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A Nonsubstituted Primary Hydroxyl Group in Position 6′ of Free Lipid A Is Required for Binding of Lipid A Monoclonal Antibodies

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Lipid A monoclonal antibodies, which require for binding the presence of the bisphosphorylated D-glucosamine disaccharide lipid A backbone, were tested against synthetic lipid A precursor Ia and compound B 1047 by enzyme immunoassay. The last-named compound is a precursor Ia analog with a methoxy instead of a hydroxy group at C6′ and was chosen to determine why these antibodies failed to recognize the bound lipid A present in lipopolysaccharide (LPS). Whereas all antibodies tested bound to precursor Ia, none of them bound to compound B 1047 or *Escherichia coli* Re LPS to a significant extent. Compared to the natural substituent at C6′, i.e., 3-deoxy-D-*manno*-octulosonic acid (Kdo), the methoxy group is neither bulky nor charged. Thus, the data suggest that it is not hindrance by Kdo but rather the generation of a neoantigen that endows lipid A with immunoreactivity upon liberation from LPS by acid hydrolysis.

Lipopolysaccharides (LPS) (endotoxins) represent virulence factors of gram-negative bacteria and play an important role in the pathogenesis and toxic manifestations of gram-negative sepsis (17). Chemically, LPS consist of a polysaccharide and a lipid component (lipid A), the latter of which is the endotoxic principle of LPS (11). Enterobacterial lipid A and bioactive lipid A of various other bacterial genera are made up of a 1,4′-bisphosphorylated β1.6-linked D-glucosamine disaccharide, which carries acyl or acyloxyacyl groups in positions 2, 3, 2′, and 3′. The hydroxy group in position 6′ is occupied by 3-deoxy-D-*manno*-octulosonic acid (Kdo), which is the link between the polysaccharide component and lipid A. On treatment of LPS with acid, the ketosidic linkage between Kdo and lipid A is cleaved, yielding free lipid A, which thus carries a nonsubstituted primary hydroxy group in position 6′ (22).

In the search for new strategies for the control of gram-negative sepsis, polyclonal and, in particular, monoclonal antibodies (MAbs) against LPS have been developed (15, 16). Lipid A has been considered a logical target of protective antibodies for two main reasons. First, lipid A of different pathogenic gram-negative bacteria is structurally highly conserved (19, 23). One might thus expect anti-lipid A antibodies to cross-react with lipid A or LPS of different gram-negative bacteria. Second, since lipid A constitutes the toxic principle of LPS, antibodies to it could be expected to neutralize the bioactivity of lipid A or LPS.

The immunogenicity of lipid A remained unknown for a long time, as immunization of experimental animals with S- or R-form bacteria, or LPS derived from them, did not lead to the formation of lipid A antibodies. More than two decades ago, however, it was found that lipid A-specific antibodies could be engendered if free lipid A, i.e., lipid A devoid of the polysaccharide component, was used and if free lipid A was complexed with a suitable carrier (9). Polyclonal lipid A antisera were shown to be cross-reactive with polysaccharide-free lipid A of various bacterial origins (9). Such antisera, however, lacked antiendotoxic and antiinfectious properties unless very peculiar in vivo assay conditions were used (9, 18). When anti-lipid A MAbs became available (1, 7), their serological properties were studied and their epitopes were characterized with various partial structures of LPS and, in particular, of synthetic lipid A (3, 4). The most important results of these studies can be summarized as follows. (i) The epitopes recognized by various lipid A MAbs reside, without exception, in the hydrophilic backbone region, i.e., in the region of the phosphorylated glucosamine disaccharide (2, 5, 13). (ii) Fatty acids may modulate the exposure of these epitopes but are not part of the determinants (3). (iii) Lipid A MAbs cross-react with a large variety of free lipid A preparations of distinct bacterial origins (9, 13). (iv) Lipid A MAbs, however, do not cross-react under the experimental conditions used by us with LPS or even with Re LPS, i.e., with lipid A carrying only Kdo residues as the polysaccharide component (2, 9). The conditions applied in our studies favor high-affinity binding. Only under other assay conditions, such as radioimmunoassay (1), application of higher concentrations of antigen and antibody, more-gentle washing steps (12), long incubation times (1), and other modifications of test parameters (8a), could binding to Re LPS be observed.

