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Molecular Structures That Influence the Immunomodulatory Properties of the Lipid A and Inner Core Region Oligosaccharides of Bacterial Lipopolysaccharides

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The relationship between chain length as well as the position of fatty acyl groups to the ability of lipid A to abolish the expression of suppressor T-cell (Ts) activity was examined. Fatty acyl chain lengths of C_{12} to C_{14}, as in the lipid A of *Escherichia coli* and *Salmonella minnesota*, appear to be optimal for this bioactivity, since lipid A preparations with fatty acyl groups of relatively short chain length (C_{10} to C_{12} for *Pseudomonas aeruginosa* and *Chromobacterium violaceum*) or predominantly long chain length (C_{18} for *Helicobacter pylori*) are without effect. The presence of an acyloxyacyl group of appropriate chain length at the 3′ position of the glucosamine disaccharide backbone of lipid A also plays a decisive role. By contrast, the lipid A proximal inner core region oligosaccharides of some bacterial lipopolysaccharides increase the expression of Ts activity; this is due mainly to the capacity of such oligosaccharides, which are relatively conserved in structure among gram-negative bacteria, to enlarge or expand upon the population of $CD8^+$ Ts generated during the course of a normal antibody response to unrelated microbial antigens. The minimal structure required for the expression of the added immunosuppression observed appears to be a hexasaccharide containing one 2-keto-3-deoxyoctonate residue, two glucose residues, and three heptose residues to which are attached two pyrophosphorylethanolamine groups. The relevance of these findings to virulence and to the pathogenesis of gram-negative infections is discussed.

It has been established that the adjuvant effects of bacterial lipopolysaccharides (LPSs) are mediated by the lipid A portion of the LPS macromolecule (reviewed in reference 59) and can be attributed, at least in part, to its ability to abolish the expression of suppressor T-cell (Ts) activity, without adversely influencing cytotoxic T-cell, amplifier T-cell (Ta), and helper T-cell functions (3, 4, 16). Since the magnitude of the antibody response to the capsular polysaccharide (PS) antigen of type III *Streptococcus pneumoniae* (SSS-III) is controlled in positive and negative manners by the competitive interaction of $CD4^+$ $CD8^-$ Ta and $CD4^-$ $CD8^+$ Ts, respectively, treatment with LPS, or lipid A and its analogs, eliminates the negative effects of Ts and permits the positive effects of Ta to be more fully expressed, thereby resulting in an increased antibody response (reviewed in reference 3). Other studies showed that, to be able to abolish the expression of Ts function, lipid A (i) must be a glucosamine disaccharide, (ii) may have either one or two phosphate groups, and (iii) must have at least five fatty acyl groups (6). The chemical structures responsible for the toxicity of lipid A differ from those required for its capacity to abolish Ts function and to induce the polyclonal activation of B cells (reviewed in reference 6).

Although much attention has been focused on the well-known immunomodulatory effects elicited by the lipid A fraction of LPS, the PS portion of some, but not all, preparations of LPS also has been reported to have immunostimulatory properties (18). This has been attributed to the capacity of such PS fractions to induce the synthesis of various cytokines, to stimulate B-cell mitogenesis, to facilitate the binding of LPS to macrophages, and to activate the alternative complement pathway (reviewed in reference 22). By contrast, the administration of other preparations of LPS, or their PS fractions, induces significant immunosuppression which is (i) T cell dependent, (ii) mediated by the PS, but not the lipid A, portion of the LPS molecule, and (iii) independent of the capacity to activate B cells polyclonally (22). Such immunosuppression appears to be due to the capacity of PS to expand or enlarge the size of the population or pool of Ts, normally generated after exposure to antigens such as SSS-III (22). Since neither the immunostimulatory nor the immunosuppressive effects induced are general properties of PS fractions derived from all types of LPS, one may assume that they are dependent upon the chemical structure and composition of the PS molecule per se as well as the manner in which it was isolated.

In the present work, which is an extension of previous studies on molecular structures that influence the immunomodulatory properties of lipid A and the PS fraction of LPS (6, 22), we examine the role of fatty acid chain length and the lipid A proximal inner core region oligosaccharides (OS) in the expression of the aforementioned bioactivities of LPS. The

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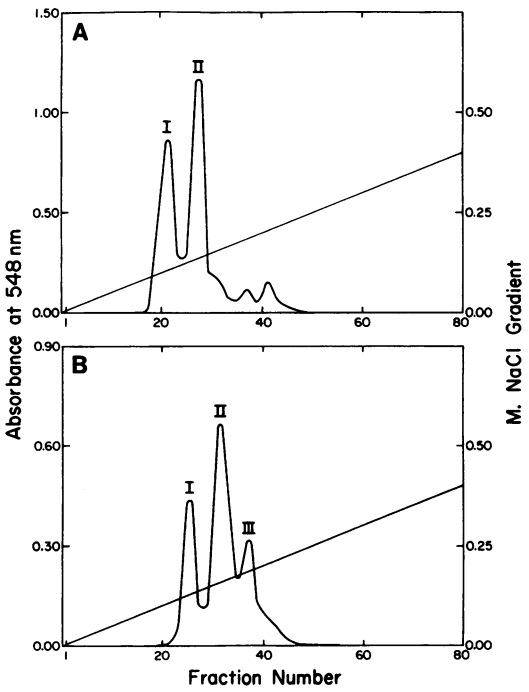


FIG. 1. DEAE-cellulose column fractionation of OS/Rd-LPS and OS/Rc-LPS. (A) OS/Rd-LPS (170 mg) on a column (2 by 18 cm) with a linear gradient of sodium chloride in a total volume of 400 ml. Four-milliliter fractions were collected and assayed for Kdo (A_{548}). Peaks I and II represent the free Kdo and OS/Rd-LPS(II), respectively. (B) OS/Rc-LPS (200 mg) treated as described for panel A. Peak I represents the free Kdo fraction. Peaks II and III represent OS fractions OS/Rc-LPS(II) and OS/Rc-LPS(III), respectively.

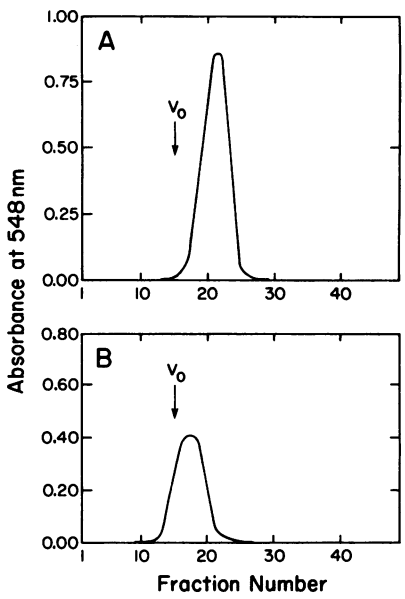


FIG. 2. Bio-Gel P-2 column fractionation of OS/Rc-LPS(II) (A) and OS/Rc-LPS(III) (B) on a column (2 by 62 cm) in 0.05 M sodium chloride. Samples were dissolved in 2 ml of 0.05 M sodium chloride and applied to the column. Two-milliliter fractions were collected and assayed for Kdo. V_0, void volume.

possible significance of the results obtained to the pathogenesis of infections caused by certain gram-negative bacteria is discussed.

MATERIALS AND METHODS

Mice. Female BALB/cByJ mice, 8 to 10 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, Maine. Female athymic nude (*nu/nu*) and thymus-bearing (*nu/+*) littermate control BALB/cAnNCr mice, 6 to 7 weeks of age, were obtained from the Frederick Cancer Research Center, Frederick, Md.

Preparation of LPS and inner core region OS of Rc and Rd mutant strains of *Escherichia coli*. Cells of *E. coli* D31m3 (an Rd mutant) and *E. coli* B (an Rc mutant) were grown in a 28-liter New Brunswick fermentor at 37°C in Luria-Bertani broth medium (53). LPS from these strains (Rd-LPS and Rc-LPS) was extracted from bacterial cells as described by Galanos et al. (19) with modifications (45). The properties and characteristics of the resulting Rc-LPS and Rd-LPS obtained have been documented (13, 28, 43).

For the preparation of OS, 1 g of Rc-LPS or Rd-LPS was suspended in 200 ml of 1% acetic acid and incubated at 100°C for 30 min; the preparation was cooled and then extracted with 500 ml of chloroform-methanol (2:1 [vol/vol]). The upper aqueous layer was recovered, and the organic solvents were removed, by means of a rotary evaporator at 45°C, until the volume was reduced to 100 ml. Nine hundred milliliters of ethanol was then added (with mixing), and the material was cooled to 4°C. The resulting precipitate was recovered by centrifugation (12,000 × *g* for 15 min), washed three times with 15 ml of cold 90% ethanol, and then dried under high vacuum. The yields of OS obtained from Rd-LPS and Rc-LPS were 170 and 200 mg, respectively. For convenience, these preparations are referred to as OS/Rd-LPS and OS/Rc-LPS.

