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Structural Features That Influence the Ability of Lipid A and Its Analogs To Abolish Expression of Suppressor T Cell Activity

PHILLIP J. BAKER,[1]* TOMAS HRABA,[1] CHRISTOPHER E. TAYLOR,[1] KENT R. MYERS,[2] KUNI TAKAYAMA,[3] NILOFER QURESHI,[3] PETER STUETZ,[4] SHOICHI KUSUMOTO,[5] AND AKIRA HASEGAWA[6]

Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Twinbrook-II Research Facility, 12441 Parklawn Drive, Rockville, Maryland 20852[1]; Ribi ImmunoChem Research, Inc., Hamilton Montana 59840[2]; Mycobacteriology Research Laboratory, William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin 53705[3]; Sandoz Forschungsinstitut, Gesellschaft m.b.H., A-1235 Vienna, Austria[4]; and Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka 560,[5] and Department of Applied Bioorganic Chemistry, Gifu University, Gifu 501-11,[6] Japan

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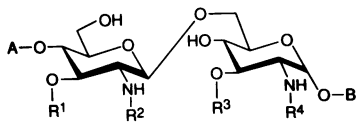
Lipid A preparations derived from the lipopolysaccharides of several gram-negative bacteria, as well as chemically defined synthetic lipid A's and their analogs (both glucosamine mono- and disaccharides), were used to establish the chemical structures required for (i) abolishing the expression of suppressor T cell (Ts) function and (ii) inducing polyclonal activation of B cells. *Salmonella minnesota* R595 lipid A (diphosphoryl lipid A) possesses both of these activities. Decreasing the number of phosphate groups in lipid A from two to one (monophosphoryl lipid A) as well as decreasing the fatty acyl content, primarily by removing the residue at the 3 position, resulted in a progressive reduction in toxicity; however, these structural modifications did not influence its ability to abolish the expression of Ts function. Reducing the fatty acyl content from five to four (lipid A precursor IV_A or I_a) eliminated the capacity to influence Ts function but not to induce polyclonal activation of B cells. None of the monosaccharide analogs of lipid A examined influenced the expression of Ts activity, although some were able to activate B cells polyclonally. Thus, in order 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. Also, the chain length of the nonhydroxylated fatty acid, as well as the location of acyloxyacyl groups (2′ versus 3′ position), may play an important role. These findings indicate that the chemical structures responsible for the toxicity of lipid A differ from those that influence its capacity to abolish the expression of Ts function and to induce polyclonal activation of B cells.

It has been established that the magnitude of the antibody response to the capsular polysaccharide antigen (SSS-III) of type III *Streptococcus pneumoniae* is influenced in both a negative and positive manner by two types of thymus-derived (T) lymphocytes with opposing functions; such regulatory T cells have been called suppressor T cells (Ts) and amplifier T cells (Ta), respectively. Ts limit the extent to which populations of bone marrow-derived precursors of antibody-forming cells (B cells) enlarge in response to antigen, whereas Ta drive clones of B cells to expand further upon antigenic stimulation. Because the activities of $CD4^-$ $CD8^+$ Ts and $CD4^+$ $CD8^-$ Ta are normally counterbalanced, the effect of one type of regulatory T cell is not usually apparent unless the activity of the other is either eliminated or altered significantly. Consequently, in the absence of Ts activity, Ta function is more fully expressed, thereby resulting in an increased or enhanced antibody response. These findings have been reviewed in detail elsewhere (2, 3, 7). Since the endotoxins or lipopolysaccharides (LPSs) of gram-negative bacteria are potent immunological adjuvants (13, 16, 22, 25, 43), we sought to determine whether some, though certainly not all, of their immunomodulatory properties might be attributed to a modification in regulatory T cell function, i.e., to an increase or decrease in Ta or Ts activity, respectively.

It has been demonstrated that the immunomodulatory, toxic, and pharmacological effects of LPS are mediated by the bis(phospho)lipid A or diphosphoryl lipid A (DPL) moiety of the LPS molecule (17, 26, 44); however, it is difficult to evaluate the role of lipid A as an immunomodulator, or to exploit its potential as an adjuvant, because lipid A, like LPS, is extremely toxic to most animal species (26, 44). This problem was resolved when it was shown that removal of the glycosidic phosphate from the reducing end of lipid A to yield monophosphoryl lipid A (MPL) reduced the toxicity but did not diminish the adjuvant properties of lipid A and LPS (33–35, 43). It has since been established that treatment with MPL abolishes the inhibitory effects of antigen-specific Ts without adversely influencing the expression of other T cell functions, e.g., helper, amplifier, and cytotoxic T cell activity (6, 15). Thus, MPL acts selectively on activated Ts, which appear to possess a receptor-linked biochemical pathway that is extremely sensitive to inactivation by MPL and its analogs (6, 15).