The lack of cross-reactivity of high-affinity antibodies obtained upon free lipid A immunization with LPS remained an unresolved problem, and two main hypotheses were discussed for its explanation. First, within LPS, the lipid A epitopes are not accessible because Kdo or other LPS constituents may sterically shield the determinants and thereby hinder the binding of antibodies to lipid A. These epitopes, however, are accessible in free lipid A, i.e., after the removal of Kdo and the polysaccharide component. Second, free lipid A is a neoantigen which exposes determinants not present in LPS. Such a newly expressed functional group, which may constitute part of a neoantigen, is represented by the primary hydroxy group at position 6′ of lipid A.

In this study, we performed experiments to clarify this problem using lipid A structures carrying a free or modified primary hydroxy group in position 6′.

The chemical structures of the compounds used in this study

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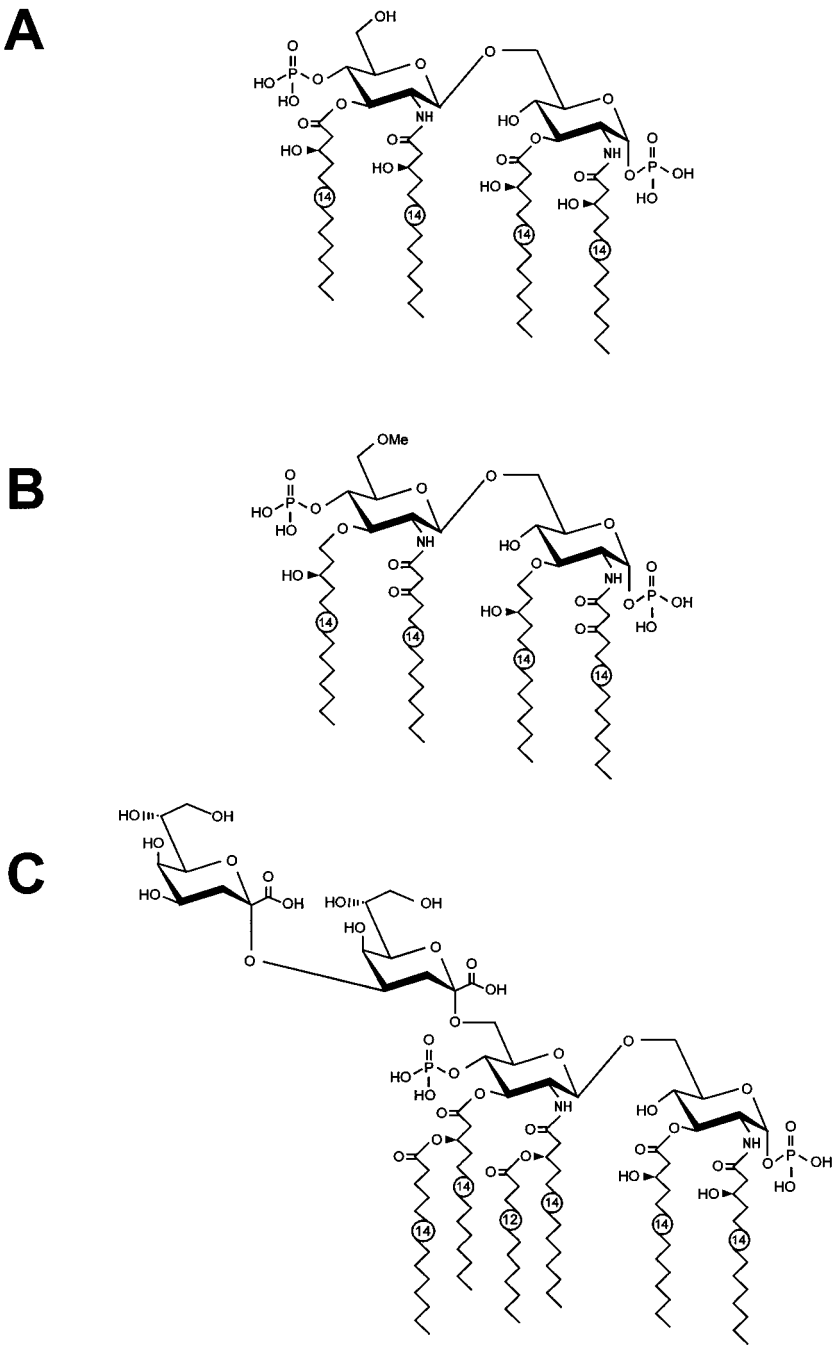

