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Synthetic *Salmonella*-Type Lipid A Antigen with High Serological Specificity

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A synthetic compound (compound 516), β(1-6)-linked D-glucosamine disaccharide 1,4′-bisphosphate, which is acylated by (*R*)-3-hexadecanoyloxytetradecanoyl, (*R*)-3-hydroxytetradecanoyl, (*R*)-3-dodecanoyloxytetradecanoyl, and (*R*)-3-tetradecanoyloxytetradecanoyl groups at positions 2, 3, 2′, and 3′, respectively, exhibited in vitro antigenic reactivity of high specificity comparable to that of free lipid A from *Salmonella minnesota* R595. This was confirmed by an enzyme-linked immunosorbent assay and an enzyme-linked immunosorbent assay inhibition test with monoclonal and conventional antibodies. The results of comparative analysis performed with several synthetic lipid A analogs as well as three monosaccharide derivatives suggested that the complete structure involving both phosphate groups at the C-1 and C-4′ positions and the 3-acyloxyacyl groups at the C-2, C-2′, and C-3′ positions of the glucosamine disaccharide are required for the expression of the serological specificity of *Salmonella*-type lipid A. This was deduced from the observations that compound 506, a synthetic *Escherichia coli*-type lipid A which has the same structure as that of compound 516, except that 3-hydroxytetradecanoyl group is substituted for an acyloxyacyl residue at the C-2 position, exhibited significantly reduced antigenic reactivity as compared with compound 516 and that the replacement by the hydrogen atom of the phosphoryl group at the C-1 position or by 3-hydroxytetradecanoyl or tetradecanoyl groups of acyl residues at the 2, 3, 2′, and 3′ positions of compound 516 results in a marked reduction of reactivity with monoclonal antibodies 5G and 36G. Similar results were obtained by assays with conventional rabbit antibodies, but the structural difference between compounds 516 and 506 could not be distinguished by these polyclonal antibodies. The results of cross-reactions among synthetic analogs with monoclonal antibodies 161M and 1-9M, which have been confirmed to exhibit different serological specificities from the 5G or 36G antibody, also suggested that the nature and linkage of fatty acyl residues as well as the backbone structure of lipid A play an important role in determining serological specificity of the lipid A molecule.

Since the conventional antibodies to lipid A, the endotoxic component of gram-negative bacterial lipopolysaccharide, were prepared by Galanos et al. (10), the immunodominant group has been investigated mainly with enterobacterial lipid A. Results of previous studies (4, 10, 19; N. Kasai, N. Nagumo, T. Satomi, J. Mishimo, and S. Okubo, Jpn. J. Bacteriol. **30**:179, 1975) have suggested that glucosamine disaccharides that carry amide-linked 3-hydroxytetradecanoic acid are involved in the serological determination of lipid A. It has also been shown (4, 7) that the antibodies against *Salmonella minnesota* or *Escherichia coli* lipid A cross-react with the lipid A of many gram-negative bacteria that share common structural features, but they do not cross-react with the lipid A of species that have different structures, such as those of *Rhodopseudomonas* species (11).

Recently, Galanos et al. (5) have shown by use of degradation products of natural lipid A that the minimum structure that expresses full antigenic activity is a glucosamine disaccharide containing one amide-linked 3-hydroxytetradecanoic acid; hence, ester-linked fatty acids and phosphate groups are not essential for antigenic activity. Lüderitz et al. (18) have examined the antigenic reactivity of synthetic lipid A analogs prepared by Kusumoto et al. (16) with respect to the proposed structure of lipid A as a provisional model. They have shown that the amide-bound 3-hydroxyacyl residues can be replaced by nonhydroxylated fatty acids without a reduction in antigenic reactivity and that the monosaccharide *N*-(3-hydroxytetradecanoyl)glucosamine-1-phosphate exhibits reactivity toward lipid A antiserum. Thus, they concluded that the immunodeterminant structure of lipid A comprises the linkage region of amide-linked fatty acid and D-glucosamine.