Little is known concerning the structural components of the MPL molecule that contribute to its ability to interact with Ts and influence the expression of Ts function. In the present work, several naturally derived lipid A structures, as well as a number of chemically defined synthetic glucosamine mono- and disaccharide analogs, were used to

* Corresponding author.



Analog	R1	R2	R3	R4	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
S. minnesota R595 4'-minophosphate, 3-O-deacylated	$C_{14}OC_{14}$	$C_{12}OC_{14}$	H	$C_{16}OC_{14}$	P	H
R. sphaeroides 1,4'-diphosphate	HOC_{10}	$C_{14}O\Delta^7C_{14}$	HOC_{10}	OC_{14}	P	P

FIG. 1. Chemical structures of naturally derived diglucosamine analogs of lipid A used. All structures are depicted in their most acylated forms. The lipid A from *R. sphaeroides* is primarily a mixture of two compounds with variations in the presence or absence of unsaturation in R^2. Abbreviations: P, phosphate; HOC_{10}, 3-hydroxydecanoyl; HOC_{14}, 3-hydroxytetradecanoyl; OC_{14}, 3-oxotetradecanoyl; $C_{12}OC_{14}$, 3-dodecanoyloxytetradecanoyl; $C_{14}OC_{14}$, 3-tetradecanoyloxytetradecanoyl; $C_{16}OC_{14}$, 3-hexadecanoyloxytetradecanoyl; $C_{14}O\Delta^7C_{14}$, 3-(Δ^7-tetradecenoyloxy)tetradecanoyl.

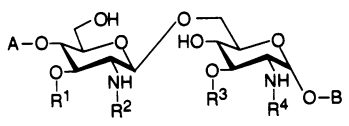
determine the structural basis for its ability to abolish the expression of Ts function.

MATERIALS AND METHODS

Mice. Female BALB/cByJ mice, 8 to 10 weeks of age, were purchased from Jackson Laboratories, Bar Harbor, Maine.

Naturally derived LPS, lipid A, and lipid A analogs. *Salmonella minnesota* R595 LPS and its corresponding bis(phospho)lipid A or DPL, and MPL fractions were obtained from Ribi ImmunoChem Research, Inc., Hamilton, Mont.; their method of preparation, immunomodulatory properties, and structural features have been documented (6, 16, 30, 33–35, 43). The 3-O-deacylated MPL of *S. minnesota* R595 was prepared as described previously (27). DPL was isolated from the nontoxic LPS of *Rhodobacter sphaeroides* as described previously (32, 38, 41). The chemical structures of the most acylated forms of these naturally derived glucosamine disaccharide lipid A preparations and their analogs are presented in Fig. 1. All were dissolved to a final concentration of 1 mg/ml in distilled water containing 0.2% triethylamine. After thorough mixing, they were sonicated briefly (10 to 20 s) to obtain a stock solution, which was stored at 4°C until used. Such stock solutions were slightly turbid with no insoluble material or opalescence. They were diluted with saline to obtain the desired concentration for intraperitoneal (i.p.) injection in a volume of 0.2 ml.

Synthetic glucosamine disaccharide analogs of lipid A or MPL. Synthetic tetraacyl lipid A precursor IV_A or I_a (LA-14-PP), mono- and diphosphoryl hexaacyl lipid A's of *Escherichia coli* (LA-15-PH and LA-15-PP, respectively), and heptaacyl *Salmonella* MPL (LA-16-PH) were purchased from ICN Biochemicals Inc., Cleveland, Ohio; their structures have been described elsewhere (37, 40). Pentaacyl LA-20-PH and LA-21-PH were prepared by one of us (S. Kusumoto); their structural and biological properties have been described (40). These preparations likewise were dissolved in distilled water containing 0.2% triethylamine as described above.



Analog	R1	R2	R3	R4	A	B
LA-14-PP, Precursor	HOC_{14}	HOC_{14}	HOC_{14}	HOC_{14}	P	P
LA-15-PH, *E. coli*	$C_{14}OC_{14}$	$C_{12}OC_{14}$	HOC_{14}	HOC_{14}	P	H
LA-15-PP, *E. coli*	$C_{14}OC_{14}$	$C_{12}OC_{14}$	HOC_{14}	HOC_{14}	P	P
LA-16-PH, Salmonella	$C_{14}OC_{14}$	$C_{12}OC_{14}$	HOC_{14}	$C_{16}OC_{14}$	P	H
LA-20-PH	HOC_{14}	HOC_{14}	HOC_{14}	$C_{16}OC_{14}$	P	H
LA-21-PH	HOC_{14}	$C_{16}OC_{14}$	HOC_{14}	HOC_{14}	P	H
SDZ 880.690-LY	HOC_{14}	HOC_{14}	OH	HOC_{14}	H	P-LY_2
SDZ 89.706-LY	HOC_{14}	HOC_{14}	HOC_{14}	HOC_{14}	H	P-LY

FIG. 2. Chemical structures of synthetic glucosamine disaccharide analogs of lipid A used. Abbreviations are the same as in Fig. 1, with the following additions: LY, lysine; LY_2, lysyllysine.