FIG. 1. Chemical structures of tetraacyl lipid A precursor Ia (synthetic compound 406) (A), a synthetic lipid A precursor Ia analog (compound B 1047) (B), and *E. coli* Re LPS (C).

are shown in Fig. 1. Tetraacyl lipid A precursor Ia (synthetic compound 406, kindly provided by S. Kusumoto, Osaka, Japan) constitutes a partial structure of *Escherichia coli* lipid A (10) lacking dodecanoic and tetradecanoic acids (Fig. 1A). In the human system, this compound is a potent LPS antagonist capable of inhibiting LPS-induced cytokine production in peripheral monocytes (for reviews, see references 8 and 14). Synthetic compound B 1047 (Fig. 1B) is a lipid A precursor Ia analog carrying a methoxy group in position 6′. Also, compound B 1047 is a potent LPS antagonist (6). In our hands, its potency in inhibiting the LPS-induced production of tumor necrosis factor alpha, interleukin 1, and interleukin 6 by human monocytes is essentially identical to that of compound 406 (data not shown). The chemical structure of LPS of the *E. coli* Re mutant F515 is shown in Fig. 1C. It is made up of lipid A carrying an α2.4-linked Kdo disaccharide at the primary hydroxy group in position 6′ of the nonreducing D-GlcN residue of lipid A (23). All compounds were tested as solid-phase antigens immobilized on polyvinyl plates in an enzyme immunoassay (EIA) as described previously (13).

Four different lipid A MAbs were selected, the epitopes of which have been studied and previously described in detail

TABLE 1. Reactivities in an EIA of lipid A MAbs with synthetic compound 406 (precursor Ia), synthetic compound B 1047, and *E. coli* Re LPS

MAb	Antibody concn (ng/ml) yielding an optical density of >0.2 in an EIA with preparation:		
	406	B 1047	*E. coli* Re LPS
A6	10	2,500	1,250
8A1	1	5,000	10,000
S1-15	10	2,500	1,250
A43	10	2,500	2,500

(13). MAbs A6 (immunoglobulin G2b [IgG2b]) and 8A1 (IgG1) are anti-lipid A antibodies, as they require for binding the bisphosphorylated GlcN disaccharide backbone. In addition, we analyzed MAb S1-15 (IgG2b), which requires the 4′-monophosphoryl GlcN disaccharide, and MAb A43 (IgM), which recognizes the GlcN II residue of lipid A. Furthermore, MAb A25 (IgG3), which specifically binds to the Kdo disaccharide present in the Re LPS preparation, was included (20).