On the other hand, results of recent studies of Mashimo et al. (J. Mashimo, S. Arata, K. Egawa, N. Kasai, K. Okuda, and Y. Aihara, Jpn. J. Med. Sci. Biol. **37**:179, 1984) and Arata et al. (S. Arata, J. Mashimo, K. Egawa, N. Kasai, K. Okuda, Y. Aihara, T. Shiba, and S. Kusomoto, Jpn. J. Bacteriol. **39**:466, 1984) on the antigenic structure of *S. minnesota* R595 lipid A indicate that ester- and amide-linked fatty acyl groups as well as the glucosamine disaccharide phosphate backbone of the proposed structure of lipid A are involved in the determination of the serological specificity of the lipid A molecule. This was demonstrated by an enzyme-linked immunosorbent assay (ELISA) and an ELISA inhibition test with both conventional and monoclonal antibodies. This finding has also been supported by our previous results (15) regarding the antigenic reactivities of a synthetic *E.*

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Compound	R^2	$R^{2'}$	R^3	$R^{3'}$	X	Y
514	C_{14}-O-(C_{16})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	H	P
516	C_{14}-O-(C_{16})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	P	P
LA-17-PH	C_{14}	C_{14}	C_{14}	C_{14}	H	P
LA-17-PP	C_{14}	C_{14}	C_{14}	C_{14}	P	P
LA-18-PH	C_{14}-OH	C_{14}-OH	C_{14}	C_{14}	H	P
LA-18-PP	C_{14}-OH	C_{14}-OH	C_{14}	C_{14}	P	P
506	C_{14}-OH	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	P	P
404	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	P
405	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	H
406	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	P
401	C_{14}-OH		C_{14}-OH		P	H
408	C_{14}-O-(C_{16})		C_{14}-OH		P	H
410	C_{14}-OH		C_{14}-OH		H	P

FIG. 1. Chemical structure of synthetic lipid A analogs. Abbreviations: P, $PO(OH)_2$; C_{14}, tetradecanoyl; C_{14}-OH, (*R*)-3-hydroxytetradecanoyl; C_{14}-O-(C_{12}), (*R*)-3-dodecanoyloxytetradecanoyl; C_{14}-O-(C_{14}), (*R*)-3-tetradecanoyloxytetradecanoyl; C_{14}-O-(C_{16}), (*R*)-3-hexadecanoyloxytetradecanoyl.

coli-type lipid A, compound 506, which has full endotoxic properties and the analogs of lipid A (12).

In this study, we describe the in vitro antigenic reactivity of the recently synthesized *Salmonella*-type lipid A and its analogs.

MATERIALS AND METHODS

Synthetic lipid A analogs. The chemical structures of a synthetic *Salmonella*-type lipid A (compound 516) and its analogs are shown in Fig. 1. Compound 506, a synthetic *E. coli*-type lipid A, was prepared as described previously (12) and served as one of the reference compounds. Compounds 401 and 408 were synthesized as described previously (17). Details of the synthesis of other compounds will be described elsewhere and the following is an outline of the procedure used. Briefly, 1,4′-bisphosphate (compound 516) corresponding to *Salmonella*-type lipid A was synthesized by a procedure described previously for *E. coli*-type lipid A (13). Thus, two appropriately functionalized monosaccharide components, i.e., 2-deoxy-4-diphenylphosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranosyl bromide and allyl 3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxytetradecanoylamino]-α-D-glucopyranoside, were prepared. Condensation of them afforded a β(1,6)-linked disaccharide containing five of the seven acyl groups required and one of the phosphate groups. Next, the remaining (*R*)-3-dodecanoyloxytetradecanoyl group for the 2′-amino function and the glycosidic phosphate, which were not introduced at the monosaccharide stage for previously described reasons (13), were introduced in a stepwise fashion. After hydrogenolytic deprotection, the product was purified by silica gel column chromatography. Acid treatment followed by lyophilization from dioxane yielded pure 1,4′-bisphosphate (compound 516) as a colorless powder. The corresponding 4′-monophosphate (compound 514) was prepared by omitting the phosphorylation step in the procedure described above. For the synthesis of LA-17 and LA-18, on the other hand, a β(1-6)-disaccharide containing 4′-phosphate and two ester-bound tetradecanoyl groups on 3 and 3′ positions was prepared as a common intermediate. The two amino groups of the disaccharide were simultaneously acylated with either tetradecanoic acid or (*R*)-3-benzyloxytetradecanoic acid. Deprotection after or without glycosyl phosphorylation yielded 1,4′-bisphosphates or 4′-monophosphates, respectively.