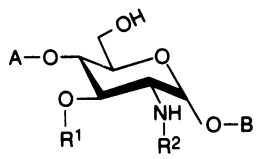
SDZ 880.690-LY and SDZ 89.706-LY were synthesized as described previously (39). These lysine (LY)-containing monophosphoryl analogs with phosphate groups attached at the 1 rather than the 4' position were handled differently because of the instability of the phosphate group at that position. Ethanol (40 μl) was added to a glass tube (10 by 75 mm) containing about 5 mg of dry material. The mixture was warmed to 45°C and thoroughly mixed. Then, 0.4 ml of warm (45°C) 0.15 M glucose solution was added, and the sample was sonicated for 10 to 20 s, after which sufficient warm glucose solution was added to provide a final concentration of 1 mg/ml. This stock solution was sonicated once more and then stored at 4°C; it was diluted with 0.15 M glucose to the desired concentration for injection (i.p.) in a volume of 0.2 ml. The chemical structures of all of the synthetic glucosamine disaccharides used are given in Fig. 2.

Synthetic glucosamine monosaccharide analogs. The structures and biological properties of synthetic GLA-27, GLA-47, GLA-57, GLA-58, GLA-59, GLA-60, GLA-113, and GLA-147 have been described (21, 24). They were dissolved in distilled water containing 0.2% triethylamine as described above.

SDZ 880.421 and SDZ MRL 953, which unlike the synthetic monosaccharide analogs described above, are 1-phospho derivatives, were synthesized as described previously (39); they were dissolved in 0.15 M glucose as described above. The chemical structures of all of the synthetic monosaccharide analogs used are shown in Fig. 3.

Antigen and immunization procedure. The immunological properties of the preparation of 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 injection (i.p.) of an optimally immunogenic dose (0.5 μg) of SSS-III in 0.5 ml of saline. The magnitude of the antibody response elicited was determined 5 days after immunization.

Immunological methods. Numbers of splenic antibody-producing plaque-forming cells (PFC) making antibody specific for SSS-III (SSS-III-specific PFC) provided a measure of the peak antibody response made by individual mice, i.e., at 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 (SRBC) coated with SSS-III by the $CrCl_3$



Monoglucosamine	R1	R2	A	B
GLA-47	C_{14}	$C_{14}OC_{14}$	P	H
GLA-47	$C_{14}OC_{14}$	$C_{14}OC_{14}$	P	H
GLA-57	C_{14}	$C_{12}OC_{14}$	P	H
GLA-58	C_{14}	$C_{16}OC_{14}$	P	H
GLA-59	HOC_{14}	$C_{14}OC_{14}$	P	H
GLA-60	$C_{14}OC_{14}$	HOC_{14}	P	H
GLA-113	$C_{10}C_{14}$	HOC_{14}	P	H
GLA-147	$C_{14}C_{14}$	HOC_{14}	P	H
SDZ 880.421	$C_{14}OC_{14}$	HOC_{14}	H	P
SDZ MRL 953	HOC_{14}	HOC_{14}	HOC_{14}	P

FIG. 3. Chemical structures of synthetic glucosamine monosaccharide analogs of lipid A used. Abbreviations are the same as in Fig. 1, with the following additions: C_{14}, tetradecanoyl; $C_{10}C_{14}$, 2-decytetradecanoyl; $C_{14}C_{14}$, 3-undecylheptadecanoyl.

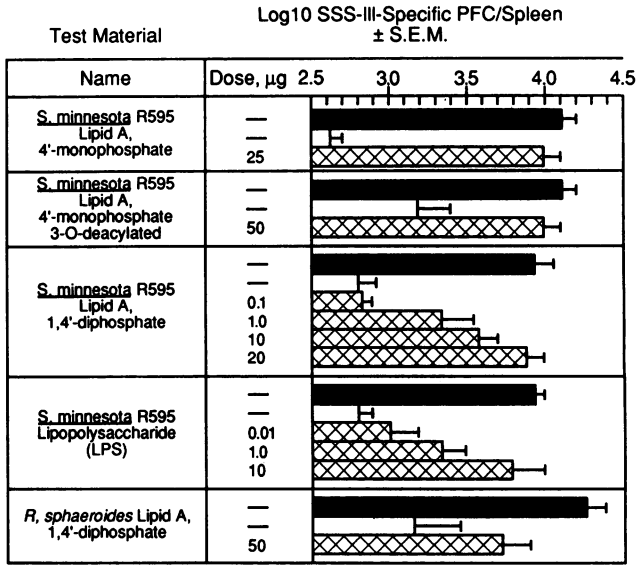


FIG. 4. Effects of treatment with two injections of different amounts of LPS or lipid A analogs on expression of low-dose immunological paralysis. ■, immunized (0.5 μg of SSS-III); □, primed (5 ng of SSS-III) and immunized (0.5 μg of SSS-III); ⊠, primed (5 ng of SSS-III), immunized (0.5 μg of SSS-III), and treated with analog.