Samples of OS/Rd-LPS and OS/Rc-LPS (170 to 200 mg) were applied to a DEAE-cellulose column (2 by 18 cm) in the chloride form; the column was washed with 100 ml of water, and a linear gradient of 0 to 0.5 M NaCl, in a total volume of 400 ml, was used to elute the sample. Four-milliliter fractions were collected, and each fraction was analyzed for 2-keto-3-deoxyoctonate (Kdo) content as described previously (30). The peak fractions were pooled and lyophilized. Figure 1 shows the fractionation results obtained for OS/Rd-LPS and OS/Rc-LPS. The preparations of interest in this work are fractions OS/Rd-LPS(II) and the more purified preparations of OS/Rc-LPS(II) and OS/Rc-LPS(III) which were isolated as described below.

Peak fractions isolated from the DEAE-cellulose column were fractionated further on a Bio-Gel P-2 column (2 by 62 cm) in 0.05 M NaCl. Samples were dissolved in 2 ml of 0.05 M NaCl, applied to the column, and then eluted from the column with the same solvent. Two-milliliter fractions were collected and analyzed for Kdo content. Figure 2 shows the fractionation results obtained for OS/Rc-LPS(II) and OS/Rc-LPS(III). Peak fractions were pooled, lyophilized, desalted on a small Bio-Gel P-2 column in water, and then dried. The yields were 46.8, 72.0, and 26.5 mg for OS/Rd-LPS(II), OS/Rc-LPS(II), and OS/Rc-LPS(III), respectively. Total phosphorus was determined by the method of Bartlett (12), whereas the sugar content of each preparation was determined by gas-liquid chromatography of the alditol acetate derivative by the method of Albersheim et al. (1). Heptose content was determined by the cysteine-sulfuric acid method (40). Analytical high-performance liquid chromatography (HPLC) of the OS was performed on an HPLC instrument (Dionex Corp., Sunnyvale, Calif.) with a Dionex CarboPac PA-1 anion-exchange column and a Dionex

pulsed amperometric detector. A linear gradient of 0.1 to 1.0 M sodium acetate (pH 8.0) at a flow rate of 1.0 ml/min was used. The presence of pyrophosphorylethanolamine ($PPEtNH_2$) in the OS preparations examined was affirmed by means of an amino acid analyzer (automated ninhydrin detection system; Becton Dickinson, Inc., Mountain View, Calif.) by using analytical-grade phosphorylethanolamine ($PEtNH_2$) as a standard.

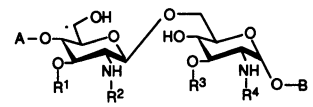
Liquid secondary-ion mass spectra (LSIMS) data for purified preparations of OS were acquired by using the Concept 1H double-focusing electromagnetic deflection mass spectrometer (Kratos Analytical, Manchester, United Kingdom). The DS-90 data system was used to acquire the data, whereas the MACH 3 data system was used to process the data obtained. A beam of 12-keV cesium ions was used for desorption of the samples from the liquid matrix. Positive-ion spectra were recorded at an accelerating potential of 8 kV, a scan rate of 10 s/decade, and a resolution of 1,700. Negative-ion spectra were executed at an accelerating potential of 4 kV. Mass calibration was achieved with cesium iodide. The samples were dissolved in water and then mixed with a matrix of monothioglycerol at a ratio of 1:1.

Preparation of LPS and lipid A from other bacteria. LPS preparations derived from *Pseudomonas aeruginosa* 07 (Fisher type 2) and *P. aeruginosa* PAC 605 were prepared as described previously (32); they were dissolved in saline to a final concentration of 1 mg/ml and stored at −20°C until used. Three different preparations of lipid A were extracted from *P. aeruginosa* PAC 605 and used in this work. They differ mainly in the number and the position of phosphate groups present and are referred to as (i) lipid A-HCl, which was obtained after hydrolysis with HCl and is a 4′-monophosphate, (ii) lipid A-HOAc, which was obtained after hydrolysis with acetic acid and contains both 4′-phosphate (10 to 25%) and 1-phosphate (75 to 90%), and (iii) lipid A-AcP, which was obtained after hydrolysis with acetate buffer and contains both 1- and 4′-phosphate in stoichiometric amounts (22). These preparations of lipid A were dissolved in distilled water to a final concentration of 500 μg/ml; in some cases, 0.2% triethylamine was added (dropwise with mixing) as needed to eliminate opalescence and facilitate solubility. The resulting stock solutions were stored at −20°C until used.

Salmonella minnesota R595 LPS and its corresponding 1,4′-diphosphoryl and 4′-monophosphoryl lipid A were purchased from Ribi ImmunoChem Research, Inc., Hamilton, Mont.; their method of preparation, immunomodulatory properties, and structure have been documented (3, 4, 16, 17, 44, 46–48). All were dissolved to a final concentration of 1 mg/ml in 0.2% triethylamine as described previously (6).

The lipid A of *Chromobacterium violaceum* was prepared as described previously (60), whereas LPS preparations from *Helicobacter pylori* 84-180 and 84-183 were prepared by a phenol-water extraction procedure (34). The chemical structure of the lipid A of *H. pylori* LPS has not yet been established; however, in contrast to the remaining preparations of lipid A used in this work, it contains a high percentage of long-chain fatty acyl groups. About 66 to 67% of all fatty acids and 3-OH fatty acids are octadecanoic (18:O) and 3-hydroxy octadecanoic (3-OH-18:O) acids, respectively (34). The predominant structures of the remaining preparations of lipid A used in this work are given in Fig. 3.

Antigen and immunization procedure. The immunological properties of the SSS-III used as well as the method by which it was prepared have been described (3, 7–10). For immunization, mice were given a single intraperitoneal (i.p.) injection of an optimally immunogenic dose (0.5 μg) of SSS-III in 0.5 ml of saline. The magnitude of the resulting antibody response elicited was determined at the peak, 5 days after immunization.



Preparation	R^1	R^2	R^3	R^4	A	B
S. minnesota R595 1, 4′- diphosphate	$C_{14}OC_{14}$	$C_{12}OC_{14}$	HOC_{14}	$C_{16}OC_{14}$	P	P
S. minnesota R595 4′ - monophosphate	$C_{14}OC_{14}$	$C_{12}OC_{14}$	HOC_{14}	$C_{16}OC_{14}$	P	H
P. aeruginosa 1, 4′ - diphosphate	HOC_{10}	$C_{12}OC_{12}$	H	$C_{12}OC_{12}$	P	P
C. violaceum 1, 4′ - diphosphate	HOC_{10}	$C_{12}OC_{12}$	HOC_{10}	$C_{12}OC_{12}$	P	P

FIG. 3. Chemical structure of naturally derived glucosamine disaccharide analogs of lipid A used in this study. All structures are depicted in their most acylated form. Abbreviations: P, phosphate; HOC_{10}, 3-hydroxydecanoyl; HOC_{14}, 3-hydroxytetradecanoyl; $C_{12}OC_{12}$, 3-dodecanoyloxydodecanoyl; $C_{12}OC_{14}$, 3-dodecanoyloxytetradecanoyl; $C_{14}OC_{14}$, 3-tetradecanoyloxytetradecanoyl; $C_{16}OC_{14}$, 3-hexadecanoyloxytetradecanoyl. R^1, R^2, R^3, and R^4 groups are attached at the 3′, 2′, 3, and 2 positions, respectively.

Immunological methods. The numbers of splenic plaque-forming cells (PFC) making antibody specific for SSS-III (SSS-III-specific PFC) provided a measure of the maximal antibody response made by individual mice 5 days after immunization. SSS-III-specific PFC making antibody of the immunoglobulin M (IgM) class (>90% of all PFC found [8]) were detected by a slide version of the technique of localized hemolysis-in-gel, using indicator sheep erythrocytes coated with SSS-III by the $CrCl_3$ method (11). Corrections were made (by subtraction) for the small numbers of background sheep erythrocyte-specific PFC found so that only values for SSS-III-specific PFC were considered for immunized mice. The values obtained (SSS-III-specific PFC per spleen), which are log normally distributed (20), are expressed as the geometric mean (antilog) of the mean $\log_{10}$ number of SSS-III-specific PFC per spleen ± the standard error of the mean (SEM) for groups of similarly treated mice. This provides a valid measure of the total antibody response produced since (i) SSS-III-specific PFC are detected only in the spleens of immunized mice, (ii) numbers of SSS-III-specific PFC per spleen are directly related to the magnitude of the serum antibody response generated, and (iii) asplenic mice are severely deficient in their ability to mount a serum antibody response to SSS-III (2, 9, 10, 29).