All the final products of synthetic analogs showed single spots on thin-layer chromatography and gave satisfactory results in elemental analysis, confirming their high purities. The structures of the all key synthetic intermediates were also confirmed by means of spectroscopic and elemental analyses.

Reference natural products. Free lipid A specimens from *S. minnesota* R595 were prepared by acid hydrolysis with 1% acetic acid of R595 glycolipid isolated by the phenol-chloroform-petroleum ether method (9) as described previously (2).

Both synthetic and natural products were dissolved or suspended as homogeneously as possible at 2 mg/ml in 0.1% triethylamine aqueous solution and diluted with either 0.1 M

TABLE 1. Antigenic reactivity of synthetic lipid A analogs in ELISA with *S. minnesota* R595 lipid A antibodies

Coating antigen[a]	Antibody titers of:				
	Monoclonal antibodies				Conventional IgG
	5G	36G	161M	1-9M	Rabbit 91
514	79,000	9,500	58,900	589,000	26,000
516	457,000	44,700	66,000	575,000	457,000
LA-17-PH	<100	<100	35,600	331,000	100
LA-17-PP	15,000	4,200	5,600	46,000	42,000
LA-18-PH	<100	<100	7,200	<100	<100
LA-18-PP	<100	<100	<100	<100	13,600
Strain R595 lipid A	204,000	25,000	59,000	724,000	550,000

[a] Microtiter plates were coated with the respective synthetic and natural compounds at a dose of 2 μg/0.2 ml per well.

carbonate-bicarbonate buffer (pH 9.6) for coating in ELISA or phosphate-buffered saline containing 0.05% Tween 20 at pH 7.2 for the ELISA inhibition test. Compounds 516, LA-17-PP, LA-18-PP, 506, 401, 408, 410, as well as natural products were completely dissolved, while compounds 514, LA-17-PH, and LA-18-PH gave slightly turbid solutions.

Conventional and monoclonal antibodies to *S. minnesota* R595 lipid A. Anti-*S. minnesota* R595 lipid A antiserum used as a conventional antibodies were obtained by intravenous injections of the *S. minnesota* R595 lipid A antigen, prepared by the method of Galanos et al. (10) into domestic white rabbits (Nippon Bio-supply Center, Tokyo, Japan). Monoclonal antibodies to *S. minnesota* R595 lipid A were prepared as reported by Mashimo et al. (Jpn. J. Med. Sci. Biol. **37**:179, 1984). ddy (Shizuoka Laboratory Animal Center, Hamamatsu) or BALB/c (Charles River Japan, Tokyo) donor mice were immunized with the *S. minnesota* R595 lipid A antigen on a schedule consisting of three intravenous injections given at 6-day intervals, as reported by Mashimo et al. (J. Mashimo, S. Arata, K. Egawa, N. Kasai, K. Okuda, and Y. Aihara, Jpn. J. Bacteriol. **38**:389, 1983). The antibody titer of sera was determined by ELISA, and animals with a high titer were selected as donors for cell fusion 3 days after the last booster. Spleen cells were fused with a BALB/c-derived myeloma cell line SP210Ag14, and the hybridoma clone producing lipid A antibody was prepared (1). The selection of hybridoma was made by *S. minnesota* R595 lipid A-ELISA system. The hybridomas that secreted anti-lipid A antibodies were then cloned twice by limiting dilution. Mouse ascites fluid produced by injecting the hybridoma line into pristane-primed BALB/c mice was used as monoclonal antibody. Four monoclonal antibodies, 5G (immunoglobulin G2b [IgG2b]) 36G (IgG3), 161 M (IgM), and 1-9M (IgM), thus obtained were used.