method (11). Corrections were made (by subtraction) for the small numbers of background SRBC-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 (18), 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 and (ii) numbers of SSS-III-specific PFC per spleen are directly related to the magnitude of the serum antibody response (1, 23).

Assessment of polyclonal activation. The appearance of increased numbers of cells secreting non-antigen-specific immunoglobulin of the IgM class, which is a characteristic of biologically active preparations of LPS and lipid A (19), was detected by a modification of the protein A plaque assay (20), in which indicator SRBC were coated with protein A (Pharmacia) in the presence of $CrCl_2$ (66 μg/ml) as the coupling agent. The affinity-purified rabbit anti-mouse IgM antibody used for the detection of non-antigen-specific IgM-secreting PFC was the same preparation as that used in other published studies (20). A dilution (1:200 in saline) known to reveal maximal numbers of IgM-secreting PFC (20) 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 IgM-secreting PFC per spleen ± SEM for groups of treated or untreated (control) mice. Here, all comparisons were made with respect to the baseline values for such PFC in age-matched control (untreated) groups of mice.

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 <0.05 were obtained.

RESULTS

Effects of treatment with naturally derived LPS, lipid A, and lipid A analogs on expression of Ts activity. Mice were pretreated or primed with a single injection (i.p.) of a subimmunogenic dose (5 ng) of SSS-III. Three days later, they were given (i.p.) an optimally immunogenic dose (0.5 μg) of SSS-III with or without an injection (i.p.) of a given amount of LPS, lipid A, or lipid A analog; a second injection of the same amount of LPS, lipid A, or lipid A analog also was given (i.p.) to primed mice 1 day after immunization with 0.5 μg of SSS-III. 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 LPS, lipid A, or lipid A analogs on the degree of unresponsiveness expressed. It should be noted that in these and in the remaining experiments to be described, mice were given two injections of the stated amounts of the test materials used, on the day of and on the day after immunization with 0.5 μg of SSS-III.

The data in Fig. 4 show that priming with a single injection of 5 ng of SSS-III resulted in the development of 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 weeks or months after priming with just one injection of 5 ng of SSS-III, and is mediated by $CD8^+$ Ts (3, 4, 7, 9, 42). Treatment with 0.01 to 10 μg of *S. minnesota* R595 LPS or 0.1 to 20 μg of toxic DPL derived from this LPS likewise resulted in a dose-dependent decrease in the degree of unresponsiveness expressed; here, unresponsiveness was abolished ($P < 0.001$) by treatment with two injections of 10

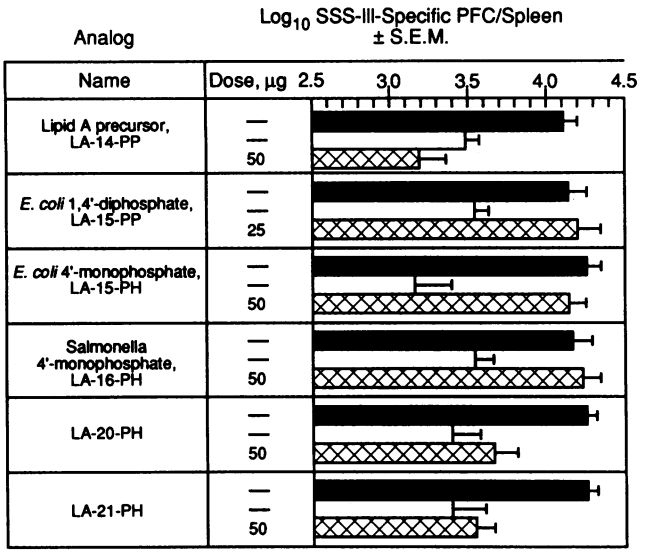


FIG. 5. Effects of treatment with two injections of 25 or 50 μg of various synthetic lipid A analogs on expression of low-dose immunological paralysis. Symbols are defined in the legend to Fig. 4.