Transfer of immunosuppression with spleen cells from mice previously exposed to SSS-III. Mice were pretreated (primed) with a single i.p. injection of 50 ng of SSS-III. A pooled suspension of washed spleen cells, containing 100×10^6 nucleated cells per ml of medium 199, was then prepared 18 to 24 h after priming, and the capacity of such cells to transfer antigen-specific suppression to recipient mice immunized with 0.5 μg of SSS-III was evaluated as described previously (5, 22, 54, 56).

To determine the type of cell responsible for inducing suppression, 50 μl of dilute (1:10) monoclonal anti-mouse CD8 antibody (NEI-006; New England Nuclear, Boston, Mass.) was added to 2.5 ml of the suspension of primed spleen cells described above. The mixture was held at 4°C for 1 h, after which the cells were washed by centrifugation ($200 \times g$ for 10

min at 4°C) and resuspended to the original volume. A 1.5-ml volume of dilute (1:40) guinea pig serum was then added as a source of complement. The mixture was placed in a 37°C water bath for 30 min, after which the cells were washed once by centrifugation and resuspended to the original volume. Known numbers of cells were injected intravenously (i.v.) in a volume of 0.2 ml to recipient mice immunized i.p. with 0.5 μg of SSS-III to assess the capacity of such cells to transfer immunosuppression (5, 22, 54, 56). Control mice were given cell suspensions to which only complement was added. Since the results of numerous experiments using larger numbers of normal (unprimed) spleen cells failed to demonstrate the transfer of immunosuppression (5, 54, 56), such controls were not deemed to be essential.

The monoclonal anti-mouse CD8 antibody used is of the IgM class. It is supplied as a 100-μl reagent in ascites fluid at a high cytotoxic titer (for BALB/c mouse thymocytes) of 4.3×10^{-4} by a ^{51}Cr release assay. In other studies, it had no reactivity against $CD4^+$ T cells as evidenced by its failure to influence the expression of $CD4^+$ $CD8^-$ Ta activity (54).

Assessment of polyclonal activation of B cells. The appearance of increased numbers of spleen cells secreting non-antigen-specific IgM, which is characteristic of the polyclonal B-cell activation induced by biologically active preparations of LPS and lipid A (21), was detected by a modification of the protein A plaque assay (23) in which indicator sheep erythrocytes were coated with protein A in the presence of $CrCl_2$ as the coupling agent. The affinity-purified anti-mouse IgM antibody used for the detection of non-antigen-specific IgM-secreting PFC was the same as that used in other published studies (23). A dilution (1:200 in saline) known to reveal maximal numbers of IgM-secreting PFC (23) was added (50 μl) to the soft-agarose reaction mixture before the addition of spleen cells. Results are expressed as the mean relative increase in numbers of IgM-secreting PFC per spleen ± SEM for groups of similarly treated mice, with reference to the mean value for age-matched untreated control mice (background controls). The latter, which served as internal controls for each experiment conducted, ranged from 143,000 to 220,000 IgM-secreting PFC during the course of these studies.

Statistics. Student's *t* test was used to assess the significance of the differences observed. Differences were considered to be significant when probability (*P*) values of <0.05 were obtained.

RESULTS

Effect of treatment with LPS or lipid A on the expression of Ts activity. Mice were pretreated or primed with a single i.p. injection of a subimmunogenic dose (5 ng) of SSS-III. Three days later (day 0), they were given i.p. an optimally immunogenic dose (0.5 μg) of SSS-III, with or without an i.p. injection of a given amount of lipid A or LPS; a second i.p. injection of the same amount of lipid A or LPS was given to primed mice 1 day after immunization with SSS-III (day +1). The magnitude of the SSS-III-specific antibody (PFC) response produced was determined 5 days after immunization. The results obtained were compared with those for unprimed mice and primed mice immunized with 0.5 μg of SSS-III to evaluate (i) the degree of unresponsiveness induced as a result of priming with 5 ng of SSS-III and (ii) the effect of treatment with lipid A or LPS on the unresponsiveness expressed.

The data of Fig. 4 show that priming with a single injection of 5 ng of SSS-III induced, as expected, significant unresponsiveness in all experimental groups considered (solid versus open bars; $P < 0.005$ in all cases). It has been established that such unresponsiveness, which has been called low-dose immunological paralysis, is antigen specific, persists for several months after priming with just one injection of 5 ng or more of SSS-III, and is mediated by $CD8^+$ Ts (3, 5, 7, 9, 56, 57); therefore, it provides a valid measure of the degree of Ts activity generated under the experimental conditions used. Treatment with either the 1,4′-diphosphoryl or the 4′-monophosphoryl lipid A of *S. minnesota* R595 completely abolishes such unresponsiveness (solid versus hatched bars; $P > 0.05$ for both types of lipid A preparations); this is in agreement with the results of previously published studies (6). By contrast, treatment with three different preparations of lipid A derived from *P. aeruginosa* PAC 605 or treatment with a structurally similar lipid A derived from *C. violaceum* (Fig. 3) had little or no significant effect ($P > 0.05$) on the expression of such unresponsiveness.

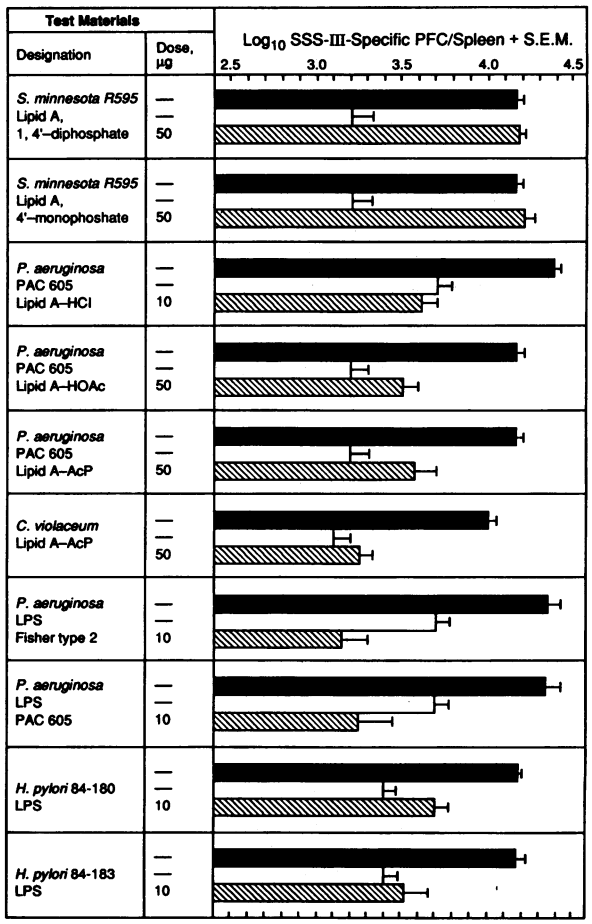


FIG. 4. Effect of treatment with two injections of stated amounts of LPS or lipid A on the expression of low-dose immunological paralysis. Symbols: ■, immunized (0.5 μg of SSS-III); □, primed (5 ng of SSS-III) and immunized (0.5 μg of SSS-III); ▨, primed (5 ng of SSS-III), immunized (0.5 μg of SSS-III), and treated with two injections of LPS or lipid A (day 0 and day +1 relative to immunization).

In previous studies, treatment with two injections of 10 μg of *S. minnesota* R595 LPS abolished the expression of low-dose paralysis under the same experimental conditions (6); however, this was not the case for primed mice given (i) LPS derived from two different strains of *P. aeruginosa* or (ii) LPS derived from two different strains of *H. pylori* (Fig. 4). Although treatment with *H. pylori* LPS had little or no effect on the degree of unresponsiveness expressed, both of the prepa-

TABLE 1. Relative increase in non-antigen-specific IgM-secreting PFC per spleen in mice given different preparations of lipid A or LPS

Type	Test material		Relative increase[a]	P[b]
	Prepn	Amt (μg)		
Lipid A	*S. minnesota* R595 1,4′-diphosphate	50	8.2 ± 1.6	<0.001
	S. minnesota R595 4′-monophosphate	50	3.7 ± 0.7	<0.001
	P. aeruginosa PAC 605 lipid A-HCl	50	2.4 ± 0.4	<0.001
	P. aeruginosa PAC 605 lipid A-HOAc	50	1.9 ± 0.3	<0.01
	P. aeruginosa PAC 605 lipid A-AcP	50	2.7 ± 0.3	<0.001
LPS	*S. minnesota* R595	10	26.2 ± 3.4	<0.001
	P. aeruginosa Fisher type 2	10	3.1 ± 0.2	<0.001
	P. aeruginosa PAC 605	10	2.4 ± 0.4	<0.01
	H. pylori 84-180	10	0.9 ± 0.1	>0.05
	H. pylori 84-183	10	1.1 ± 0.1	>0.05

[a] Mean relative increase in non-antigen-specific IgM-secreting PFC per spleen ± SEM for groups of five BALB/cByJ mice 3 days after the i.p. administration of lipid A or LPS. All comparisons were made with reference to the baseline values for such PFC in age-matched untreated control mice for each experiment conducted. The mean value for baseline controls ranged from 143,000 to 220,000 IgM-secreting PFC per spleen during the course of this study.
[b] *P* values for comparisons with respective baseline controls for a given experiment.

rations of *P. aeruginosa* LPS considered further increased ($P < 0.05$) the degree of unresponsiveness induced.