ELISA and ELISA inhibition. The serological assays were performed in microtitration plates (Linbro/Titertek; Flow Laboratories, Inc., McLean, Va.) by a modification described by Arata et al. (S. Arata, M. Sasaki, J. Mashimo, and N. Kasai, Jpn. J. Bacteriol. **37**:351, 1982) of the method of Engvall et al. (3). Goat and sheep antibodies (Cappel Laboratories, Cochranville, Pa.) to rabbit IgG (γ-chain specific), mouse IgG (γ-chain specific), and mouse IgM (μ-chain specific) were conjugated to alkaline phosphatase type VII-S (Sigma Chemical Co., St. Louis, Mo.) for these assays.

Each of the reagents mentioned below was added in 0.2-ml volumes to each well. In ELISA, the wells were coated with either synthetic or natural preparations at a dose of 2 μg/0.2 ml for 5 h at 37°C and overnight at 4°C. The wells were washed with physiological saline containing 0.05% Tween 20 and were reacted with serial dilutions of conventional or monoclonal antibodies for 2.5 h at 37°C. After washing with phosphate-buffered saline–Tween 20, a 1:400 dilution of the antibody-enzyme conjugate was added, and the plates were incubated for 2.5 h at 37°C. After washing, a 0.1% *p*-nitrophenyl phosphate (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) solution in 0.05 M carbonate buffer containing 1 mM $MgCl_2$ (pH 9.8) was added to each well. Color development was terminated at 40 min by the addition of 0.05 ml of 1 N NaOH to each well. Plates were read in a microplate photometer (Corona Electric Co., Ltd., Katsuta, Japan) at 405 nm. In the preliminary experiments, the background reading in the controls, in which coating antigen, lipid A antibody, or second antibody was omitted, of less than 0.02 were obtained. The antibody titer was expressed as the highest serum (ascites fluid) dilution giving a reaction of 0.1 by determining the optical density at 405 nm (OD_{405}). In addition, since the antibody titer varies significantly by the use of different lots of antibody-enzyme conjugates, the same lots of conjugates were used throughout the study. Usually, the results were averaged over two or three individual experiments because of the limited amounts of synthetic compounds. The mean ± standard deviations of the antibody titers of monoclonal antibodies 5G, 36G, 161M, and 1-9M and conventional antibody 91, when the ELISA wells were coated with strain R595 lipid A as a reference, were 204,000 ± 13,000, 25,000 ± 6,700, 59,000 ± 4,500, 724, 000 ± 46,000, and 550,000 ± 67,000, respectively.

In the ELISA inhibition test, strain R595 free lipid A was employed as the coating antigen. A fixed dilution of lipid A antibodies was preincubated for 30 min at 37°C with an equal volume of inhibiting ligands in different concentrations. Each 0.2 ml of these mixtures was then placed in strain R595 lipid A-coated wells and incubated for 2.5 h at 37°C. After washing, the standard ELISA procedures were followed again. The percent inhibition of binding was determined as (OD_{405} without inhibitor − OD_{405} with inhibitor/OD_{405} without inhibitor) × 100. The 50% inhibitory value was expressed as the micrograms per well of a ligand needed to obtain a 50% decrease in the optical density as compared with the control wells to which no inhibitor was added. The mean ± standard deviations of 50% inhibitory values of strain R595 lipid A were tested against the same lipid A preparation with antibodies 5G, 36G, 161M, 1-9M, and 91 were 0.16 ± 0.02, 0.15 ± 0.04, 0.24 ± 0.04, 0.08 ± 0.02, and 0.12 ± 0.03, respectively.