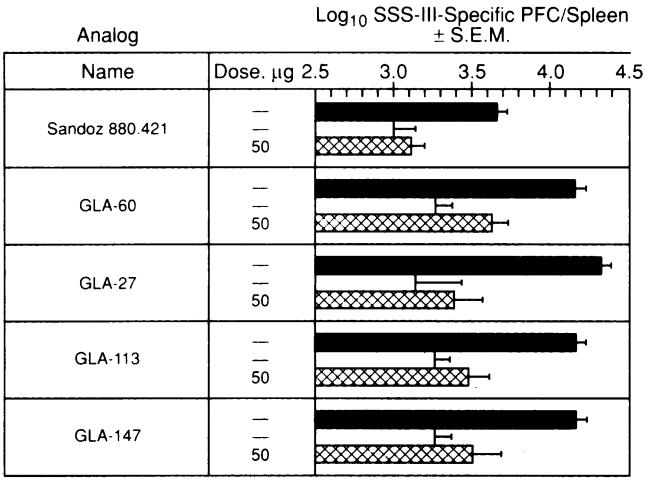


FIG. 6. Effects of treatment with two injections of 50 μg of various synthetic monoglucosamine analogs on expression of low-dose immunological paralysis. Symbols are defined in the legend to Fig. 4.

to 20 μg of each of these preparations, which are quite toxic and pyrogenic (27, 33).

The administration of two injections of 50 μg of nontoxic *R. sphaeroides* DPL (32, 38, 41) caused a significant decrease in, but not a complete abolition of, unresponsiveness; however, treatment with 25 to 50 μg of relatively nontoxic *S. minnesota* R595 MPL or its even less toxic 3-O-deacylated derivative (27) abolished unresponsiveness ($P < 0.001$). Thus, although the phosphate group at the 1 position and the acyl group at the 3 position are required for the full expression of endotoxic activity (27, 33), neither appears to be essential for adjuvanticity (27) or for the abrogation of low-dose unresponsiveness, i.e., the ability to inactivate the expression of Ts function. It is interesting that the nontoxic LPS of *R. sphaeroides* is still active in that regard (12), likewise indicating a dissociation between toxicity and an effect on the expression of Ts activity.

Effects of treatment with synthetic lipid A analogs on expression of Ts activity. Thus far, treatment with 50 μg or less of all preparations of LPS examined, as well as with naturally derived preparations of their respective lipid A and MPL fractions, had a significant effect on the expression of Ts activity (Fig. 4). Therefore, the same experimental approach also was used to evaluate the effects of treatment with two doses of 25 or 50 μg of various synthetic lipid A analogs on the expression of Ts activity. The results obtained (Fig. 5) show that treatment with synthetic hexaacyl *Escherichia coli* MPL or DPL and heptaacyl *Salmonella* MPL (LA-15-PH, LA-15-PP, and LA-16-PH, respectively) abolished ($P < 0.001$) low-dose unresponsiveness. By contrast, treatment with (i) synthetic tetraacyl lipid A precursor (LA-14-PP), which contains only HOC_{14} groups, and with (ii) pentaacyl LA-20-PH or LA-21-PH, which contain only a single $C_{16}OC_{18}$ group at the 2 or 2′ position, respectively, was without effect. The ability of synthetic preparations of MPL, which are not expected to contain LPS, to abolish the expression of Ts activity indicates that the results obtained with *S. minnesota* R595 or *Salmonella typhimurium* MPL in this and in previous studies (Fig. 4; 6, 15) cannot be attributed simply to contamination by small amounts of LPS or DPL, which likewise can influence the expression of Ts activity.

The results of previous studies indicate that once Ts are activated, they acquire a cell surface receptor for the binding of lipid A, in addition to possessing a biochemical pathway or effector function that is extremely sensitive to inactivation by lipid A and LPS (6, 12, 14). This accounts for the fact that the binding and elution of MPL-adherent cells from a solid matrix yields cell suspensions greatly enriched (>1,000-fold) in Ts activity (5) and that the in vitro treatment of antigen-primed spleen cells with nanogram amounts of MPL or LPS abolishes their capacity to transfer Ts-mediated suppression (12, 14). This raises the possibility that, although some analogs of lipid A may not be able to abolish the expression of Ts activity, they still might block the capacity of MPL to do so. Treatment with 50 to 100 μg of synthetic SDZ 880.690-LY or SDZ 89.706-LY had no effect ($P > 0.05$) on the expression of Ts activity; furthermore, the administration of 50 μg of these synthetic compounds 15 to 20 min prior to treatment with each of two doses of 50 μg of *S. minnesota* R595 MPL also did not block or interfere with the capacity of MPL to abolish Ts function (data not shown). Since tetracyl lipid A precursor IV_A or I_a (LA-14-PP) was likewise without effect (Fig. 5), it is not surprising that these triacyl analogs lack such activity. Therefore, these synthetic disaccharide analogs lack the capacity to compete with MPL for binding to relevant cell surface receptor molecules, as well as to influence the functional activity of Ts. Although the presence of lysine in these synthetic preparations might be a factor, their low fatty acyl content (Fig. 2) is most likely the main reason for their lack of activity.

