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The Immunogenicity and Antigenicity of Lipid A Are Influenced by Its Physicochemical State and Environment

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We investigated the immunogenicity and antigenicity of synthetic lipid A and partial structures thereof. Included in the study were compounds which varied in the position of phosphate (1-mono-, 4′-mono-, and 1,4′-bisphosphates) and in the acylation (type, number, and distribution of fatty acids) and, in the case of monosaccharide compounds, the nature of the backbone sugar (D-glucosamine, D-glucose, 3-amino-3-deoxy-D-glucose, and 2,3-diamino-2,3-dideoxy-D-glucose). With the aid of the passive-hemolysis and passive-hemolysis-inhibition assays and by absorption experiments, five distinct antibody specificities were detected in polyclonal rabbit antisera raised against sheep erythrocyte-coated lipid A and lipid A incorporated into the membrane of liposomes (liposome-incorporated immunogens). Three antibody specificities reacted with disaccharide antigens specific for a 1-mono-, 4′-mono-, and 1,4′-bisphosphorylated β-1,6-linked D-glucosamine disaccharide. Two antibodies reacted with either 1- or 4-phosphates of acylated D-*gluco*-configured monosaccharides and exhibited no cross-reaction with each other. However, they cross-reacted with disaccharide antigens with phosphate groups in the appropriate positions. We found that the physicochemical state and the environment of lipid A modulated its immunoreactivity. The immunogenicity was best expressed by erythrocyte-coated and liposome-incorporated immunogens. The antigenicity of lipid A was also greatly influenced by its physical surroundings. The reaction pattern of the above antibodies was highly specific in the hemolysis assay and in absorption experiments (the antibody reacted with antigen embedded in a cell membrane), whereas some cross-reactivities were observed in inhibition studies (the antibody reacts with antigen in aqueous solution). By using liposome-incorporated antigens as inhibitors, nonspecific reactions were avoided and specific ones were enhanced. Thus the antibodies described above against lipid A recognize epitopes in the hydrophilic backbone, the exposure of which depends on the intrinsic physicochemical properties of lipid A on the one hand and the physical environment on the other.

Antibodies against the lipid A component of bacterial lipopolysaccharide have attracted our interest for several reasons. The chemical structure of lipid A is particularly well conserved during bacterial evolution and exhibits a similar architecture in different gram-negative genera and families (30). Thus, antibodies to lipid A cross-react with lipid A from various bacterial sources (10). In addition, lipid A is the principle endotoxic component of lipopolysaccharide (20). This was proven by the determination of its chemical structure (34) and its complete chemical synthesis (14), which showed that the biological activities of natural and synthetic lipid A were identical (9). Therefore, antibodies against toxic lipid A could be expected to neutralize the deleterious effects of endotoxins, a finding which would in turn have enormous biomedical implications for the treatment of gram-negative infections. The few data available on the cross-protective activities of lipid A antibodies are, however, contradictory, and the potential biomedical relevance of these antibodies remains to be determined (9, 16, 17, 22, 24, 27, 31, 33).

On the other hand, we regard the immunogenicity and antigenicity of lipid A as a useful tool for studying the structure-activity or structure-function relationships of lipid A on the molecular level. The binding of lipid A to serum antibodies or surface immunoglobulin is considered here as a model for lipid A-protein interactions similar to those expected for the interactions of lipid A with other humoral or cellular protein targets.

Finally, the immunochemistry of lipid A is of particular interest. Lipid A harbors hydrophilic and hydrophobic regions and thus exhibits peculiar physicochemical properties (20, 31). Studies on the immunogenicity and antigenicity of lipid A are therefore expected to contribute to an understanding of the immunochemistry of amphiphilic glycolipids in general.

Ever since the first studies of Galanos et al. (10), it has been known that lipid A is immunogenic provided that it is present as free lipid A, i.e., after cleavage of its polysaccharide moiety. At that time, the specificity of one antibody was described which reacted similarly with all lipid A antigens regardless of the bacterial source and mode of preparation (6, 7, 10). During recent investigations, we characterized three different antibody specificities in the polyclonal antisera of rabbits immunized with natural (2) or synthetic (3) lipid A antigens. These antibodies were shown to recognize distinct epitopes, the exposure of which depended on the presence of phosphoryl residues. In these two studies it was also noted that the physicochemical properties of lipid A, e.g., its aggregation state, influence its immunochemical features. Whereas highly specific reactions were found for each of the three antibodies in the passive-hemolysis assay and in absorption experiments (in which the antigen is embedded in the membrane of erythrocytes), inhibition studies (in which the antigen is in aqueous solution) revealed a higher degree of cross-reactivity than ex-

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pected, a finding which was not understood at that time. We hypothesized that the physicochemical environment greatly influences the exposure of antigenic determinants of lipid A and thus may modulate or determine its immunoreactivity. We report here additional data that support this hypothesis.