Plates were coated with the synthetic compounds 406 and B 1047 as well as Re LPS in large amounts (up to 200 ng/well) to guarantee a high density of epitopes, and the compounds were analyzed for reactivity with MAbs. The results obtained are shown in Table 1. As expected, the lipid A MAbs A6 and 8A1 as well as MAbs S1-15 and A43 showed strong binding to compound 406 and lacked significant binding to Re LPS. None of these lipid A clones showed reactivity with compound B 1047. In order to analyze this reactivity pattern in more detail, antibody binding curves for compounds 406 and B 1047 (Fig. 2) as well as Re LPS (Fig. 3) were established by titration (twofold dilutions) of MAbs A6 and S1-15 as representative clones by using plates coated with various amounts of antigen (range,

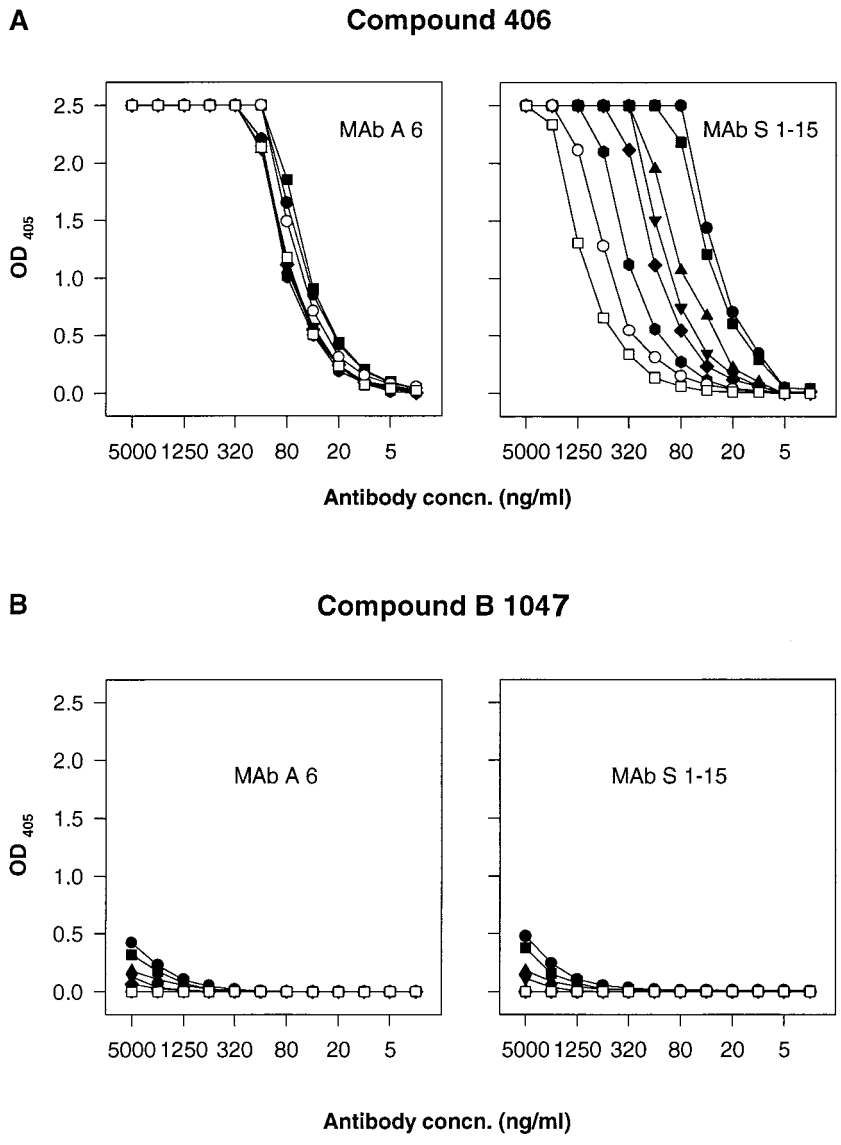

FIG. 2. Checkerboard titrations of lipid A MAbs (A6 and S1-15) in an EIA using as solid-phase antigens compound 406 (A) and compound B 1047 (B), shown in Fig. 1A and B, respectively. Plates were coated (50 μl per well) at concentrations of 4 (●), 2 (■), 1 (▲), 0.5 (▼), 0.25 (◆), 0.125 (⬢), 0.063 (○), and 0.032 (□) μg/ml. MAbs A6 and S1-15 were added at the concentrations indicated on the abscissa. The confidence limits of quadruplicate samples did not exceed 10%. OD_{405}, optical density at 405 nm.