RESULTS

Antigenic reactivity of synthetic disaccharide analogs. The antibody titers of mouse monoclonal and rabbit conventional antibodies determined by ELISA with synthetic disac-

TABLE 2. Antigenic reactivity of synthetic lipid A analogs in ELISA inhibition with *S. minnesota* R595 lipid A-anti-lipid A systems[a]

Test antigen	Amt (μg) of test antigen needed for 50% inhibition by the following:				
	Monoclonal antibodies				Conventional IgG
	5G	36G	161M	1-9M	Rabbit 91
514	7.8	7.6	1.4	0.81	1.5
516	0.38	0.10	0.18	1.3	0.33
LA-17-PH	>10	>10	0.36	0.50	>10
LA-17-PP	>10	>10	>10	>10	>10
LA-18-PH	>10	>10	0.40	0.36	>10
LA-18-PP	>10	>10	5.0	>10	>10
506	3.8	2.6	0.06	0.35	0.22
Strain R595 lipid A	0.16	0.15	0.24	0.08	0.12

[a] Microtiter plates were coated with *S. minnesota* R595 lipid A at a dose of 2 μg/0.2 ml per well.

charide lipid A analogs and strain R595 lipid A as a coating antigen are shown in Table 1.

In the cross-reactions among synthetic preparations with monoclonal antibodies 5G and 36G, the highest antibody titers of about 450,000 were noted when compounds 516 was used as the coating antigen, while the antibody titers observed with compound 514 were about one-fifth of those with compound 516. Compounds LA-17 and LA-18, with only single acyl, not acyloxyacyl, residues, exhibited no antigenic reactivity, although low titers were noted with compound LA-17-PP. These results suggest that the structure involving the phosphate group at the C-1 position and 3-acyloxyacyl groups in the backbone structure play an essential role in the complete cross-reactivity with antibodies 5G or 36G. On the other hand, antibodies 161M and 1-9M strongly cross-reacted with compounds 514 and 516, as did strain R595 lipid A, while other compounds hardly reacted with these antibodies, except compound LA-17-PH exhibited a relatively high reactivity. In the system with conventional antibodies (rabbit 91) a high antibody titer comparable to that of strain R595 lipid A was observed with compound 516, but low titers were noted with other synthetic compounds.

In the ELISA inhibition test, compound 516 very effectively inhibited all the *S. minnesota* R595 lipid A-anti-lipid A systems, especially those with monoclonal antibodies 5G and 36G, at concentrations as low as 0.1 to 0.4 μg, as did natural lipid A (Table 2). Compound 514 exhibited lower reactivity than compound 516, except in the 1-9M antibody system. Compound 506, the most antigenically active preparation among the synthetic *E. coli*-type lipid A analogs, inhibited strain R595 lipid A-161M and -1-9M antibody systems at lower concentrations than did compound 516, while concentrations approximately 10 to 20 times higher than that of compound 516 were required for the inhibition of strain R595 lipid A-5G and -36G antibody systems. In the assays of the compounds of LA-17 and LA-18 series, only LA-17-PH and LA-18-PH were inhibitory in the systems with antibodies 161M or 1-9M; the other two compounds were virtually inactive.

Antigenic reactivity of synthetic monosaccharide analogs. The results of ELISA and the ELISA inhibition test performed with synthetic compounds 401, 408, and 410 are shown in Tables 3 and 4. In the ELISA using the plates coated with either of the synthetic compounds, titers of less than 100 were obtained. In the ELISA inhibition test, these synthetic compounds were also hardly active at doses as high as 10 μg in all the systems tested, except compound 408 exhibited weak activity when the 161M antibody was used as a test reagent.