Polyclonal activation of B cells by lipid A and LPS. Mice were given a single i.p. injection of 10 to 50 μg of different preparations of lipid A or LPS. Three days later, spleen cell suspensions from individual mice were assayed for numbers of non-antigen-specific IgM-secreting PFC to evaluate the capacity of such preparations to activate B cells polyclonally. The results obtained for each experiment conducted were compared with the baseline values for such PFC in untreated age-matched control mice to calculate the mean relative increase in IgM-secreting PFC per spleen for groups of mice treated with each preparation of lipid A or LPS considered.

The data of Table 1 show that all of the preparations of lipid A examined induced significant ($P < 0.01$) polyclonal activation, even though some (e.g., all three preparations of *P. aeruginosa* lipid A) were unable to abolish the expression of low-dose paralysis mediated by Ts (Fig. 4). Although the 1,4′-diphosphoryl lipid A and 4′-monophosphoryl lipid A of *S. minnesota* R595 differ significantly ($P < 0.05$) in their capacity to induce polyclonal activation, they do not differ in their ability to abolish the expression of Ts function (Fig. 4); this is consistent with the results of previous studies (6, 23).

The LPS of *S. minnesota* R595 is an extremely potent polyclonal B-cell activator (PBA) (Table 1). Although the LPSs of *P. aeruginosa* Fisher type 2 and PAC 605 were moderately, but significantly, active in that regard ($P < 0.01$), the LPSs of *H. pylori* 84-180 and 84-183 were without effect ($P > 0.05$). This is in agreement with published data on the reduced bioactivities of *P. aeruginosa* and *H. pylori* LPS (31, 32, 37).

Immunosuppression induced by chemically defined inner core region OS. The inability of *P. aeruginosa* lipid A to abolish the expression of Ts activity as well as the much lower antibody response elicited by primed mice treated with *P. aeruginosa* LPS (Fig. 4) suggests that the PS moiety of that LPS might be stimulating, rather than reducing, the Ts activity generated after exposure to SSS-III. Since significant T-cell-mediated suppression also was noted in other studies after treatment with LPS (or its PS fraction) derived from different gram-negative bacteria (22), one might assume that it is induced by the relatively conserved inner core region OS, rather than the O-antigen-specific region, of the LPS macromolecule. Therefore, because the chemical structures of the inner core region OS of Rc and Rd mutant strains of *E. coli* have been established (28), we elected to examine whether comparable T-cell-mediated immunosuppression also might be induced by purified preparations of these OS under the same experimental conditions. To this end, groups of mice were given i.p. different amounts (1 to 10 μg) of purified inner core region OS/Rc-LPS(II), OS/Rc-LPS(III), or OS/Rd-LPS(II) at the time of i.p. immunization with 0.5 μg of SSS-III; this experimental approach was found to induce significant T-cell-mediated immunosuppression with PS fractions of LPS in other studies (22). The magnitude of the SSS-III-specific antibody response was then determined 5 days after immunization and compared with that of immunized control mice not given OS.

Although treatment with 1 μg of OS/Rc-LPS(III) decreased the magnitude of the antibody (PFC) response to SSS-III by about 36% (Table 2) ($P > 0.05$), the administration of 5 μg consistently induced significant ($P < 0.01$) suppression (57%). A similar level of immunosuppression (52%) also was noted for mice treated with 5 μg of another lot of OS/Rc-LPS(III) in a second experiment (Table 2) ($P < 0.02$). It is typical of that obtained in five separate experiments in which mice were given

TABLE 2. Effect of treatment with different preparations of purified OS on the magnitude of the antibody (PFC) response to SSS-III

Expt no.	Treatment		SSS-III-specific PFC/spleen[b]	P[c]
	Prepn[a]	Dose (μg)		
			4.378 ± 0.074 (23,864)	
1	OS/Rc-LPS(III)	1	4.183 ± 0.099 (15,255)	>0.05
	OS/Rc-LPS(III)	5	4.016 ± 0.095 (10,382)	<0.01
	OS/Rc-LPS(II)	1	4.341 ± 0.115 (21,918)	>0.05
	OS/Rc-LPS(II)	5	4.310 ± 0.091 (20,431)	>0.05
2			4.258 ± 0.044 (18,102)	
	OS/Rc-LPS(III)	1	4.308 ± 0.067 (20,343)	>0.05
	OS/Rc-LPS(III)	5	3.973 ± 0.058 (9,403)	<0.02
3			4.081 ± 0.063 (12,062)	
	OS/Rd-LPS(II)	5	4.063 ± 0.070 (11,573)	>0.05
	OS/Rd-LPS(II)	10	4.211 ± 0.123 (16,269)	>0.05

[a] Each OS preparation was given i.p. at the time of immunization with 0.5 μg of SSS-III.
[b] Values are $\log_{10}$ mean numbers ± SEMs for groups of 8 to 10 BALB/cByJ mice 5 days after i.p. immunization with 0.5 μg of SSS-III; geometric means (antilogs) are shown in parentheses.
[c] *P* values for comparisons with immunized controls not treated with OS.

TABLE 3. Effect of treatment with OS/Rc-LPS(III) on the magnitude of the antibody (PFC) response to SSS-III in athymic (*nu/nu*) and thymus-bearing (*nu/+*) mice

Mice	OS/Rc-LPS(III) (μg)[a]	SSS-III-specific PFC/spleen[b]	P[c]
nu/+	0	4.209 ± 0.059 (16,164)	
	5	3.990 ± 0.067 (9,773)	<0.05
nu/nu	0	4.186 ± 0.086 (15,355)	
	5	4.126 ± 0.054 (13,353)	>0.05

[a] OS/Rc-LPS(III) was given i.p. at the time of i.p. immunization with 0.5 μg of SSS-III.

[b] Values are $\log_{10}$ mean numbers ± SEMs for groups of 10 BALB/cAnNCr *nu/+* or *nu/nu* mice 5 days after i.p. immunization with 0.5 μg of SSS-III; geometric means (antilogs) are shown in parentheses.

[c] *P* values for comparisons with immunized mice not given OS/Rc-LPS(III).

5 μg of the same or similar preparations of OS/Rc-LPS(III) at the time of immunization with 0.5 μg of SSS-III (mean percent suppression = 58.4 ± 4.1; $P < 0.01$); treatment with 10 μg of these preparations did not increase the degree of immunosuppression obtained (data not shown). By contrast, no suppression ($P > 0.05$) was induced in mice given 1 or 5 μg of OS/Rc-LPS(II), and in four separate experiments, no significant suppression ($P > 0.05$) was noted after treatment with 1, 5, 10, or 20 μg of OS/Rd-LPS(II) (Table 2; data not shown). Thus, of all the preparations of OS examined, only OS/Rc-LPS(III) is able to induce significant and reproducible immunosuppression of the same magnitude. OS/Rc-LPS(III) does not induce significant polyclonal activation of B cells when tested at doses of 50 μg ($P > 0.05$; data not shown).

In another experiment (Table 3), groups of athymic *nu/nu* and thymus-bearing *nu/+* mice were given i.p. 5 μg of OS/Rc-LPS(III) at the time of i.p. immunization with 0.5 μg of SSS-III; the magnitude of the SSS-III-specific antibody response was determined 5 days after immunization and compared with that of immunized mice not given OS/Rc-LPS(III). Treatment with OS/Rc-LPS(III) induced significant suppression ($P < 0.05$) but only in thymus-bearing *nu/+* mice; this means that the added immunosuppression induced by OS/Rc-LPS(III) is T cell dependent.

To obtain more information on the subset of T cells mediating the immunosuppression observed, pooled spleen cell suspensions were prepared from thymus-bearing BALB/cByJ mice 18 to 24 h after prior exposure (priming) with a single i.p. injection of a suboptimal dose (50 ng) of SSS-III, with or without the i.p. administration of 5 μg of OS/Rc-LPS(III) at the time of priming with SSS-III. The donor cell suspension was adjusted, with medium 199, to an appropriate concentration of nucleated cells which then were administered i.v. to recipient mice (in a volume of 0.2 ml) at the time of i.p. immunization with 0.5 μg of SSS-III. The magnitude of the SSS-III-specific antibody response was determined 5 days after immunization and compared with that of (i) immunized mice not given primed spleen cells and (ii) immunized mice given primed spleen cells from donor mice also given OS/Rc-LPS(III) at the time of priming.