MATERIALS AND METHODS

Synthetic disaccharide antigens. The structure and components of the synthetic disaccharide antigens discussed here are given in Fig. 1 and Table 1. Synthetic *Escherichia coli* lipid A and its 1- and 4′-dephosphoryl derivatives (compounds 506, 504, and 505, respectively) were synthesized as described previously (14, 19); they are also known as LA15-PP, LA15-PH, and LA15-HP, respectively. (In the present study the designations PP and PH refer to bisphosphorylated and 1-dephosphoryl derivatives, respectively.) Compound 516 (LA16-PP) is the synthetic counterpart of a naturally occurring hepta-acyl species of lipid A from *Salmonella minnesota* (8), and compounds 406, 404, and 405 (15) correspond to a biosynthetic precursor of lipid A (precursor Ia [13]) and its 1- and 4′-dephosphoryl derivatives (LA14-PP, LA14-PH, and LA14-HP, respectively). Synthetic compounds of the 600 series represent des-*O*-acyl derivatives of lipid A (which correspond to alkali-treated lipid A [10]) and are available as the bisphosphorylated and 1- and 4′-monodephosphoryl derivatives (compounds 606, 604, and 605, respectively). Compounds LA20-PP and LA21-PP are a synthetic penta-acyl partial structure of lipid A corresponding to another precursor of lipid A (precursor Ib [13]) and an isomer carrying the palmitoyl residue on the nonreducing glucosamine (cf. Fig. 1), respectively. Both compounds have been synthesized as the bisphosphorylated and the 1-dephosphoryl derivatives. LA17-PP and LA18-PP are artificial structures with an acylation pattern that is not yet known from natural lipid A preparations. The synthesis of bisphosphorylated and the 1-dephosphoryl derivatives was as described elsewhere (T. Shiba and S. Kusumoto, manuscript in preparation).

Synthetic monosaccharide antigens. The structure and components of the synthetic monosaccharide antigens discussed here are given in Fig. 2 and Table 2. The synthetic counterparts of the naturally occurring monosaccharide precursors lipid X and lipid Y (29), as well as the 4-phosphoryl isomer of lipid X (compounds 401, 408, and 410, respectively) were synthesized as described previously (18). The other monosaccharide derivatives listed in Fig. 2 represent lipid A monosaccharide analogs in which the patterns of acylation and phosphorylation were varied. In addition, in some of the compounds the D-glucosamine was replaced by D-glucose, 3-amino-3-deoxy-D-glucose, or 2,3-diamino-2,3-dideoxy-D-glucose, the latter representing the backbone sugar of lipid A in some photosynthetic bacteria (23). The synthesis of these compounds has been reported elsewhere (20a, 21). The compounds are referred to by their numbers in the present study.

Liposomes. Dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), distearoyl-L-α-phosphatidylcholine (DSPC), diacetylphosphate (DCP), and cholesterol were obtained from Sigma Chemical Co. (Munich, Federal Republic of Germany). Diacyl phosphatidylcholines, DCP, and cholesterol were each dissolved in chloroform-methanol (9:1 [vol/vol]) at a concentration of 10 mM and mixed in the molar ratio of 1:0.11:0.75 (1). This solution (1 ml) was added to the respective synthetic compound (500 μg [dry weight]), which was obtained by successive evaporations from water, water-methanol, and chloroform-methanol), followed by evaporation of the solvents. The residue was dissolved in chloroform-methanol (3 ml) and evaporated; this procedure was repeated twice. A

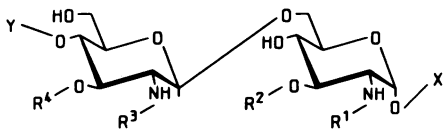

FIG. 1. Chemical structure of disaccharide lipid A antigens. See also Table 1.

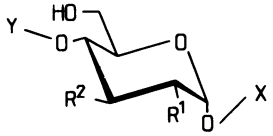

FIG. 2. Chemical structure of monosaccharide partial structures of lipid A. See also Table 2.

TABLE 1. Synthetic disaccharide antigens[a]

Compound	R, X, and Y components					
	R^1	R^2	R^3	R^4	X	Y
516	C_{14}-O-C_{16}	C_{14}-OH	C_{14}-O-C_{12}	C_{14}-O-C_{14}	P	P
506	C_{14}-OH	C_{14}-OH	C_{14}-O-C_{12}	C_{14}-O-C_{14}	P	P
505	C_{14}-OH	C_{14}-OH	C_{14}-O-C_{12}	C_{14}-O-C_{14}	P	H
504	C_{14}-OH	C_{14}-OH	C_{14}-O-C_{12}	C_{14}-O-C_{14}	H	P
LA20-PP	C_{14}-O-C_{16}	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	P
LA20-PH	C_{14}-O-C_{16}	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	P
LA21-PP	C_{14}-OH	C_{14}-OH	C_{14}-O-C_{16}	C_{14}-OH	P	P
LA21-PH	C_{14}-OH	C_{14}-OH	C_{14}-O-C_{16}	C_{14}-OH	H	P
406	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	P
405	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	H
404	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	P
606	C_{14}-OH	H	C_{14}-OH	H	P	P
605	C_{14}-OH	H	C_{14}-OH	H	P	H
604	C_{14}-OH	H	C_{14}-OH	H	H	P
LA18-PP	C_{14}-OH	C_{14}	C_{14}-OH	C_{14}	P	P
LA17-PP	C_{14}	C_{14}	C_{14}	C_{14}	P	P

[a] See structure in Fig. 1. P, Phosphoryl group.