DISCUSSION

Results of this study demonstrate that the synthetic *Salmonella*-type lipid A, compound 516, exhibits strong reactivity with monoclonal and conventional antibodies raised against the lipid A derived from *S. minnesota* R595. The reactivity was practically indistinguishable from that of the natural product, *S. minnesota* R595 lipid A. These and our previous results (15) also show that there are significant differences regarding the antigenic reactivities between the synthetic *Salmonella*-type lipid A (compound 516) and synthetic *E. coli*-type lipid A (compound 506). We have found (15) that the antigenic reactivity of the latter was lower than that of a natural *S. minnesota* R595 lipid A, although it was indistinguishable from that of a natural *E. coli* F515 lipid A in the cross-reactions with same antibody preparations used in this study. This may be due to differences in the structure of the two compounds: the amino group of the reducing end of the glucosamine disaccharide of compound 516 is substituted by the (*R*)-3-hexadecanoyloxytetradecanoyl group, while the corresponding amide-bound acyl group of the 506 is replaced by the (*R*)-3-hydroxytetradecanoyl group.

It has been suggested by Mashimo et al. (Jpn. J. Med. Sci. Biol. **37**:179, 1984) and Arata et al. (Jpn. J. Bacteriol. **39**:466, 1984) that the glycosidic and ester-linked phosphate groups

TABLE 3. Antigenic reactivity of synthetic monosaccharide analogs of lipid A in ELISA with *S. minnesota* R595 lipid A antibodies

Coating antigen[a]	Antibody titers of:				
	Monoclonal antibodies				Conventional IgG
	5G	36G	161M	1-9M	Rabbit 91
401	<100	<100	<100	<100	<100
408	<100	<100	<100	<100	<100
410	<100	<100	<100	<100	<100
Strain R595 lipid A	204,000	25,000	59,000	724,000	550,000

[a] Microtiter plates were coated with the respective synthetic and natural compounds at a dose of 2 μg/0.2 ml per well.

and ester- and amide-bound fatty acyl residues, especially 3-acyloxyacyl groups, are involved in the antigenic structure of the lipid A molecule. The important role of fatty acyl residues including the 3-acyloxyacyl group together with the glucosamine disaccharide 1,4′-bisphosphate backbone structure for the expression of antigenic reactivity of the *Salmonella*-type lipid A was also confirmed in this study. As mentioned above, compound 514, which lacks the glycosidic phosphate group, exhibited an apparently lower reactivity than compound 516 in the cross-reactions with the monoclonal antibodies 5G and 36G, although the former was most active among the three compounds that lacked the glycosidic phosphate. Compound LA-17-PP, which has tetradecanoyl groups at the C-2, C-2′, C-3, and C-3′ positions of glucosamine disaccharide 1,4′-bisphosphate, was much less active than compound 516. Similar results were observed with compound LA-18-PP, which has (*R*)-3-hydroxytetradecanoyl groups at the C-2 and C-2′ positions and tetradecanoyl groups at the C-3 and C-3′ positions. Furthermore, monosaccharide analogs (compounds 401, 408, and 410) showed no detectable activity when tested by both ELISA and the ELISA inhibition test, although compound 408 exhibited weak reactivity in the ELISA inhibition test. In addition to these observations, we have found (15) that a synthetic counterpart of a biosynthetic precursor of *Salmonella*-type lipid A, compound 406, which has the same (*R*)-3-hydroxytetradecanoyl group at positions C-2, C-2′, C-3, and C-3′ of the glucosamine disaccharide 1,4′-bisphosphate (Fig. 1) exhibited apparently low reactivity compared with that of compound 506 in the cross-reactions with monoclonal antibodies 5G, 36G, and 161M. These results suggest that the complete structure, including both phosphate groups at the C-1 and C-4′ positions and 3-acyloxyacyl groups at the C-2, C-2′, and C-3′ positions of the disaccharide, are required for the expression of the serological specificity of *Salmonella*-type lipid A. The important roles of phosphate groups and fatty acyl residues were also suggested by the assays with conventional antibodies. However, the differences between compounds 516 and 506 in the reactivity with monoclonal antibodies could not be detected by these polyclonal antibodies.