The transfer of 2×10^7 primed spleen cells from donor mice not given OS/Rc-LPS(III) caused significant suppression as expected (Table 4) (group A versus group C, $P < 0.05$); it has been established that such suppression is antigen specific and mediated by $CD8^+$ Ts, activated following exposure to SSS-III (5, 54). The failure of smaller numbers (2×10^6) of primed spleen cells to transfer significant suppression (group A versus group B, $P > 0.05$) is typical of the results of previous studies using spleen cells from mice primed with 5 ng of SSS-III (22, 57). It indicates that, for donor mice primed with either 5 or 50 ng of SSS-III, at least 2×10^7 primed spleen cells are required to transfer significant suppression under the experimental conditions used. By contrast, significant suppression could be transferred with as few as 2×10^6 primed spleen cells from donor mice also given 5 μg of OS/Rc-LPS(III) at the time of priming (group A versus group E, $P < 0.05$), and some suppression was demonstrable even after the transfer of 2×10^5 primed spleen cells although it was not statistically significant (group A versus group D, $P > 0.05$). These findings indicate that treatment with OS/Rc-LPS(III) results in a significant increase in the number of Ts activated after exposure to SSS-III. The results of another similarly conducted experiment (Table 5) establish that the increased suppression generated after priming with SSS-III and treatment with OS/Rc-LPS(III) is in fact mediated by $CD8^+$ Ts since treatment with monoclonal anti-mouse CD8 antibody plus complement completely abolishes the capacity of 2×10^6 primed spleen cells to transfer suppression to recipient mice immunized with 0.5 μg of SSS-III.

Chemical analysis of core region OS preparations. Total phosphorus and glucose analyses showed that OS/Rd-LPS(II) was devoid of both components, whereas both OS/Rc-LPS(II) and OS/Rc-LPS(III) were phosphorus and glucose positive. All three OS preparations contained Kdo and heptose. Amino acid analysis of OS/Rc-LPS(II) and OS/Rc-LPS(III) after mild acid hydrolysis (1.0 M HCl, 100°C, 5 h) confirmed the presence of $PEtNH_2$. We were unable to quantitate the amounts of $PEtNH_2$ present because of variable amounts of salts in the samples. Nevertheless, these findings are consistent with published data on the chemical composition of such OS preparations (28).

Mass spectral analysis of core region OS preparations. OS/Rd-LPS(II) was analyzed by both positive- and negative-ion LSIMS. Figure 5 shows the positive-ion LSIMS spectrum. Molecular ions $(M+Na)^+$ and $(M-H+2Na)^+$ were observed at *m/z* 645.1 and 667.1, respectively. The dimers of molecular ions $(2M+Na)^+$, $(2M-H+2Na)^+$, and $(2M-2H+3Na)^+$ were observed at *m/z* 1,267.3, 1,289.2, and 1,311.2, respectively. Negative-ion LSIMS spectrum showed molecular ions $(M-H)^-$, $(M-2H+Na)^-$, and $(M-H+Na+Cl)^-$ at *m/z* 621.2, 643.2, and 679.1, respectively (data not shown). The molecular ions $(M+Na)^+$ at *m/z* 645 and $(M-H)^-$ at *m/z* 621 were further investigated by the collision-induced dissociation method (data not shown). The positive-ion collision-induced dissociation spectrum of $(M+Na)^+$ showed the cleavage of the glycoside bond between Kdo and heptose, giving a fragment ion at *m/z*

TABLE 4. Effect of prior treatment with OS/Rc-LPS(III) on the ability of primed spleen cells to transfer immunosuppression

Expt group	Treatment of donor mice[a]		No. of cells transferred	SSS-III-specific PFC/spleen[b]
	SSS-III	OS/Rc-LPS(III)		
A	−	−		4.255 ± 0.079 (18,002)
B	+	−	2×10^6	4.191 ± 0.053 (15,510)
C	+	−	2×10^7	3.926 ± 0.064 (8,438)
D	+	+	2×10^5	4.156 ± 0.077 (14,320)
E	+	+	2×10^6	3.947 ± 0.126 (8,846)

[a] Donor mice were given 5 μg of OS/Rc-LPS(III) i.p. at the time of i.p. priming with 50 ng of SSS-III. Suspensions of donor spleen cells were prepared 18 to 24 h after priming with SSS-III; they were given i.v. to recipient mice at the time of i.p. immunization with 0.5 μg of SSS-III.

[b] Values are $\log_{10}$ mean numbers ± SEM for groups of 8 to 10 BALB/cByJ mice 5 days after i.p. immunization with 0.5 μg of SSS-III; geometric means (antilogs) are shown in parentheses.

TABLE 5. Effect of treatment with monoclonal anti-mouse CD8 antibody on the capacity of spleen cells from primed mice also given OS/Rc-LPS(III) to transfer suppression

Expt group	No. of cells transferred[a]	Treatment of transferred cells	SSS-III-specific PFC/spleen[b]	% Suppression[c]
A	None		4.250 ± 0.053 (17,762)	
B	2×10^6	Complement	3.888 ± 0.082 (7,726)	57
C	2×10^6	Anti-CD8 antibody + complement	4.255 ± 0.043 (17,978)	0

[a] A pooled suspension of spleen cells was prepared from donor BALB/cByJ mice 18 to 24 h after i.p. priming with 50 ng of SSS-III and i.p. treatment with 5 μg of OS/Rc-LPS(III). Spleen cells were given i.v. at the time recipient mice were immunized with 0.5 μg of SSS-III.

[b] Values are $\log_{10}$ mean numbers ± SEM for groups of 9 to 10 BALB/cByJ mice 5 days after i.p. immunization with 0.5 μg of SSS-III; geometric means (antilogs) are in parentheses.

[c] Calculated with reference to immunized controls (group A) not given primed spleen cells.

407 by the loss of Kdo. The negative-ion collision-induced dissociation spectrum of $(M\text{-}H)^-$ also showed the cleavage of the glycoside bond between Kdo and heptose, giving a fragment ion at *m/z* 383. These results suggest that OS/Rd-LPS(II) is a trisaccharide with an M_r of 622 as shown in Fig. 6. Holst and Brade (24) recently obtained the same product upon mild acid hydrolysis of the LPS of *S. minnesota* R7 (an Rd chemotype).

OS/Rc-LPS(II) was analyzed by both negative-ion LSIMS and by positive-ion electrospray methods. In the LSIMS analysis, molecular ions $(M\text{-}H)^-$ and $(M+Na\text{-}2H)^-$ were observed at *m/z* 1,516.2 and 1,538.2, respectively (Fig. 7). A loss of water and a carboxyl group gave $(M\text{-}H\text{-}18)^-$ and $(M\text{-}H\text{-}46)^-$ at *m/z* 1,498.2 and 1,470.2, respectively. The loss of a $PEtNH_2$ group gave $(M\text{-}H\text{-}123)^-$ at *m/z* 1,393.2 and $(M\text{-}H\text{-}123\text{-}18)^-$ at *m/z* 1,375.2. The loss of one hexose by cleavage at both sides of the glycoside bond gave fragments $(M\text{-}H\text{-}162)^-$ and $(M\text{-}H\text{-}180)^-$ at *m/z* 1,354.2 and 1,336.2, respectively. The loss of Kdo by the same kind of cleavages gave fragments $(M\text{-}3H\text{-}220)^-$ and $(M\text{-}H\text{-}238)^-$ at *m/z* 1,294.2 and 1,278.2, respectively. The peak at *m/z* 1,220.2 was not identified. Losses of two hexoses, $(M\text{-}H\text{-}324)^-$ and $(M\text{-}H\text{-}342)^-$, were also observed at *m/z* 1,192.2 and 1,174.2 (data not shown).

In the positive-ion electrospray method, the molecular ions $(M+H)^+$ at *m/z* 1,518.4 and $(M+Na)^+$ at *m/z* 1,540.6 were observed (data not shown). Double-charged molecular ions $(M+H+Na)^{2+}$ at *m/z* 770.7 and $(M+2Na)^{2+}$ at *m/z* 781.6 were also observed (data not shown). These results suggest that the major component of the isolated core OS fraction OS/Rc-LPS(II) is a heptasaccharide with an M_r of 1,517 and is consistent with the presence of a single $PPEtNH_2$ group as shown in Fig. 6. There appears to be a methyl group at an undetermined position.

