subcutaneously with deaggregated BSA together with various LPSs were compared by ELISA (Table 4). Subcutaneous injection of deaggregated BSA alone did not initiate any detectable anti-BSA antibody response. However, a strong anti-BSA response was induced by injection of BSA mixed with various LPSs tested. Recombinant LPS possessing mannose homopolymer exhibited the strongest antibody responses to BSA in this experimental system. Wild-type LPS from *Klebsiella* O3, *E. coli* O8, and *E. coli* O9 also exhibited the strong adjuvant action. There was no marked difference in the intensity of the adjuvant action between recombinant LPS and wild-type LPS possessing the mannose homopolymer. On the other hand, LPS from *E. coli* O111 or K12 did not show such strong adjuvant activity. LPS from the *E. coli* O9 derivative exhibited intermediate adjuvant activity.

TABLE 4. Adjuvant action of various LPSs on primary antibody response to deaggregated BSA

LPS	Antibody titer[a]
Recombinant *E. coli* O8	>2,000[b]
Recombinant *E. coli* O9	>2,000[b]
Recombinant *E. coli* O9 derivative	1,200[b]
E. coli O8	>2,000[b]
E. coli O9	>2,000[b]
Klebsiella O3	>2,000[b]
E. coli O111	500
E. coli K-12	400
None[c]	<50

[a] Serum dilution showing an OD of 0.500.
[b] $P < 0.01$ versus *E. coli* O111 and *E. coli* K-12 by Student's *t* test.
[c] BSA alone was injected.

TABLE 5. Activity of recombinant LPS to enlarge the regional lymph node

LPS	Mean wet weight of the regional lymph node ± SD (mg)
Recombinant *E. coli* O8	28.17 ± 13.9[a]
Recombinant *E. coli* O9	25.50 ± 15.26[a]
Recombinant *E. coli* O9 derivative	17.56 ± 1.84[a]
E. coli O8	28.17 ± 13.9[a]
E. coli O9	23.67 ± 14.12[a]
Klebsiella O3	47.45 ± 7.16[a]
E. coli O111	7.31 ± 1.13
E. coli K-12	6.84 ± 2.6

[a] $P < 0.01$ versus *E. coli* O111 and *E. coli* K-12 by Student's *t* test.

The regional lymph node-enlarging activity of recombinant LPS. Previously we reported that subcutaneous injection of LPS possessing the mannose homopolymer resulted in a marked enlargement of the regional lymph node, whereas injection of LPS possessing the heteropolysaccharide did not (7, 26, 27). Mice were injected subcutaneously with LPS (100 μg), and 2 weeks later the regional inguinal lymph nodes were weighed. Recombinant LPS was tested for the ability to enlarge the regional lymph node (Table 5). The injection of recombinant LPS possessing the mannose homopolymer induced marked swelling of the regional inguinal lymph node. The swelling of the regional lymph node produced by LPS from *E. coli* O111 or K-12 was only slight. LPS from *Klebsiella* O3 exhibited the strongest regional lymph node-enlarging activity among wild-type LPSs possessing the mannose homopolymer.

The ability of recombinant LPS to activate SRBC-primed B cells polyclonally in vivo. Mice were injected i.p. with 2×10^8 SRBC, and 2 weeks later the effective dose of LPS (100 μg) was injected i.p. The anti-SRBC PFC number in the spleen was estimated 2 days after injection of LPS. The experimental results are shown in Table 6. There was no significant difference in anti-SRBC PFC numbers among various LPSs tested. Recombinant LPS exhibited the same high-level activity as LPS possessing the heteropolysaccharide, such as *E. coli* O111 LPS or rough-type *E. coli* K-12 LPS.

The ability of recombinant LPS to activate SRBC-primed B cells polyclonally in vitro. Mice were injected i.p. with 2×10^8 SRBC, and 2 weeks later the spleens were removed. Since

TABLE 6. In vivo polyclonal B-cell activation by recombinant LPS

LPS	No. of anti-SRBC PFCs[a]
Recombinant *E. coli* O8	8,800 ± 908
Recombinant *E. coli* O9	7,120 ± 733
Recombinant *E. coli* O9 derivative	9,980 ± 843
E. coli O8	7,600 ± 1,208
E. coli O9	9,920 ± 1,524
Klebsiella O3	8,640 ± 1,680
E. coli O111	8,480 ± 923
E. coli K-12	9,760 ± 1,527
None	1,040 ± 367

[a] Mean number of PFCs per spleen with standard deviation. Statistically not significant.