Effects of treatment with glucosamine monosaccharide analogs of lipid A on expression of Ts activity. Using the same experimental approach, we found that treatment with two doses of 50 μg of various synthetic monosaccharide analogs, e.g., Sandoz (SDZ) 880.421, GLA-27, GLA-113, or GLA-147, had a slight, but not significant ($P > 0.05$), effect on the expression of Ts activity (Fig. 6); this was likewise the case for primed mice given two doses of 50 μg of SDZ MRL 953, GLA-47, GLA-57, GLA-58, or GLA-59 ($P > 0.05$; data not shown). Furthermore, treatment with 50 or 100 μg of GLA-47, GLA-60, GLA-113, or GLA-147 15 to 20 min before

TABLE 1. Relative increase in non-antigen-specific IgM-secreting PFC in BALB/cByJ mice treated with different naturally derived and synthetic test materials

Test material		Dose (μg)	Mean relative increase ± SEM[a]	P value[b]
Type	Prepn			
Naturally derived	*S. minnesota* R595 DPL	50	15.32 ± 1.92	<0.001
	S. minnesota R595 MPL	50	5.46 ± 0.87	<0.001
	S. minnesota R595 MPL	100	10.43 ± 0.66	<0.001
	S. minnesota R595 3-O-deacylated MPL	50	9.05 ± 3.17	<0.01
	R. sphaeroides DPL	50	1.78 ± 0.24	<0.02
Synthetic disaccharide analogs	LA-14-PP (precursor IV_A or I_a)	50	8.20 ± 0.48	<0.001
	LA-15-PH	50	5.13 ± 0.78	<0.001
	LA-15-PP	50	6.87 ± 0.34	<0.001
	LA-20-PH	50	1.39 ± 0.13	>0.05
	LA-21-PH	50	1.09 ± 0.14	>0.05
Synthetic monosaccharide analogs	GLA-47	100	1.49 ± 0.34	>0.05
	GLA-59	100	2.79 ± 0.28	<0.001
	GLA-60	20	0.94 ± 0.09	>0.05
	GLA-60	100	2.06 ± 0.24	<0.01
	GLA-113	20	1.16 ± 0.14	>0.05
	GLA-113	100	2.80 ± 0.61	<0.001
	GLA-147	20	1.22 ± 0.15	>0.05
	GLA-147	100	1.62 ± 0.23	>0.05
	SDZ 880.421	100	0.83 ± 0.12	>0.05
	SDZ MRL 953	50	2.85 ± 0.50	<0.001
	SDZ MRL 953	100	2.21 ± 0.69	<0.001

[a] Mean relative increase in non-antigen-specific IgM-secreting PFC ± SEM for groups of 5 to 10 mice 3 days after the administration (i.p.) of test material. The mean number of non-antigen-specific IgM-secreting PFC per spleen for age-matched control mice not treated with test material was 158,000 ± 14,000.
[b] Based on comparisons with control mice not treated with test material.

treatment with each of two doses of 50 μg of *S. minnesota* R595 MPL did not block or antagonize ($P > 0.05$) the capacity of the MPL to abrogate unresponsiveness (data not shown). Sufficient amounts of these compounds were not available to permit testing at larger (>100 μg) doses.

GLA-60 had a significant ($P < 0.05$) influence on the expression of unresponsiveness; however, the effect observed after treatment with two doses of 50 μg of GLA-60 was partial at best and represents only a 25 to 30% decrease in the degree of unresponsiveness expressed (Fig. 6). Treatment with two doses of a larger amount (100 μg) of GLA-60 likewise resulted in the same (25 to 30%) reduction in unresponsiveness (data not shown). Since the administration of 50 μg of GLA-60 15 to 20 min prior to treatment with each of two doses of *S. minnesota* R595 MPL did not block or interfere with the capacity of MPL to abolish the expression of Ts activity (Fig. 4; data not shown), it is possible that the effect of GLA-60 on unresponsiveness is mediated by a different mechanism. None of the remaining synthetic glucosamine monosaccharide analogs tested had a significant effect on the expression of Ts activity (Fig. 6; $P > 0.05$ in all cases).

Polyclonal activation of B cells by naturally derived and synthetic lipid A and analogs. Mice were given a single injection (i.p.) of 20 to 100 μg of the various naturally derived or synthetic test materials used in this work. Three days later, spleen cell suspensions from individual mice were assayed for numbers of non-antigen-specific IgM-secreting PFC. The results obtained were compared with the baseline values for such PFC in untreated control mice to determine the mean relative increase in IgM-secreting PFC per spleen for groups of mice treated with each preparation examined.