Differences in physical properties such as solubility or micellar state are known to influence the expression of biological activities of lipid A. Therefore, the decreased serological reactivity of compounds 514, LA-17-PH, and LA-18-PH, as described above, might result from their low solubility in the aqueous system in ELISA or the ELISA inhibition test. From this consideration, tests should be performed to demonstate that the material, even highly soluble compounds, coats the ELISA wells.

In connection with this problem, it is assumed that different assay systems also may affect the efficiency of expression of serological activity of such lipid A analogs. By passive hemolysis and passive hemolysis inhibition assays, Galanos et al. (6) recently have shown that synthetic analogs of compound 406 and compounds 404 and 405, which have only one phosphoryl group at the C-4′ and C-1 positions, respectively, express antigenicity comparable to that of compound 406 or natural lipid A, indicating that phosphate groups play no role. More recently, Galanos et al. (8) have reported that compound 506 exhibits the same antigenic reactivity with natural *Salmonella*-type lipid A. On the other hand, Kanegasaki et al. (14) have reported that compounds 404, 405, and 406 exhibit very strong and comparable reactivity to that of natural *Salmonella* lipid A by the serological assays employing liposomal systems in which lipid A and synthetic analogs were introduced into liposomes containing a fluorescent probe. Since monophosphorylated compound 405, which has a phosphate group at the C-1 position, showed stronger reactivity than the other compounds in the assay, these investigators suggest that phosphate groups, especially at the C-1 position of the disaccharide, contribute to the antigenic reactivity in liposomal systems. The discrepancies among our results and those of Galanos et al. (6, 8) and Kanegasaki et al. (14) may be due to differences in antibody preparations and serological system used in the assays.

TABLE 4. Antigenic reactivity of synthetic monosaccharide analogs of lipid A in ELISA inhibition with *S. minnesota* R595 lipid A-anti-lipid A systems[a]

Test antigen	Amt (μg) of test antigen needed for 50% inhibition by the following:				
	Monoclonal antibodies				Conventional IgG
	5G	36G	161M	1-9M	Rabbit 91
401	>10	>10	>10	>10	>10
408	>10	>10	2.1	>10	>10
410	>10	>10	>10	>10	>10
Strain R595 lipid A	0.16	0.15	0.24	0.08	0.12

[a] Microtiter plates were coated with *S. minnesota* R595 lipid A at a dose of 2 μg/0.2 ml per well.

Results of the present study reconfirm that the serological specificity between monoclonal antibodies 161M or 1-9M and 5G and 36G are apparently different; the latter seems to be considerably more specific for *Salmonella*-type lipid A, while the former recognizes a part or a common structure of lipid A. This impression has been indicated previously by results of studies of Mashimo et al. (Jpn. J. Med. Sci. Biol. **37**:179, 1984) and Arata et al. (Jpn. J. Bacteriol. **39**:466, 1984), in which cross-reactivity among natural lipid A from various species or degradation products with these monoclonal and conventional antibodies were examined. The distinct reactivity between the monoclonal antibodies 161M and 5G has also been demonstrated (S. Arata, A. Hashimura, and N. Kasai, unpublished data) by the serological analysis of *Salmonella*-free lipid A components by a thin-layer chromatographic-ELISA technique described by Mattsby-Baltzer and Alving (20). Results of our experiments (N. Kasai, S. Arata, J. Mashimo, A. Hasegawa, and M. Kiso, manuscript in preparation) in which antigenic reactivity of a number of monosaccharide lipid A analogs were tested with monoclonal antibodies, especially 161M, suggest that the chemical structure of hydrophilic and hydrophobic regions of lipid A were involved in determining the serological specificity.

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