TABLE 2. Synthetic monosaccharide antigens[a]

Compound	R, X, and Y components			
	R^1	R^2	X	Y
401	NH-C_{14}-OH	O-C_{14}-OH	P	H
408	NH-C_{14}-O-C_{16}	O-C_{14}-OH	P	H
880.319	NH-C_{14}-O-C_{14}	O-C_{14}-OH	P	H
880.421	NH-C_{14}-OH	O-C_{14}-O-C_{14}	P	H
880.201	NH-C_{14}-OH	O-C_{14}	P	H
880.244	NH-C_{14}	O-C_{14}	P	H
89.397	NH-C_{14}-OH	NH-C_{14}-OH	P	H
89.695	O-C_{14}-OH	O-C_{14}-OH	P	H
880.124	O-C_{14}-OH	NH-C_{14}-OH	P	H
410	NH-C_{14}-OH	O-C_{14}-OH	H	P
880.475	NH-C_{14}-OH	NH-C_{14}-OH	H	P
89.575	NH-C_{14}-OH	NH-C_{14}-OH	Me	P
89.589	NH-C_{14}-O-C_{14}	NH-C_{14}-O-C_{14}	Me	P

[a] See also Fig. 2. NH and O indicate amide- and ester-bound fatty acids, respectively, depending on the nature of the backbone sugar. Compounds 410 and 880.475 are mixtures of the α and β anomers; compounds 89.575 and 89.589 are β-methyl glycosides. Me, Methyl group. P, Phosphoryl group.

solution of sodium chloride (0.15 M; 1 ml) was added to the dry residue, and swelling was allowed to occur as described below until a macroscopically homogeneous suspension was obtained. In this way, antigens were incorporated into the liposomal membrane; they are referred to here as liposome-incorporated compounds. Liposomes prepared in this way had a medium size of a few micrometers, as determined by dark-field microscopy. Different particle sizes were prepared by the following procedures: (i) incubation at 55°C for 2 h with gentle agitation every 30 min, (ii) shaking in a Vortex mixer for 3 min at room temperature, and (iii) ultrasonication at room temperature (Branson microtip sonifier, model B15; 1 min at 40 W), yielding approximate particle sizes of up to 10, 1, and 0.1 μm in diameter, respectively (11).

Antisera and immunogens. Polyclonal antisera against compounds 404, 405, 406 and against 504, 505, and 506 were obtained in rabbits after immunization with the respective antigens incorporated into liposomes (3) or coated onto sheep erythrocytes (SRBC) as described earlier (4). Antisera against compound 516 were prepared only with liposome-incorporated antigens. Antisera from animals 64, 81, and 46 were as described in a previous study (3); they were prepared against liposome-incorporated compounds 504, 505, and 506, respectively. Only rabbits with no preimmunization titer against compound 506 were used for immunization.

Immunogens prepared as follows did not yield measurable antibody titers. (i) Calcium-precipitated immunogens were obtained by adding calcium chloride to a final concentration of 20 mM to a solution of the respective immunogen (2 mg/ml in water). After incubation (37°C for 30 min), the precipitate was centrifuged, washed twice in distilled water, and finally suspended in water at a concentration of 1 mg/ml. (ii) Immunogen-coated latex beads were prepared by incubating latex beads (500 μl of Bacto-Latex 0.81; Difco Laboratories, Detroit, Mich.) with the respective immunogen (250 μg in 1.0 ml of phosphate-buffered saline) at room temperature for 1 h, followed by two cycles of washing and centrifugation (10,000 × *g* for 30 min). (iii) Complexation of immunogens to bovine serum albumin was achieved by mixing equal volumes (1 mg/ml in phosphate-buffered saline) with the respective immunogen (1 mg/ml in water), followed by three cycles of evaporation and solubilization in water (1 ml).

Serological assays. The experimental details of the passive-hemolysis and passive-hemolysis-inhibition assays and of the absorption experiments have been described before (2–4). Sensitization of SRBC was performed as follows. SRBC (200 μl of packed cells) were suspended in phosphate-buffered saline (4 ml), and various amounts of antigen (1 to 200 μg at 1 mg/ml) were added. After incubation at 37°C for 30 min (suspending the settled SRBC every 10 min), the coated cells were washed three times in phosphate-buffered saline and finally suspended in Veronal-buffered saline (40 ml) to 0.5%. Optimal sensitization is defined as the amount of antigen yielding the highest antibody titer in hemolysis whereby amounts of 200 μg/200 μl of packed cells were never exceeded.

RESULTS

Immunogenicity and antigenicity of disaccharide compounds. To determine the influence of the physicochemical environment on the immunogenicity of lipid A, various synthetic lipid A preparations were injected into rabbits in the following forms: (i) as aqueous solutions, (ii) in an insoluble form after precipitation with calcium chloride, (iii) complexed with bovine serum albumin, (iv) adsorbed to polystyrene particles (latex beads), (v) adsorbed onto homologous erythrocytes or SRBC, and (vi) incorporated into liposomes as an integral part of their phospholipid matrix. The antisera obtained were tested in the passive-hemolysis assay against *E. coli*-type lipid A (compound 506) and against synthetic precursor Ia (compound 406), as well as with their 1- and 4′-monodephosphoryl derivatives. Only immunization with SRBC-coated and liposome-incorporated immunogens yielded significant antibody titers. The results are shown in Table 3, with each serum being representative for at least two independent immunizations. After immunization with liposome-incorporated antigens of the 400 series, maximal titers of only 32 were observed (data not shown in Table 3). If, however, the animals were immunized with SRBC-coated antigens, high levels of antibody were obtained, with titers of up to 2,048; similar results were seen with compounds of the 400 or 500 series used as test antigens coated onto SRBC. The opposite result was obtained with compounds of the 500 series: higher titers were observed after immunization with liposome-incorporated instead of SRBC-coated antigens (data not shown). Antisera against 404 and 504 reacted with antigens carrying a 4′-phosphate (compounds 404, 406, 504, and 506); those against 405 and 505 reacted with antigens having the 1-phosphoryl group (compounds 405, 406, 505, and 506), except for the negative reaction of anti-405 with compound 505. Antisera against 406, 506, and 516 only gave a positive reaction with bisphosphorylated antigens (i.e., compounds 406 and 506; compound 516 could not be tested as an antigen since it did not sensitize SRBC properly). The antisera were then absorbed with the same antigens used in the hemolysis assay and tested for residual reactivity. The activity of antisera against 1- and 4′-monodephosphoryl derivatives could be absorbed completely with antigens carrying 4′- or 1-phosphoryl residues, respectively, whereas the titers of anti-406, anti-506, and anti-516 antisera could be only abolished by absorption with either compound 406 or 506. Thus, anti-404 and anti-504 antibodies recognized an antigenic determinant requiring a 4′-phosphoryl residue, and anti-405 and anti-505 reacted with a 1-phosphate-associated determinant, whereas anti-406, anti-506, and anti-516 antibodies were specific for an epitope only expressed by antigens containing the 1,4′-bisphosphorylated β-1,6-linked D-glucosamine disaccharide.