OS/Rc-LPS(III) was analyzed by both negative-ion LSIMS and by positive-ion electrospray methods. In the LSIMS analysis, molecular ions $(M\text{-}2H+Na)^-$ and $(M\text{-}H)^-$ at *m/z* 1,565.2 and 1,543.2, respectively, were observed (Fig. 8). The loss of $PEtNH_2$ $(M\text{-}2H\text{-}123+2Na)^-$, $(M\text{-}2H\text{-}123+Na)^-$, $(M\text{-}H\text{-}123)^-$, and $(M\text{-}H\text{-}123\text{-}18)^-$ at *m/z* 1,464.1, 1,442.2, 1,420.2, and 1,402.2, respectively, was observed. The loss of both $PEtNH_2$ and carboxyl group $(M\text{-}H\text{-}123+46)^-$ was observed at *m/z* 1,374.2. The loss of the $PEtNH_2$ groups $(M\text{-}2H\text{-}123\text{-}123+Na)^-$, $(M\text{-}H\text{-}123\text{-}123)^-$, and $(M\text{-}H\text{-}123\text{-}18)^-$ at *m/z* 1,319.2, 1,297.2, and 1,279.2, respectively, was observed. The loss of both $PEtNH_2$ and hexose groups $(M\text{-}H\text{-}123\text{-}162)^-$ and $(M\text{-}H\text{-}123\text{-}162\text{-}18)^-$ at *m/z* 1,258.2 and 1,240.2, respectively, was observed. The loss of both $PEtNH_2$ and Kdo groups $(M\text{-}H\text{-}123\text{-}220)^-$, $(M\text{-}3H\text{-}123\text{-}220)^-$, and $(M\text{-}H\text{-}123\text{-}220\text{-}18)^-$ at *m/z* 1,200.2, 1,198.2, and 1,182.1, respectively, was also observed. A fragment containing two hexoses $(M\text{-}H\text{-}162\text{-}162)^-$ at *m/z* 1,096.1 was observed.

The presence of the major component of Os/Rc-LPS(II) in this preparation was indicated by peaks representing molecular ion $(M'\text{-}H)^-$ at *m/z* 1,516.2 and several fragmentation ions. These included $(M'\text{-}H\text{-}18)^-$ and $(M'\text{-}H\text{-}46)^-$ at *m/z* 1,498.2 and *m/z* 1,470.2, respectively. The loss of $PEtNH_2$ groups $(M'\text{-}H\text{-}109)^-$, $(M'\text{-}H\text{-}123)^-$, and $(M'\text{-}H\text{-}123\text{-}18)^-$ at *m/z* 1,407.2, 1,393.2, and 1,375.2, respectively, was observed. The

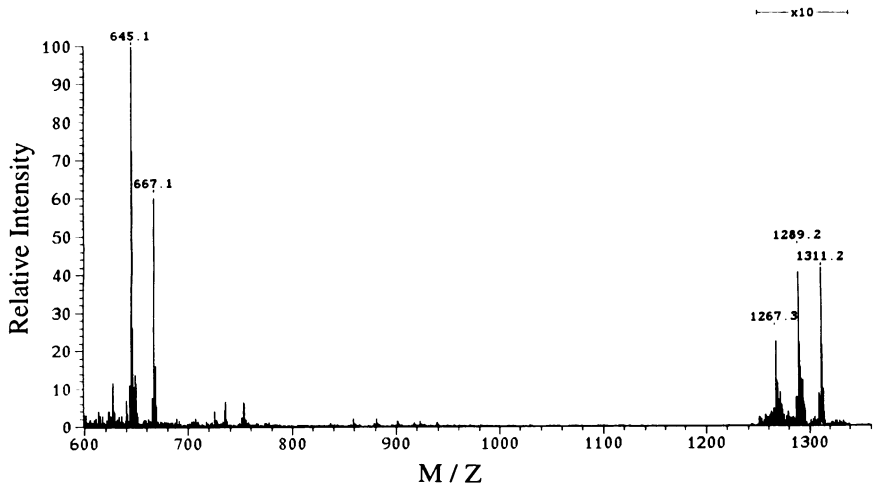


FIG. 5. Partial positive-ion LSIMS spectrum of OS/Rd-LPS(II).

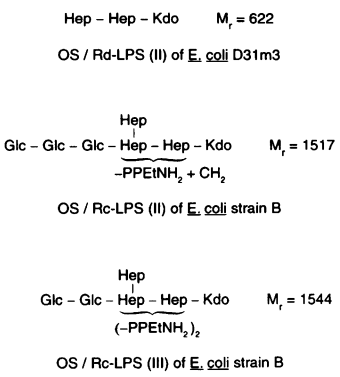


FIG. 6. Proposed general structure of the OS preparations used in this study. They were derived from the rough chemotypes of LPS from *E. coli*. The structural determinations were based on mass spectral analysis, chemical compositional analysis, and what is presently known concerning the structure of the core region of *E. coli* LPS (28). Abbreviations: Hep, heptose; Glc, glucose; CH_2, methyl group. The location of $PPEtNH_2$ has not been established.

loss of $PEtNH_2$ and carboxyl groups $(M'\text{-}123\text{-}46)^-$ gave an ion at *m/z* 1,347.2. Finally, the loss of both $PEtNH_2$ and Kdo group $(M'\text{-}2H\text{-}123\text{-}220)^-$ gave an ion at *m/z* 1,171.2. This is consistent with the expected overlapping of the small OS/Rc-LPS(III) peak with the large OS/Rc-LPS(II) peak in the DEAE-cellulose column fractionation and the HPLC analysis (Fig. 1 and 9).

In the positive-ion electrospray method, double-charged molecular ions were revealed by $(M+Na+H)^{2+}$ and $(M+2H)^{2+}$ at *m/z* 784.2 and 773.1, respectively (data not shown). The loss of $PEtNH_2$ showed fragments $(M+2Na\text{-}123)^{2+}$ at *m/z* 733.4, $(M+Na+H\text{-}123)^{2+}$ at *m/z* 722.7, and $(M+2H\text{-}123)^{2+}$ at 711.7. A single, charged ion, $(M\text{-}123+Na)^+$ at *m/z* 1,444.4, was also observed. These results suggest that the major component of the isolated core OS fraction designated OS/Rc-LPS(III) is a hexasaccharide with an M_r of 1,544 and is consistent with the presence of two $PPEtNH_2$ groups as shown in Fig. 6.

Compositional analysis of OS/Rc-LPS(II) and OS/Rc-LPS (III). Analytical HPLC with an anion-exchange column was used to assess the composition of purified OS/Rc-LPS(II) and OS/Rc-LPS(III) preparations. OS/Rc-LPS(II) showed a major component (peak 1), a minor component (peak 2), and a trace component (peak 3) as shown in Fig. 9A. We have designated peak 1 to be the heptasaccharide whose structure is shown in Fig. 6. Since peak 2 has a greater charge based on elution time than peak 1, it might represent a heptasaccharide containing only one phosphate group instead of one $PPEtNH_2$ group. This is consistent with the presence of a peak at *m/z* 1,393.2, which may represent the molecular ion $(M_2\text{-}H)^-$ of Fig. 7. Peak 3 appears to represent the hexasaccharide, a major component of OS/Rc-LPS(III), whose structure is shown in Fig. 6 (see below).

HPLC of OS/Rc-LPS(III) showed one major component (peak 3) and four trace to minor components (Fig. 9B). We have designated peak 3 to be the hexasaccharide whose structure is shown in Fig. 6. Peaks 1, 2, and 3 were found in the OS/Rc-LPS(II) preparation (Fig. 9A). We were unable to relate the component representing the unlabeled peak between peaks 2 and 3 to the two OS structural series. The components representing peaks 4, 5, and 6 have progressively greater charges. This might indicate that the component in peak 4 represents a structural series of hexasaccharide containing one $PPEtNH_2$ and one phosphate group instead of two $PPEtNH_2$ groups. This is consistent with the presence of peaks at *m/z* 1,464.1, 1,442.2, and 1,420.2 which may represent the molecular ions $(M_4\text{-}2H+2Na)^-$, $(M_4\text{-}H+Na)^-$, and $(M_4\text{-}H)^-$, respectively (Fig. 8). Peak 5 might represent another structural series of hexasaccharide containing two phosphate groups instead of two $PPEtNH_2$ groups. This is consistent with the presence of peaks at *m/z* 1,319.2 and 1,297.2, which may represent the molecular ions $(M_5\text{-}H+Na)^-$ and $(M_5\text{-}H)^-$, respectively (Fig. 8). The nature of the trace component in peak 6 cannot be evaluated. Since a pulsed amperometric detector was used in HPLC, it is not possible to quantitate the relative abundance of each of these components.