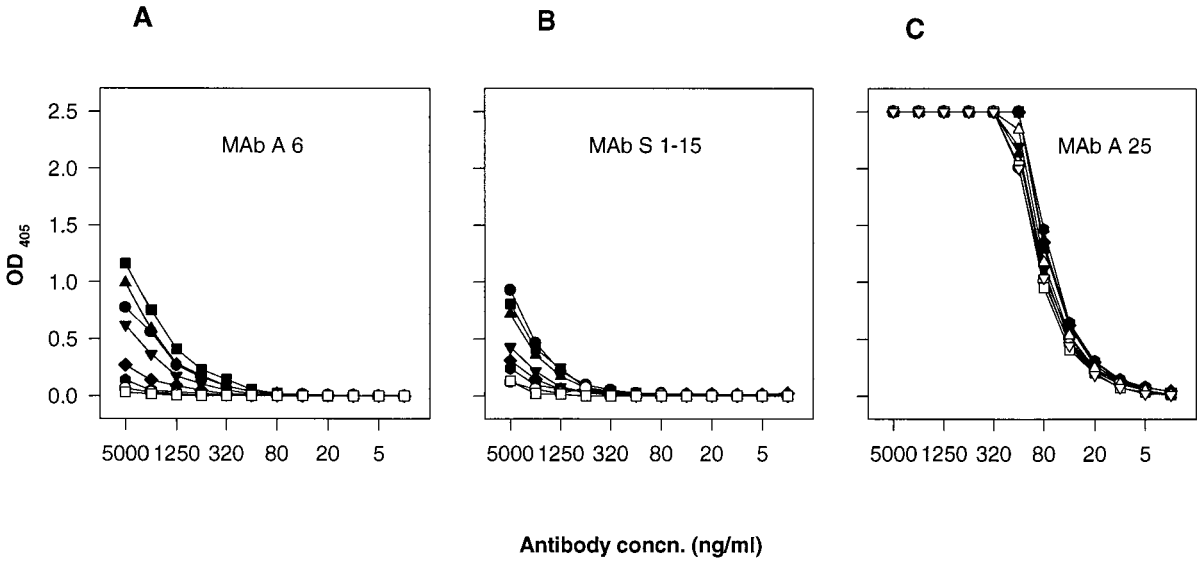

FIG. 3. Checkerboard titrations of lipid A MAbs A6 (A) and S1-15 (B) and Kdo disaccharide-recognizing MAb A25 (C) in an EIA using as solid-phase antigens *E. coli* Re LPS. Plates were coated at the same concentrations as noted in the legend to Fig. 2. OD_{405}, optical density at 405 nm.

0.032 to 4 μg/ml; 50 μl/well). The results obtained clearly show that MAbs A6 and S1-15 bind with high affinity to compound 406 (Fig. 2A) but only at high concentrations to preparation B 1047 at all antigen concentrations tested (Fig. 2B). Lipid A MAb A6 (Fig. 3A) and clone S1-15 (Fig. 3B) reacted with *E. coli* Re LPS only if high concentrations of antigen (2 to 4 μg/ml) and antibody (2.5 to 5 μg/ml) were applied, in contrast to results with MAb A25, which recognized the Kdo disaccharide and which showed excellent LPS binding (Fig. 3C). In agreement with these data, neither compound B 1047 nor Re LPS inhibited the reaction of antibody A6 with compound 406 (data not shown).

The lack of reactivity of a MAb could theoretically be due to the absence of antigen on the EIA plates. To exclude this possibility, the following experiments were performed.