TABLE 3. Hemolytic antibody titers in rabbits after immunization with synthetic disaccharide antigens

Animal no.	Immunogen compound[a]	Hemolytic titer against SRBC coated with compound[b]:					
		404	405	406	504	505	506
73	404 (SRBC)	128	<4	256	64	<4	256
75	405 (SRBC)	<4	32	32	<4	<4	64
61	406 (SRBC)	8	16	2,048	32	32	1,024
64	504 (L)	1,024	<4	2,048	1,024*	<4*	2,048*
81	505 (L)	<4	1,024	2,048	<4*	1,024*	1,024*
46	506 (L)	16	64	4,096	16*	128*	2,048*
116	516 (L)	16	16	512	16	16	256

[a] The indicated compounds were absorbed onto SRBC or incorporated into liposomes (L).
[b] *, Data obtained from reference 3.

To provide further evidence for the phosphate-restricted specificity of these antibodies, three groups of synthetic antigens were tested for their SRBC-coating efficiency (optimal sensitization) and for their interaction with antibodies in the passive-hemolysis assay. The antigens used were 1-

TABLE 4. Reactivity of three different antibody specificities with synthetic antigens with different acylation patterns

Sensitizing antigen (compound)	Amt (μg) of antigen/200 μl of SRBC	No. of acyl chains	Hemolytic antibody titer obtained with[a]:		
			Anti-504	Anti-505	Anti-506
4′-Phosphates					
504	200	6	1,024	<4	16
LA20-PH	200	5	1,024	<4	<4
LA21-PH	200	5	1,024	<4	<4
404	5	4	1,024	<4	<4
604	5	2	2,048	<4	128
1-Phosphates					
505	200	6	<4	1,024	128
405	10	4	<4	1,024	64
605	10	2	<4	1,024	128
1,4-Bisphosphates					
506	100	6	2,048	1,024	2,048
LA20-PP	200	5	2,048	1,024	2,048
LA21-PP	100	5	2,048	1,024	2,048
LA17-PP	200	4	1,024	1,024	512
LA18-PP	200	4	512	2,048	2,048
406	5	4	2,048	2,048	4,096
606	10	2	1,024	256	512

[a] Anti-504, anti-505, and anti-506 antisera were from animals 64, 81, and 46, respectively (see Table 3).

and 4′-monodephosphoryl derivatives and bisphosphorylated compounds with the same hydrophilic backbone but with different acylation patterns (type, number, and distribution of fatty acids). The results obtained with anti-504, anti-505, and anti-506 antisera are listed in Table 4. The amount of antigen needed for optimal sensitization varied: compounds with five and six fatty acids required 100 to 200 μg/200 μl of packed cells, whereas preparations with only two or four acyl residues yielded optimal results with 5 to 10 μg. With compounds LA17-PP and LA18-PP, 200 μg was necessary for optimal sensitization even though they contained only four acyl residues. However, in contrast to compound 406, in LA17-PP the two ester-linked 3-hydroxymyristic acid residues were replaced by myristic acid, and in LA18-PP all four fatty acids were nonhydroxylated myristic acid, which obviously led to an increased hydrophobicity. Notably, the hemolytic titers obtained with optimally sensitized SRBC did not show significant differences and the phosphate-related specificity was not affected at all. Comparable data were obtained with antisera against compounds of the 400 series (data not included in Table 4).

We knew from our previous work (2–4) that inhibition experiments (using the inhibitor in a free aqueous solution) may lead to unexpected cross-reactions, which we attributed to the conformational state (supramolecular organization) of lipid A in solution, that could be different from that of lipid A embedded in a cell membrane. To test this hypothesis, the bisphosphorylated compounds listed in Table 5 were tested as native antigens (solubilized in water) and after incorporation into liposomes for their capacity to inhibit the 506–anti-506 hemolytic system. These antigens were chosen because they all had both phosphate groups but differed in their substitutions with fatty acids. The 506–anti-506 hemolytic system was used since 506-specific antibodies exhibit the highest specificity. The results are shown in Table 5. Whereas the inhibition values for the native antigens varied from 2 to 500 ng, they were identical and significantly lower (0.5 to 1 ng) when antigens were incorporated into liposomes. The phase transition temperatures (melting point of the hydrocarbon chains) of these compounds were determined by infrared spectroscopy (26) as an objective physicochemical parameter for the anticipated conformational changes of the antigens in solution and in liposomes. Although the native preparations exhibited phase transition temperatures of between 18 and 50°C, they yielded similar values (35 to 40°C) after incorporation into liposomes containing DMPC (data not shown).