DISCUSSION

More precise information on the structure of lipid A as well as the complete synthesis of biologically active preparations of lipid A of known structure now permits one to examine relationships between its chemical structure and the expression

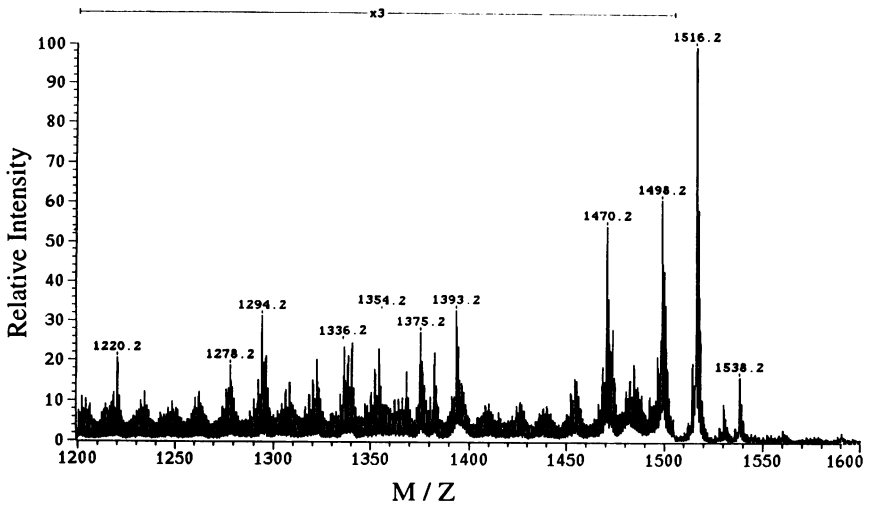


FIG. 7. Partial negative-ion LSIMS spectrum of OS/Rc-LPS(II).

of several bioactivities characteristic of lipid A and LPS. In studies on the lipid A of *Salmonella* spp., both the number of attached phosphate groups and the position of acyloxyacyl groups have been shown to greatly influence toxicity. For example, the 1,4′-diphosphoryl lipid A of *Salmonella* spp. is more than 1,000 times more toxic than its corresponding 4′-monophosphoryl lipid A (34, 46–48), and the deletion of a fatty acyl group from the 3 position of the glucosamine disaccharide backbone of the 4′-monophosphoryl lipid A results in an even further decrease in toxicity (36, 38). Since the naturally derived lipid A of *P. aeruginosa* lacks a fatty acyl group at the 3 position (Fig. 3) (32), this could contribute to its lower toxicity in comparison with that of lipid A and LPS derived from other gram-negative bacteria (31, 32). By contrast, neither the number of phosphate groups nor the presence of a fatty acyl group at the 3 position influences the capacity of lipid A to abolish the expression of Ts activity (6). Thus, different structural components of lipid A determine toxicity as well as its ability to abrogate Ts function.

Both the number and the position of phosphate groups also have been reported to influence the capacity of naturally derived and synthetic lipid A to activate B cells polyclonally (6); such variables have no effect on the ability of lipid A to abolish the expression of Ts function (6). The 1,4′-diphosphoryl lipid A of *S. minnesota* is a more potent PBA than its corresponding 4′-monophosphoryl lipid A; however, they do not differ in their effect on the expression of Ts function (6). Also, some synthetic monoglucosamine subunits of lipid A (e.g., GLA-59, GLA-60, GLA-113, and SDZ MRL 953) are good PBAs, yet none are able to influence Ts function in a significant manner (6). Some synthetic monophosphoryl lipid A preparations, which are phosphorylated at the 1 position, are more mitogenic and more active PBAs than those phosphorylated at the 4′ position (26); this does not influence their capacity to alter the expression of Ts function (6). In the present work, the lipid A of *P. aeruginosa* and the 4′-monophosphoryl lipid A of *S. minnesota* are comparable PBAs (Table 1); only the latter is able to abolish the expression of Ts activity (Fig. 4). From these findings, one may conclude that different chemical structures influence the capacity of lipid A to act as a PBA as well as to abrogate the expression of Ts function.

It has been established that the synthetic glucosamine monosaccharide analogs of lipid A (lipid A subunit analogs) induce significant nonspecific protection against microbial infections and possess potent antitumor activity (26, 50); none of these well defined synthetic compounds alter Ts function since only glucosamine disaccharides with a minimum of five fatty acyl groups (e.g., the lipid A of *Rhodobacter sphaeroides*, formerly called *Rhodopseudomonas sphaeroides*) are able to abolish the expression of Ts activity (6). These bioactivities likewise must be governed independently by different chemical structures.

The relationship between fatty acyl chain length, the position of acyl or acyloxyacyl groups, and the expression of various bioactivities of lipid A has been examined by some investigators (26, 27, 39, 52). Fatty acyl chain length influences the ability of lipid A to induce nonspecific protection against bacterial infections (26), and the antiviral activity induced by lipid A increases as fatty acyl chain length decreases from C_{16} to C_{12} (26, 27). In the present work, lipid A preparations with fatty acyl groups of relatively short chain length (C_{10} to C_{12} for *P. aeruginosa* and *C. violaceum* lipid A) (Fig. 3) or fatty acyl groups of predominantly long chain length (C_{18} for *H. pylori* lipid A) are unable to abolish the expression of Ts function (Fig. 3). In view of the results of these and previous studies (6), it appears that fatty acyl chain lengths of C_{12} to C_{14} (as in the lipid A of *E. coli* and *Salmonella* spp.) may be optimal for lipid A to abolish the expression of Ts activity. Besides chain length, the position or location of fatty acyl (or acyloxyacyl) groups on the glucosamine disaccharide backbone of lipid A also may play a critical role in the expression of this and other bioactivities. The fatty acyl or acyloxyacyl groups are attached at the 2, 3, 2′, and 3′ positions of the glucosamine disaccharide backbone (Fig. 3). The deletion of a fatty acyl group from the 3 position of *Salmonella* lipid A reduces toxicity but has no influence on its adjuvant properties or ability to abolish Ts function (6, 38). The synthetic precursor lipid A (IV_A or I_a) of *E. coli* (LA-14-PP) has only fatty acyl (HOC_{14}) groups at the 2, 3, 2′, and 3′ positions. Although LA-14-PP is a potent PBA (6), it has no effect on Ts function; this indicates that acyloxyacyl rather than fatty acyl groups are essential for the ability of lipid A to abolish the expression of Ts activity. By contrast, the synthetic diphosphoryl lipid A (LA-15-PP) and monophosphoryl lipid A (LA-15-PH) of *E. coli* have $C_{14}OC_{14}$ and $C_{12}OC_{14}$ groups at only the 2′ and 3′ positions, respectively. Since both

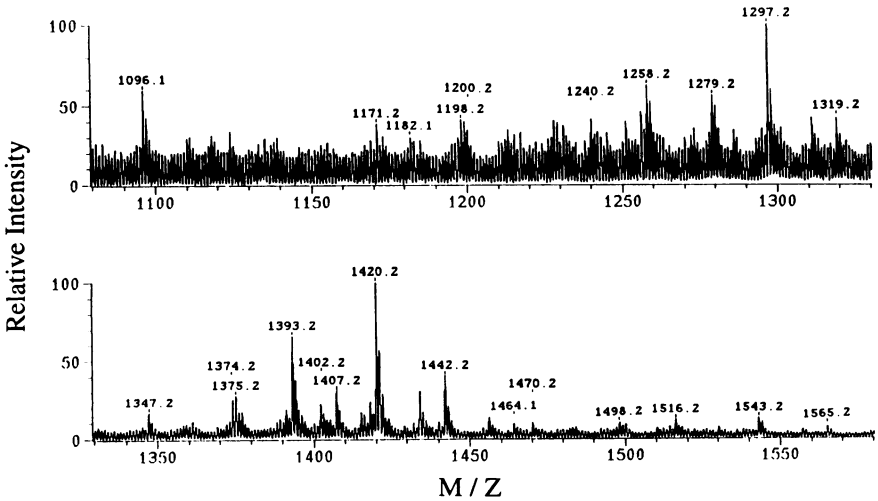


FIG. 8. Partial negative-ion LSIMS spectrum of OS/Rc-LPS(III).

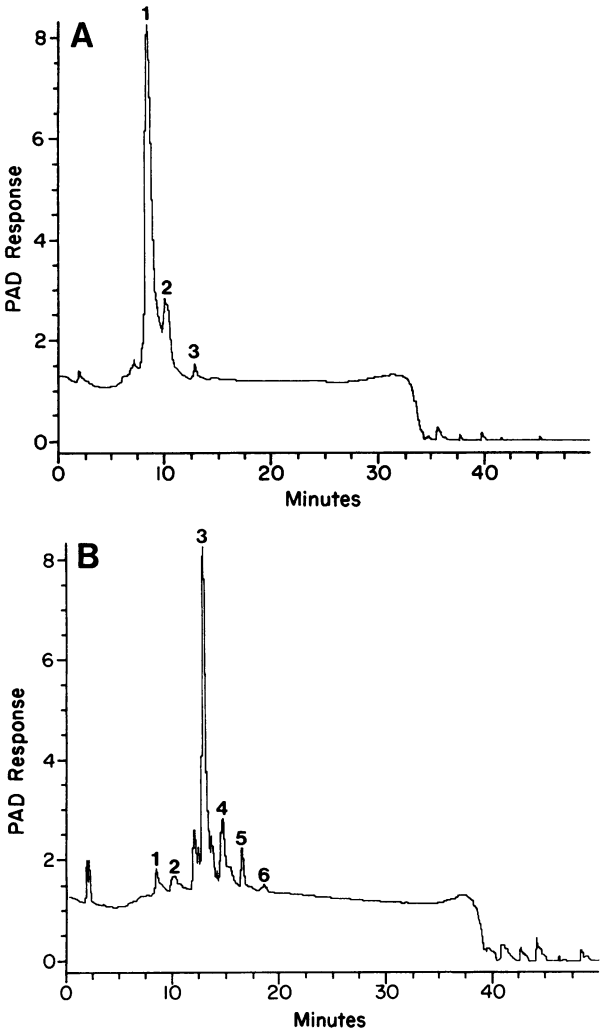


FIG. 9. Analytical HPLC, using an anion-exchange column, of OS/Rc-LPS(II) (A) and OS/Rc-LPS(III) (B). A sample (20 μg) of 20 μl of water was injected into the instrument, and the Dionex CarboPac PA-1 column was eluted with a linear gradient of 0.1 to 1.0 M sodium acetate (pH 8.0) at a flow rate of 1.0 ml/min for 50 min. A Dionex pulsed amperometric detector (PAD) was used to detect the sample.