The data in Table 1 show that all of the naturally derived preparations that were capable of abolishing the expression of Ts activity (Fig. 4) also were able to induce significant polyclonal activation, although to differing extents. *S. minnesota* R595 DPL appeared to be about three times more potent than its less toxic MPL analog, suggesting that polyclonal activation may be influenced by the number of phosphate groups attached to the molecule; however, synthetic LA-15-PP and LA-15-PH also differ in the number of phosphate groups present yet induced the same degree of polyclonal activation, indicating that other factors must be involved. In addition, the potency of 3-O-deacylated MPL was equal to, or slightly greater than, that of fully acylated MPL; this suggests that the acyl group at the 3 position, though important for the expression of toxicity (27), is not essential for the induction of polyclonal activation. Both synthetic LA-15-PH and LA-15-PP were able to abolish the expression of Ts activity (Fig. 5), as well as to induce significant polyclonal activation (Table 1); although the degree of polyclonal activation induced by these synthetic preparations was similar to that induced by synthetic LA-14-PP, LA-14-PP had no effect on the expression of Ts activity (Fig. 5). By contrast, LA-20-PH and LA-21-PH neither influenced the expression of Ts activity (Fig. 5) nor induced polyclonal activation.

Except for the marginal effect of GLA-60, none of the other synthetic glucosamine monosaccharide analogs tested abolished the expression of Ts activity (Fig. 6); however, some (e.g., GLA-59, GLA-60, GLA-113, and SDZ MRL 953) were able to induce polyclonal activation, though perhaps not to the same degree as that noted for some of the glucosamine disaccharides considered. Except for the position of the phosphate group, GLA-60 and SDZ 880.421 are identical in chemical structure (Fig. 3); however, GLA-60 induces significant ($P < 0.01$) polyclonal activation, whereas SDZ 880.421 does not ($P > 0.05$; Table 1). This suggests that

the position of the phosphate group may play an important role in the ability of these compounds to induce polyclonal activation. Although some of the synthetic glucosamine monosaccharide analogs tested were able to induce significant polyclonal activation, none were able to abolish the expression of Ts activity (Fig. 5).

DISCUSSION

Although the LPSs or endotoxins of gram-negative bacteria evoke a variety of toxic, pharmacologic, and immunomodulatory effects in most animal species (for reviews, see references 16, 26, and 44), past attempts to relate the expression of such bioactivities to chemical structures present in these complex macromolecules have generated much controversy. During the early period of endotoxin research, much attention was focused upon the purity of the LPS preparations employed, as well as upon the particular chemical extraction procedures used to isolate biologically active material from various species or strains of bacteria grown under different culture conditions. Such concerns were not without foundation and certainly merit attention today since all of these variables influence the bioactivity and potency of the final product obtained (28). The recognition that the bioactivities of LPS are mediated by the lipid A portion of the molecule (for a review, see reference 44) has led to attempts to define precise structure-function relationships for the various activities of lipid A. Progress in this regard has been advanced greatly by the availability of chemically defined synthetic analogs of lipid A with biological properties comparable to that of extracted or naturally derived lipid A (21, 31, 40). Unfortunately, few precise statements can be made concerning structure-function relationships since not all of the synthetic preparations considered to date have been tested under similar experimental conditions. Furthermore, dose-response relationships of amphiphilic materials such as lipid A might also be influenced by the three-dimensional properties of their aggregated structures, the formation of which is governed by the number, length, and location of the long-chain fatty acid residues, as well as by the number and positions of charged groups on the glucosamine disaccharide backbone of lipid A or its synthetic analogs (36). Despite these complexities, a general scheme for classifying certain structure-function relationships for lipid A has been proposed (40). This scheme is based on the association of acylation patterns, in addition to other structural components of various synthetic glucosamine mono- and disaccharide analogs examined, with the expression of (i) lethality for chicken embryos, (ii) elicitation of the Shwartzman reaction, (iii) lethality for galactosamine-loaded mice, (iv) induction of alpha/beta interferon, interleukin-2, and tumor necrosis factor, (v) adjuvanticity, (vi) mitogenicity, (vii) increased macrophage function, and (viii) activation of the complement cascade. The present work adds to this growing body of knowledge since it provides, for the first time, information on the minimal structure that influences the ability of MPL to abolish the expression of Ts activity without adversely affecting either the induction or expression of other T cell functions (6, 14, 15).