TABLE 5. Inhibition values of native and liposome-incorporated synthetic bisphosphorylated disaccharide antigens in the hemolytic antigen-antibody system of 506–anti-506

Inhibitor (compound)	Inhibition value (ng) obtained with:	
	Native antigen	Liposome-incorporated antigen[a]
516	32	0.5
506	8	0.5
LA20-PP	8	1
LA21-PP	8	1
406	2	0.5
606	2	1
LA18-PP	125	0.5
LA17-PP	500	0.5

[a] These liposomes contained DMPC.

Liposomes were thus prepared which varied in size and composition in order to evaluate the influence of fluidity of the liposomal membranes on the exposure of antigenic determinants. Compound 506 was incorporated as a model substance and tested for its ability to inhibit anti-506 antiserum in the passive-hemolysis assay. When the antigen was present in small (0.1-μm) liposomes, lower inhibition values (2 to 8 ng) were observed compared to those seen with larger (10-μm) liposomes (16 to 32 ng). Medium-sized liposomes (1 μm) resulted in intermediate inhibition values of 8 to 16 ng. Different inhibition values were also obtained with liposomes with different lipid compositions. If vesicles of the same size but with different compositions were used as a matrix for compound 506, those with a higher fluidity (i.e., shorter acyl chains in diacyl phosphatidylcholine [myristoyl, palmitoyl, and stearoyl]) exhibited more efficient inhibition. Obviously, the smaller radius of curvature on the one hand and the higher fluidity on the other favored the exposure of the hydrophilic backbone of lipid A incorporated into the liposomal membrane (Table 6).

Immunogenicity and antigenicity of monosaccharide compounds. Immunization of rabbits with SRBC-coated monosaccharide compounds (401, 408, and 410) yielded low titers of antisera (32 to 64), whereas no immune response was

TABLE 6. Influence of size and fluidity of liposomes on the antigenicity of incorporated synthetic lipid A (compound 506)

Liposome size (μm)[a]	Inhibition value (ng) obtained with compound 506 incorporated into liposomes with[b]:		
	DMPC (C_{14})[b]	DPPC (C_{16})	DSPC (C_{18})
Small (0.1)	2	4	8
Medium (1)	8	16	16
Large (10)	16	16	32

[a] Based on the approximate upper diameter.

[b] Inhibition values were determined using the 506–anti-506 hemolytic system. To prepare the liposomes, the indicated diacyl phosphatidylcholine, DCP, cholesterol, and compound 506 were combined in the molar ratio of 1:0.11:0.75:0.003. The nature of the acyl residues in the respective diacyl phosphatidylcholine is indicated in parentheses.

TABLE 7. Hemolytic antibody titers of rabbit antisera against monosaccharide antigens

Animal no.	Immunogen compound[a]	Hemolytic antibody titer against SRBC coated with compound:	
		401	410
64	504	<10	1,280
65	504	<10	320
79	504	<10	320
66	505	640	40
67	505	320	80
81	505	1,280	80
82	505	640	20
46	506	640	20

[a] Liposome-incorporated immunogens were used.

observed with liposome-incorporated immunogens. However, during these experiments we observed that antisera against disaccharide antigens also reacted with monosaccharide antigens. As shown below, this reactivity was due to the presence of a second antibody specificity and not to a cross-reaction of disaccharide antibodies with monosaccharide antigens. We screened a total of 45 anti-lipid A antisera in the passive-hemolysis assay for the presence of such monosaccharide-specific antibodies by using compounds 401 and 410 as representatives of the 1- and 4-phosphate specificities, respectively. The screening also included antisera against acid-treated (hydrochloric or acetic acid [3]) Re-mutant *S. minnesota*, *Proteus mirabilis*, and *E. coli*. None of 19 antisera against acid-treated bacteria had detectable titers against compound 401, and only two of them exhibited reactivity (titers of 160 and 320) to compound 410. Of the antisera against synthetic compounds, only those which had been elicited with liposome-incorporated antigens displayed titers of ≥320 (Table 7). Antisera against preparation 504 only reacted with the 4-phosphoryl lipid A partial structure 410 and not with compound 401. On the other hand, antisera against compounds 505 and 506 exhibited titers of up to 1,280 against the 1-phosphoryl compound 401 but only marginal titers against compound 410. The data suggested that these reactivities could be ascribed to the presence of antibodies recognizing an antigenic determinant which required the presence of a 1- or a 4-phosphate group, respectively, for its expression in addition to the acylated glucosamine monosaccharide residue. The antisera from animals 64, 81, and 46 were selected for further experiments designed to sustain the phosphate-restricted reaction pattern. The antisera were tested against different synthetic antigens, all of which contained a *gluco*-configured monosaccharide backbone sugar but which carried either a 1- or a 4-phosphoryl residue and, in addition, differed in the acylation pattern (Fig. 2 and Table 2). Each antiserum was titrated against SRBC coated with various amounts of antigen. The optimal amounts of antigen to sensitize SRBC and the antibody titers so obtained are listed in Table 8. Independent of the acylation pattern and the backbone sugar (D-glucosamine, D-glucose, 3-amino-3-deoxy-D-glucose, or 2,3-diamino-2,3-dideoxy-D-glucose), anti-505 and anti-506 reacted with 1-phosphoryl compounds, whereas anti-504 reacted only with 4-phosphate antigens, except for the reaction of anti-504 with compound 880.244 (titer of 320).