are able to abolish the expression of Ts function (6), it appears that acyloxyacyl groups of appropriate chain length (C_{12} to C_{14}) at the 2′ and/or the 3′ position(s) are critical for the expression of this bioactivity. It should be noted that the pentaacyl lipid A of *R. sphaeroides* also is able to inactivate Ts function but not to the same degree as either the hexaacyl (3-O-deacylated) or heptaacyl lipid A derivatives of *S. minnesota* (6); the main structural differences are that *R. sphaeroides* lipid A possesses a HOC_{10} group at the 3′ position and a $\Delta^7C_{14}OC_{14}$ group at the 2′ position. If one assumes that, to abolish the expression of Ts activity, (i) an acyloxyacyl rather than an acyl group of appropriate chain length at the 3′ position is required and (ii) the bioactivity of a $\Delta^7C_{14}OC_{14}$ group at the 2′ position is similar to that of the $C_{12}OC_{14}$ group of *S. minnesota* lipid A at the same position, then a glucosamine disaccharide having an acyloxyacyl group from C_{12} to C_{14} in length at the 3′ position might be the minimal structural unit required to abrogate the expression of Ts function. In this context, the lipid A's of *P. aeruginosa* and *C. violaceum* possess a HOC_{10} group at the 3′ position but a $C_{12}OC_{12}$ group at the 2′ position (Fig. 3). Since neither is able to abolish the expression of Ts function (Fig. 4), this lends further support to the view that an acyloxyacyl group of appropriate chain length at the 3′ position indeed plays a decisive role in the expression of this bioactivity. This does not exclude the possibility that acyl or acyloxyacyl groups at other positions might contribute indirectly by stabilizing the configuration of such a molecule.

In other studies (22), the immunomodulatory properties of several LPSs derived from *P. aeruginosa*, *Branhamella catarrhalis*, and *Bordetella pertussis* were evaluated for their capacity to influence the magnitude of the antibody response to SSS-III which, as noted above, is known to be regulated by Ts and Ta (reviewed in reference 3). Treatment with preparations of LPS derived from these bacteria, at the time of immunization with SSS-III, induced significant suppression of the SSS-III-specific antibody response; such immunosuppression is (i) not induced by the LPS or lipid A of *E. coli* or *S. minnesota*, which is able to abolish the expression of Ts function (6) (Fig. 4), and (ii) independent of the capacity of LPS or lipid A to activate B cells polyclonally (22). Further studies on the LPS of *P. aeruginosa* showed that the immunosuppression induced is T cell dependent and mediated by the PS rather than the lipid fraction of the LPS macromolecule; it is due, at least in part, to the capacity of PS to expand or enlarge upon the size of the population or pool of Ts normally generated after immunization or exposure to SSS-III (22). Since the lipid A of *P. aeruginosa*, unlike the lipid A of *E. coli* and *S. minnesota*, is not able to abolish the expression of Ts function (6) (Fig. 4), the net effect of treatment with *P. aeruginosa* LPS or its PS fraction would be to increase further the degree of Ts activity generated in response to SSS-III. The resulting increased immunosuppression that is induced is manifested either by a decrease in the magnitude of the antibody response to an optimal dose of the immunizing antigen, when *P. aeruginosa* LPS or PS is given at the time of immunization with SSS-III (22), or by an increase in the degree of low-dose unresponsiveness expressed after prior exposure to SSS-III (Fig. 4).

Although we attempted to obtain more precise information on the chemical composition and structure of PS preparations found to induce significant immunosuppression in other studies (22), this was difficult to accomplish since many of the PS fractions used, which were derived from clinical isolates, were quite complex and somewhat heterogeneous. Therefore, because the inner core region OS of enterobacterial and nonenterobacterial LPSs are relatively conserved and display limited diversity in structure (28, 49, 58) and since the structures of such OS have been determined for *E. coli* (28), we elected to examine whether comparable immunosuppression might also be induced following the administration of well-characterized, highly purified preparations of lipid A proximal inner core region OS derived from Rc and Rd mutant strains of *E. coli*. That indeed was found to be the case. Treatment with some preparations of inner core region OS derived from the LPS of Rc, but not Rd, mutants of *E. coli* induced significant immunosuppression that was T cell dependent and mediated by $CD8^+$ Ts (Tables 2 to 5). The ability to transfer the increased suppression observed with smaller numbers of primed spleen cells (Table 4) and to abolish such immunosuppression by prior treatment with monoclonal anti-CD8 antibody plus complement (Table 5) demonstrates that it is indeed the result of an expansion of the pool of $CD8^+$ Ts, activated in response to SSS-III; this is consistent with the results of other studies (22). Also, the results of the present work, using purified preparations of inner core region OS (Fig. 1 and 2) of known composition (Fig. 6), indicate that the minimal structure

required to induce the added immunosuppression observed is a hexasaccharide containing one Kdo residue, two glucose residues, and three heptose residues to which are attached two $PPEtNH_2$ groups. The presence of two $PPEtNH_2$ groups, rather than just one, may be critical since OS/Rc-LPS(III) is immunosuppressive whereas OS/Rc-LPS(II) is not (Table 2; Fig. 6). Alternatively, other minor components present in the OS/Rc-LPS(III) preparation (i.e., OS representing peaks 4 to 6 of Fig. 9B) might also contribute to immunosuppression. The mechanism by which $PPEtNH_2$ and possibly other components contribute to such immunosuppression is not known and beyond the scope of the present work; however, it should be noted that the amount of $PPetNH_2$ present in preparations of OS is greatly influenced by the conditions of the chemical procedure used for the isolation and purification of OS. Although the immunosuppression observed was induced with OS preparations of relatively small molecular size, the results of the present work (Fig. 4) and other studies (22) indicate that suppression can be induced with larger molecules (e.g., LPS or its PS fraction) also possessing that same structure. In this context, it should be noted that molecular size is likely to influence biological half-life and thus the degree of immunosuppression induced per unit weight of OS, PS, or LPS used. Consequently, the continuous synthesis and release of small amounts of immunosuppressive LPS and its native inner core region OS or PS during the course of an infection might be expected to have a detrimental effect on the development of host immunity.

In this and other studies (6, 22), lipid A and core region OS or PSs derived from different preparations of LPS were evaluated for their effects on Ts, which, in concert with Ta, controls the magnitude of the antibody response ultimately produced after immunization with SSS-III. The results obtained by using this experimental model system are likely to have broad implications since such T-cell control mechanisms have been implicated in antibody responses to several microbial PS antigens of medical importance. They include PSs of *Neisseria meningitidis* group A and C (35, 55), *Cryptococcus neoformans* capsular PS (51), *Streptococcus mutans* PS (55), *E. coli* LPS (14, 15), *Serratia marcescens* LPS (14, 15), and *P. aeruginosa* LPS (42, 55). During the course of infections caused by gram-negative bacteria, significant amounts of LPS, which contribute to many of the pathophysiological effects observed, are synthesized and released. Since some lipid A's (e.g., the lipid A of *E. coli* and *Salmonella* spp.) possess fatty acyl groups of optimal chain length at appropriate positions (6) (Fig. 3), they are able to abolish the expression of Ts function (6) (Fig. 4), including the increased immunosuppression likely to be generated in response to the inner core region OS of their respective LPS (Table 2); this would favor the development of protective immunity. By contrast, a different outcome might be expected in the case of infections caused by bacteria such as *P. aeruginosa*. Since the LPS or lipid A of *P. aeruginosa* is not able to abolish the expression of Ts activity (Fig. 4), the added immunosuppressive effects induced by its inner core region OS would predominate, thereby resulting in a greatly reduced antibody response against the O-specific PS of *P. aeruginosa* which plays a major role in the development of protective immunity (25, 33, 41). Although this may provide the basis for a potentially important virulence mechanism for certain gram-negative bacteria lacking fatty acyl groups of appropriate chain length in their lipid A, additional studies are required to test the validity of this hypothesis.

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