None of the synthetic glucosamine monosaccharide analogs examined in the present work were able to abolish the expression of Ts activity (Fig. 6) or to block the capacity of MPL to abrogate Ts function (data not shown); this was true even though they possessed acyl or acyloxyacyl groups also present in preparations of MPL having such activity. Therefore, a glucosamine disaccharide backbone appears to be essential in order to abrogate Ts function. Other studies have shown that elimination of a phosphate group from the reducing end (the 1 position) of DPL results in a large reduction in endotoxic activity (33) and the removal of an acyl group from the 3 position results in a further reduction in endotoxic activity (27). For example, the toxicity of heptaacyl *S. minnesota* R595 LPS, as measured by 50% chicken embryo lethal dose ($CELD_{50}$) is about 0.0032 μg; however, the $CELD_{50}$ values for the corresponding MPL and the 3-O-deacylated derivative are 30 and >100 μg, respectively (27). None of these chemical modifications alter either the adjuvanticity of these materials or their capacity to abolish the expression of Ts function, since 3-O-deacylated MPL is fully active with regard to those bioactivities (27 and Fig. 4). Therefore, it is apparent that the structural components required for the expression of toxicity by lipid A differ from those that influence adjuvanticity and its ability to influence the expression of Ts function.

Experiments conducted with various synthetic glucosamine disaccharide analogs revealed that only those compounds having acyloxyacyl groups at the 2′ and 3′ positions (e.g., hexaacyl LA-15-PH, LA-15-PP, and heptaacyl LA-16-PH) were able to abolish the expression of Ts activity (Fig. 2 and 5). Except for the pentaacyl DPL of *R. sphaeroides*, the results obtained with the remaining naturally derived lipid A preparations, or their analogs, support such a view (Fig. 4). This suggests that two acyloxyacyl groups, at the 2′ and 3′ positions of the glucosamine disaccharide, are essential for lipid A to abolish the expression of Ts activity; however, such a conclusion should be accepted with some reservations. The ability of the DPL of *R. sphaeroides* to decrease, but not abolish, the expression of Ts function (Fig. 4) implies that the acyloxyacyl amino group at the 2′ position may play a critical role in influencing Ts function and that the chain length of the nonhydroxylated fatty acid at that position also may have a decisive effect on functional activity. For example, the synthetic analogs (LA-15-PH, LA-15-PP, and LA-16-PH) and lipid A with a $C_{12}OC_{14}$ group at the 2′ position were able to abolish the expression of Ts function (Fig. 4 and 5). By contrast, the DPL of *R. sphaeroides*, having a $C_{14}O_{\Delta}{}^{7}C_{14}$ group at the 2′ position (Fig. 1), decreased the expression of Ts activity to a lesser, although still significant, extent (Fig. 4), whereas LA-21-PH, having a $C_{16}OC_{14}$ group at the same position, was without effect (Fig. 2 and 5). This implies that the capacity to influence the expression of Ts activity may decrease as the chain length of the nonhydroxylated fatty acid of the acyl group at the 2′ position increases from C_{12} to C_{16}. Obviously, one must examine the activity of a pentaacyl or 3-O-desacyl tetraacyl preparation having a $C_{12}OC_{14}$ group at the 2′ position to test the validity of this hypothesis. Unfortunately, such a compound is not available at this time.

Despite the fact that none of the synthetic glucosamine monosaccharide analogs examined abolished the expression of Ts activity (Fig. 6), some were able to induce the polyclonal activation of B cells (Table 1); this was likewise the case for tetraacyl LA-14-PP, a synthetic disaccharide analog (Table 1; Fig. 5). This implies that different structures are involved in the expression of these activities. For example, GLA-60 and SDZ 880.421 are identical in structure, except for the position of the phosphate group (Fig. 3); however, GLA-60 is able to stimulate significant polyclonal activation, whereas SDZ 880.421 does not (Table 1), indicating that the position of the phosphate group plays an important role in the polyclonal activation of B cells. It

should be noted that, although SDZ 880.421 is able neither to abrogate the expression of Ts activity nor to induce polyclonal activation of B cells, it has been shown to stimulate significant nonspecific protection against infection (39). Thus, of all the monosaccharide analogs examined in this work, SDZ 880.421 may be the most selective with respect to the development of nonspecific protection, suggesting that it possesses the minimal structural components required for the expression of that bioactivity.

In an other study, it was shown that treatment with various synthetic acylated glucosamine monosaccharides (e.g., GLA-27, GLA-60, GLA-78, GLA-115, or GLA-147), before immunization with SRBC results in nonspecific suppression of the antibody response (29). Although the mechanism responsible for the suppression induced by these monosaccharides is not known, it does not appear to be mediated by the Ts described in this work, whose effects are known to be antigen specific (3, 4). The results of structure-function experiments (29) indicate that the nonspecific immunosuppression induced by these synthetic analogs (i) requires a single HOC_{14} group at the 2 position, (ii) is negated by the substitution of a tetradecanoyloxy group in the HOC_{14} group at either the 2 or 3 position, and (iii) is influenced by the chain length of the hydroxylated fatty acid of the acyl group at the 2 position (29). In view of these and the other observations described above, it appears that we are close to being able to attribute many of the diverse bioactivities of LPS to specific chemical structures in the lipid A moiety.

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