To test whether the reactivity with monosaccharide antigens was attributable to separate antibody specificities present in antisera or to a cross-reaction of disaccharide antibodies with monosaccharide antigens, absorption experiments were performed (Table 9). Absorption of anti-504

TABLE 8. Hemolytic antibody titers of anti-504, anti-505, and anti-506 antisera with 1- and 4-phosphate monosaccharide antigens

Sensitizing antigen (compound)	Amt (μg) of antigen/ 200 μl of SRBC	No. of acyl chains	Hemolytic antibody titer obtained with antiserum[a]:		
			Anti-504	Anti-505	Anti-506
1-Phosphates					
880.201	20	2	20	1,280	1,280
880.244	60	2	320	2,560	1,280
89.397	80	2	<10	1,280	640
89.695	40	2	40	1,280	640
880.124	40	2	40	320	320
880.319	200	3	40	1,280	640
880.421	200	3	<10	640	320
401	10	2	<10	640	640
408	200	3	<10	1,280	640
4-Phosphates					
410	5	2	640	10	20
880.475	40	2	640	80	160
89.575	40	2	1,280	<10	160

[a] Anti-504, anti-505, and anti-506 antisera were from animals 64, 81, and 46, respectively (see Table 3).

with compound 401 or 505 (both lacking a 4-phosphoryl group) did not reduce the reactivity with compounds 410, 504, and 506. Absorption with compound 410 completely abolished the titer of antibody against the monosaccharide compound 410 but not those of antibodies against disaccharide compound 504 or 506. Absorption with compound 504 or 506 resulted in the loss of both hemolytic activities. These data show that anti-504 antiserum contained two distinct antibody specificities, one of which was specific for a disaccharide antigen and the other for a monosaccharide antigen. Both antibodies required the presence of a 4-phosphoryl group. Since the monosaccharide antibody could be ab-

TABLE 9. Hemolytic antibody titers of anti-504, anti-505, and anti-506 after absorption with mono- and disaccharide antigens

Antiserum[a]	Hemolytic titer against SRBC coated with compound:				
	401	410	504	505	506
Anti-504					
Before absorption	<4	512	1,024	<4	2,048
Absorbed 401	<4	512	1,024	<4	2,048
Absorbed 410	<4	<4	512	<4	1,024
Absorbed 504	<4	<4	<4	<4	<4
Absorbed 505	<4	512	1,024	<4	1,024
Absorbed 506	<4	<4	<4	<4	<4
Anti-505					
Before absorption	512	8	<4	1,024	2,048
Absorbed 401	<4	<4	<4	512	1,024
Absorbed 410	512	<4	<4	1,024	2,048
Absorbed 504	512	<4	<4	1,024	2,048
Absorbed 505	<4	<4	<4	<4	<4
Absorbed 506	<4	<4	<4	<4	<4
Anti-506					
Before absorption	512	16	16	128	2,048
Absorbed 401	<4	<4	<4	<4	2,048
Absorbed 410	512	<4	<4	64	2,048
Absorbed 504	512	<4	<4	128	1,024
Absorbed 505	<4	<4	<4	<4	1,024
Absorbed 506	<4	<4	<4	<4	<4

[a] The antisera were from animals 64, 81, and 46, respectively (see Table 3).

TABLE 10. Inhibition values of native and liposome-incorporated synthetic monosaccharide antigens

Inhibitor (compound)	No. of acyl chains	Inhibition value (ng) obtained in the hemolytic antigen-antibody system[a]:			
		410–Anti-504		401–Anti-505	
		Native antigen	LI antigen	Native antigen	LI antigen
1-Phosphates					
880.201	2	500	>1,000	63	16
880.244	2	250	500	63	16
89.397	2	>1,000	1,000	125	63
89.695	2	>1,000	>1,000	125	63
880.124	2	250	1,000	63	63
880.319	3	500	500	125	8
880.421	3	250	250	125	4
401	2	500	500	32	8
408	3	1,000	250	125	16
4-Phosphates					
410	2	16	8	250	125
880.475	2	500	250	1,000	500
89.575	2	125	32	>1,000	500
89.589	4	500	4	>1,000	1,000

[a] Anti-504 and anti-505 antisera were from animals 64 and 81, respectively (see Table 3). LI, Liposome-incorporated.

sorbed with compound 504 and 506, it cross-reacted with disaccharide antigens containing a 4-phosphorylated D-glucosamine.

Anti-505 and anti-506 antisera behaved similarly with regard to their monosaccharide reactivities, both exhibiting a titer of 512 against the 1-phosphoryl compound 401. Absorption of anti-505 and anti-506 antiserum with compound 401 removed the monosaccharide-reactive antibodies completely without affecting the titers of antibodies against the disaccharide antigens 504 and 506, respectively, whereas absorption with compounds 410 and 504 (both of which lack a 1-phosphoryl group) had no effect on either activity. Since anti-505 reacts with the disaccharide antigens 505 and 506, this activity could be absorbed with one of both antigens, whereas the disaccharide-specific antibodies in anti-506 could only be absorbed with compound 506 (3). These results indicate that anti-505 and anti-506 antisera contain, in addition to their respective disaccharide-reactive antibodies, monosaccharide-reactive antibodies which recognize an antigenic determinant requiring the presence of a 1-phosphate. As described above for the 4-phosphate-reactive monosaccharide antibody, the antibody reacting with 1-phosphate monosaccharide antigens also exhibited cross-reaction in absorption with disaccharide antigens carrying a 1-phosphoryl group.