To ensure that compound B 1047 had bound to the EIA plate, the amount of lipid fatty acids associated with the surface of the well and the amount of unbound material in the supernatant were determined by gas-liquid chromatography–mass spectrometry analysis. Several wells of an EIA plate were coated in the usual manner with the highest amount of compound B 1047 used in the serological assays (200 ng/50 μl). After the coating step, the supernatant was collected and the well was subsequently washed with methanol. From both samples (of five different wells) appropriate aliquots were taken. To each aliquot, 3-hydroxy-(2,2,3,4,4-*d*)-tetradecanoic acid (100 ng; a kind gift of S. K. Maitra) was added as an internal standard. Compound 406 was treated in the same way so that we would be able to compare results. The amide-bound 3-oxotetradecanoic acids of B 1047 were first carbonyl reduced with $NaBD_4$ to 3-hydroxy-3-*d*-tetradecanoic acid. The fatty acids were then liberated by acid hydrolysis (4 M HCl, 4 h, 100°C), and an aliquot was analyzed as a trimethylsilylether-methyl ester derivative by gas-liquid chromatography mass spectrometry under standard conditions (21).

With B 1047, 87% was recovered, of which 13% was detected in the bound fraction (corresponding to 21 ng) and 74% was detected in the supernatant. With compound 406, 91% was recovered, of which 31% was detected in the bound fraction (corresponding to 55 ng) and 60% was detected in the supernatant. Since we know that even 1.6 ng of compound 406 per cup results in excellent binding of MAb A6 (Fig. 2), the slightly lower efficiency of coating of B 1047 cannot be responsible for the lack of reactivity of lipid A MAb A6.

We interpret our findings in the following way. As high-affinity antibodies to lipid A are not obtained on immunization with intact LPS, Kdo-free or polysaccharide-deprived lipid A, which is prepared by acid hydrolysis of LPS, must be applied. Acid treatment separates the polysaccharide components and free lipid A, leading to the exposure of a free hydroxy group at position 6′ of the lipid A backbone. With regard to the substitution of this hydroxy group, free lipid A, therefore, differs structurally from the lipid A present in LPS.

Using free lipid A as an immunogen, we engendered antibodies which react as was previously shown and as was also demonstrated in this study with a lipid A structure harboring a free primary hydroxy group but not with preparations which carry at this position a Kdo residue (Re LPS). This lack of cross-reactivity is due neither to steric hindrance by a space-demanding glycosyl group nor to the negative charge provided by Kdo residues, since as demonstrated here, high-affinity lipid A antibodies also do not interact with compound B 1047, which carries at position 6′ a noncharged and nonbulky methoxy group. Rather, it appears that for binding lipid A antibodies require a free hydroxy group at C6′, suggesting that the primary hydroxy group is part of their epitopes and indicating that free lipid A is antigenically different from lipid A as it is present in LPS. In serological terms, therefore, free lipid A constitutes a neoantigen.

Our data thus provide an explanation for the previous results showing that polyclonal antibodies to free lipid A do not cross-react with LPS (9). We conclude that this absence of cross-reactivity with LPS is not due to hindrance of antibody access to lipid A but to differences in epitope structures, in particular, the substitution of the primary hydroxy group at C6′.

Our study deals with four MAbs which require, at least in part, the hydroxy group in position 6′ of free lipid A as their epitope. However, we cannot exclude at present the possibility that in polyclonal antisera to free lipid A or even to LPS,

specificities which recognize other regions of lipid A are present. In our own previous studies, such antibodies were not detected under the test conditions used. These conditions, however, would not have revealed the presence of antibodies that bind with low affinity, the existence of which, therefore, cannot be excluded. As high-titer polyclonal anti-lipid A antisera are not active in suppressing free lipid- or LPS-induced fever in rabbits (18), it is unlikely that such cross-reactive antibodies, if they are present, provide cross-protection. Nevertheless, it may well be possible that cross-reactive high-affinity antibodies to lipid A can be raised. This conclusion remains an intriguing possibility, as non-Ig proteins (such as BPI and ENP) which interact with and neutralize both free lipid A and LPS are known.

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