TABLE 7. In vitro polyclonal B-cell activation by recombinant LPS

LPS	No. of anti-SRBC PFCs[a]
Recombinant *E. coli* O8	108 ± 17
Recombinant *E. coli* O9	84 ± 17
Recombinant *E. coli* O9 derivative	96 ± 23
E. coli O8	124 ± 17
E. coli O9	100 ± 6
Klebsiella O3	112 ± 6
E. coli O111	116 ± 6
E. coli K-12	86 ± 11
None	4 ± 4

[a] Mean number of PFCs per 2×10^6 cells with standard deviation. Statistically not significant.

there was no significant difference among the abilities of LPSs to activate B cells in vitro at concentrations from 5 to 50 μg/ml, spleen cells were cultured at an LPS concentration of 10 μg/ml for 2 days. The anti-SRBC PFC number per dish was estimated. The experimental results are shown in Table 7. The ability of recombinant LPS to activate B cells polyclonally in vitro was as strong as that of LPS from *E. coli* O111 or K-12. There was no significant difference in the abilities of various LPSs tested to activate B cells polyclonally in vitro.

The ability of recombinant LPS to produce TNF-α and IFN-γ in vivo. Mice were injected i.p. with LPS (100 μg). The sera were tested at 1 and 6 h after the injection of LPS for TNF-α and IFN-γ, respectively, by ELISA. A typical experimental result is shown in Table 8. There was no marked difference in production of TNF-α among various LPSs tested. Similarly, no marked differences in the production of IFN-γ were seen. Rough-type LPS from *E. coli* JA221 caused the release of smaller amounts of circulating TNF-α and IFN-γ than did smooth-type LPS. TNF-α and IFN-γ levels in untreated control or saline-injected mice were lower than the detection limit of the assay (125 pg/ml for IFN-γ and 15 pg/ml for TNF-α).

DISCUSSION

In the present study we isolated a series of *E. coli* K-12 strains carrying *rfb* genes capable of synthesizing the mannose homopolymer as the O-specific polysaccharide and prepared recombinant LPS possessing the mannose homopolymer as the O-specific polysaccharide. Recombinant LPS exhibited strong anticomplement activity, adjuvant activity, and regional lymph node-enlarging activity. These activities of recombinant LPS were almost as strong as those of wild-type LPSs possessing the mannose homopolymer, such as *Klebsiella* O3, *E. coli* O8, and *E. coli* O9 LPS. Previously we reported that LPS possessing the mannose homopolymer exhibits characteristic activities, such as strong adjuvant action, extraordinarily high-level anticomplement activity, and potent regional lymph node-enlarging activity (7, 9, 16, 21, 22, 26, 27). The present study demonstrated that those characteristic activities are exhibited by recombinant LPS. Once again, it was confirmed that the mannose homopolymer as the O-specific polysaccharide clearly enhanced some of the lipid A activities. This might be a typical example of the modulation of the lipid A activities by the O-specific polysaccharide moiety of LPS molecules. Recently we have reported that the mannose-binding protein may be crucial for the expression of strong anticomplement activity by LPS possessing the mannose homopolymer (6). On the basis of the findings of the present study, mannose-binding protein may be involved in the expression of strong adjuvant action and the marked swelling of regional lymph nodes as well as anticomplement activity.

TABLE 8. In vivo production of TNF-α and IFN-γ by recombinant LPS

LPS	Concn in serum ± SD (pg/ml)	
	TNF-α	IFN-γ
Recombinant *E. coli* O8	555 ± 155	925 ± 125
Recombinant *E. coli* O9	475 ± 50	1,050 ± 350
Recombinant *E. coli* O9 derivative	690 ± 255	1,250 ± 350
E. coli O8	505 ± 165	1,050 ± 250
E. coli O9	745 ± 210	1,400 ± 350
Klebsiella O3	510 ± 90	1,650 ± 350
E. coli O111	590 ± 180	1,000 ± 150
E. coli K-12	265 ± 35[a]	475 ± 225[a]
None	<15	<125

[a] $P < 0.01$ versus smooth-type LPS by Student's *t* test.

Recombinant LPS possessing the mannose homopolymer did not affect the ability to activate B cells polyclonally in vitro and in vivo. Similarly, the presence of the mannose homopolymer as the O-specific polysaccharide did not cause enhanced production of TNF-α and IFN-γ in vivo. This suggested that the ability of LPS to activate B cells polyclonally in vivo and in vitro was dependent not on the presence of the mannose homopolymer but on the lipid A moiety alone. This is consistent with a finding reported by Nakashima et al. (15). The cytokine-inducing activity of LPS also seemed to depend on the lipid A moiety. In addition, rough-type LPS from *E. coli* K-12 produced smaller amounts of TNF-α and IFN-γ in vivo than did smooth-type LPS.

Immunological activities of LPS could be augmented by introducing *rfb* genes capable of synthesizing the mannose homopolymer into *E. coli* K-12. This indicated that the lipid A activities could be modulated through the alteration of the O-specific polysaccharide moiety in LPS molecules. Some bacterial species, such as those of the family *Rhodospirillaceae*, have an unusual lipid A structure which expresses few endotoxic activities, such as lethality and pyrogenicity (11). Therefore, it is not unlikely that recombinant LPS with strong adjuvant action but less endotoxic activity can be manufactured by genetic manipulation. Thus, the results of the present study suggest that genetically manufactured LPS may be useful as an artificial immunomodulator.

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