The monosaccharide antigens of Fig. 2 were then tested in the passive-hemolysis-inhibition assay to further characterize their specificity. Two antigen-antibody systems were used, with 410–anti-504 and 401–anti-505 being specific for 4- and 1-phosphate-containing antigens, respectively. The inhibitors were used as aqueous solutions or incorporated into liposomes. The results are shown in Table 10. Antigens carrying a 1-phosphate did not inhibit the 4-phosphate-specific system (inhibition values between 250 and >1,000 ng) regardless of whether native or liposome-incorporated antigens were used. In the 1-phosphate-specific system, native antigens exhibited inhibition values between 32 and 125 ng. However, if the antigens were incorporated into liposomes, lower inhibition values (4 to 63 ng) were obtained. This behavior was more pronounced with antigens carrying three acyl residues than with those having only two fatty acids.

The 4-phosphate antigens were also strictly specific for the homologous hemolytic system (410–anti-504), and again liposome-incorporated antigens were better inhibitors than native antigens. With compound 89.589, which has four acyl residues, a 125-fold increase in inhibitory capacity was observed after incorporation into liposomes.

DISCUSSION

We have previously shown that the immunization of rabbits with synthetic lipid A antigens yields three different antibody specificities which recognize distinct antigenic determinants of lipid A (3). These three antibodies recognize the hydrophilic lipid A disaccharide backbone, their epitope specificities being determined by the phosphorylation pattern. Antibodies against the lipid A partial structures 504 and 505 (1- and 4′-monodephosphoryl derivatives, respectively) exhibit no cross-reaction with each other but cross-react with the 1,4′-bisphosphorylated compound 506 (synthetic lipid A) in a one-way pattern. Thus, immunization with monophosphoryl compounds elicits antibodies which react with the homologous antigen and with compound 506, whereas immunization with preparation 506 leads exclusively to the production of antibodies against the homologous antigen (compound 506). Therefore, it is evident that phosphate plays an important role in determining the immunoreactivity of lipid A.

During these earlier studies we noted that the physicochemical properties of lipid A greatly influenced its behavior as an immunogen and as an antigen in serological assays. Thus, reactions of high specificity were observed in the passive-hemolysis test and in absorption experiments, whereas inhibition assays led to unexpected cross-reactions which were not understood at that time. Since the biological properties of the amphipathic lipid A molecule greatly depend on its physicochemical state and environment, we hypothesized that lipid A in aqueous solution behaves in a way that is serologically different from lipid A embedded in a membrane, e.g., SRBC. To test this hypothesis, we examined here the immunogenicity and antigenicity of synthetic lipid A with special emphasis on its physicochemical environment.

First, we tested in rabbits the immunogenicity of lipid A preparations either in aqueous solution or coated to homologous or heterologous erythrocytes, adsorbed to polystyrene particles (latex beads), complexed with bovine serum albumin, and incorporated into liposomes. The best immune responses were obtained with SRBC-coated and liposome-incorporated antigens. It was found that lipid A preparations containing four fatty acids (synthetic precursor Ia) induced a stronger immune response when coated onto SRBC, whereas compounds with six and seven fatty acids (synthetic *E. coli* and *Salmonella* hepta-acyl lipid A, respectively) yielded higher antibody titers as liposome-incorporated immunogens (Table 3). Regardless of the type of immunogen, the phosphate-restricted reaction patterns of antibodies thus obtained were similar for all antisera.

We then tested whether the antigen coated to SRBC as used in the passive-hemolysis assay had an influence on the specificity of the reaction. Using a series of synthetic compounds which possess the same hydrophilic backbone but have different acylation patterns, we demonstrated that the different degrees of hydrophobicity indeed influenced the

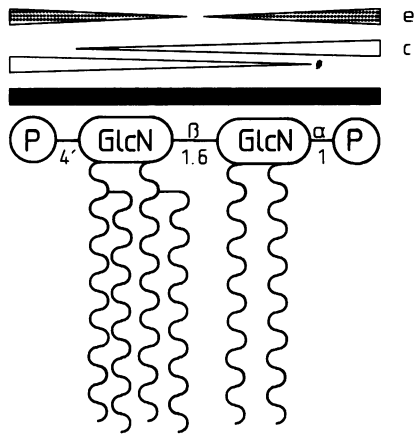

FIG. 3. Schematic representation of the proposed specificities of disaccharide (a to c)- and monosaccharide (d and e)-reactive antibodies. See also Table 11.

TABLE 11. Specificities of disaccharide- and monosaccharide-reactive antibodies[a]

Recognized hydrophilic backbone structure	Reactivity of antibody specificity:				
	a	b	c	d	e
GlcN-1-P	−	−	−	−	+
4-P-GlcN	−	−	−	+	−
GlcN-β-1,6-GlcN-1-P	−	−	+	−	+
4′-P-GlcN-β-1,6-GlcN	−	+	−	+	−
4′-P-GlcN-β-1,6-GlcN-1-P	+	+	+	+	+

[a] See also Fig. 3. Columns a to c, disaccharide reactive; d and e, monosaccharide reactive. Abbreviations: GlcN, D-glucosamine; P, phosphoryl group.

behavior of these antigenic compounds but not their specificity (Table 4): the greater the hydrophobicity (higher number of acyl chains or replacement of hydroxylated by nonhydroxylated fatty acids), the higher the amounts of antigen needed for optimal sensitization. With compound 516 (which has seven fatty acids), an efficient sensitization could not be achieved without damaging the erythrocytes.

We knew from our previous work that unexpected cross-reactions occur in the passive-hemolysis-inhibition assay when lipid A is used in aqueous solution, and we had attributed this phenomenon to the different physicochemical states and environments of the antigens. The postulated influence of the physicochemical environment on the antigenicity of lipid A was verified by the following approach (Table 5). Synthetic lipid A antigens which shared the same hydrophilic backbone (1,4′-bisphosphorylated glucosamine disaccharide) but varied in the number, type, and distribution of fatty acids were tested as native and liposome-incorporated antigens in the passive-hemolysis-inhibition assay for their reaction with anti-506 antiserum. The results showed that the epitopes residing in the hydrophilic region of lipid A were not properly—or not at all—exposed in water-solubilized antigens but became amenable to the interaction with antibody after incorporation into liposomes. Further evidence for this interpretation was provided by inhibition experiments using liposomes of different size and lipid compositions leading to different fluidities of the liposomal membranes. Small, high-fluidity liposomes favored the exposure of hydrophilic antigenic determinants. The serological data were paralleled by results obtained from measurements of the phase transition temperatures of water-solublized and liposome-incorporated antigens.

Another important finding was the detection of antibodies reacting with monosaccharide partial structures of lipid A. Since these antibodies were found together with those against disaccharide antigens, they were detected only by absorption experiments in which these antibodies were removed without affecting the respective disaccharide antibodies. Two different monosaccharide-specific antibodies were detected which distinguished between 1- and 4-phosphorylated monosaccharide antigens. Interestingly, these antibodies exhibited a cross-reactivity similar to those against disaccharide partial structures of lipid A, i.e., anti-504 and anti-505 antisera. As with these antisera, monosaccharide antibodies also cross-reacted with more complex (disaccharide) structures, provided they contained phosphate groups in the appropriate position. Many synthetic monosaccharide compounds are now available which differ in acylation pattern (type, number, and distribution of fatty acids), position of the phosphate group, and nature of the backbone sugar (see Fig. 2 and Table 2). The use of these compounds in the passive-hemolysis-inhibition assay and in absorption experiments showed that the reactivity of monosaccharide lipid A partial structures with the antibodies used was determined exclusively by the position of the phosphoryl residue (C-1 or C-4) and did not depend on either the acylation pattern or the nature of the D-*gluco*-configured backbone sugar. However, the exposure of the hydrophilic determinants was greatly influenced by the hydrophobicity of the compounds and, as shown above for disaccharide antigens, by the physicochemical environment (Table 10).

The appearance of monosaccharide antibodies in the antisera of rabbits immunized with disaccharide antigens was unexpected, particularly since all attempts to raise such antibodies by immunization with monosaccharide antigens had failed (unpublished observations). Antibodies reacting with the 1-phosphoryl compound 401 (lipid X) were only detected in antisera against the disaccharide compounds 506 and 505, whereas those against the 4-phosphate isomer (compound 410) were observed in antisera obtained after immunization with the disaccharide compound 504. Interestingly, both types of monosaccharide antibodies were only obtained after immunization with liposome-incorporated antigens. Although we have no direct experimental proof, we hypothesize that the production of monosaccharide antibodies results from the in vivo degradation of lipid A leading to immunogenic monosaccharide structures. The degradation of lipid A (deacylation and dephosphorylation) in granulocytes (12), in macrophages (25, 28), and in vivo (5) has been described. It will be interesting to determine the substrate specificities of these enzymes in order to explain why different disaccharide compounds are degraded in different ways leading to the production of either 4- or 1-phosphate-specific monosaccharide antibodies (in addition to the respective disaccharide antibodies). The fact that only liposome-incorporated immunogens yielded monosaccharide antibodies indicates the importance of the physicochemical environment of lipid A for its cellular uptake and distribution and for the action of enzymes on this amphipathic molecule.

From this and our previous work on the immunogenicity and antigenicity of lipid A, the following conclusions can be drawn. (i) The immune response against natural and synthetic lipid A is diverse. Polyclonal rabbit antisera against lipid A contain not one but several antibody specificities. Up to now, we have characterized five such specificities, all of

which recognize hydrophilic epitopes residing in the phosphorylated glucosamine disaccharide backbone of lipid A. One of these (anti-506) has the most restricted specificity since it reacts only with 1,4'-bisphosphorylated disaccharide antigens, whereas the others react with monodephosphoryl and monosaccharide partial structures thereof. These partial structures are also recognized in more complex (disaccharide) structures with the appropriate phosphorylation pattern leading to a one-way cross-reaction. Among these, two monosaccharide- and two disaccharide-reactive antibodies were found. The specificity of these antibodies is displayed schematically in Fig. 3 and Table 11. We emphasize that the usual test antigen in lipid A serology, i.e., the 1,4'-bisphosphorylated compound, cannot differentiate between mono- and disaccharide antibodies and that monosaccharide-reactive antibodies can be only detected by using monosaccharide structures as test antigens. We also note that attempts to obtain these antibody specificities as murine monoclonal antibodies have failed and, up to now, we have only been able to raise those of the 506 specificity (unpublished observation). The reason for this failure can be seen in the particular immunogenic properties of lipid A for mice (6, 7).

(ii) The immunoreactive properties of lipid A depend on its physicochemical environment. Even if the structural elements of an antigenic determinant are present in a given lipid A antigen, they may not be exposed properly for recognition by an antibody. This observation may help to explain the interaction of lipid A with humoral or cellular targets, an interaction that results in the manyfold and diverse biological effects of endotoxins. Our investigations on this aspect of lipid A led us to the assumption that, in addition to certain structural requirements, a unique conformation endows lipid A with its characteristic biological features (32). This idea is strongly supported by the results presented here, and we are currently investigating the effect and activity of liposome-incorporated synthetic lipid A preparations in other biological systems.

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