MICROBIOLOGY–1986

Editor: Loretta Leive

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MEDICAL MICROBIOLOGY AND IMMUNOLOGY

IMMUNOLOGICAL ASPECTS OF LIPOPOLYSACCHARIDE: STRUCTURE-FUNCTION RELATIONSHIPS

Introduction

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Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. It is essential for maintaining the integral form and function of the membrane and thus of the intact bacterial cell. The pathophysiological consequences of many gram-negative infections, including fever, hemodynamic changes, disseminated intravascular coagulation, and shock, once attributed to an integral toxic component of the bacterial cell wall (endotoxin), can be reproduced with purified LPS (10). The effects of LPS on host immune responses are diverse, reflecting activation of lymphocytes and macrophages, specific and nonspecific cells in host defense systems (4, 9). Our further understanding of the various biological effects of LPS on host cells depends on our knowledge of its structure and the structural variations which may exist. The basic chemical composition of LPS, particularly the LPS from the family *Enterobacteriaceae*, has been known for close to 20 years, and we have had a structural model for some 9 years (1). It is now commonly accepted that LPS units are composed of three regions: lipid A, core, and O antigen. The importance of the lipid A moiety in the endotoxic properties of LPS, as well as many of its other biological properties, has focused efforts on elucidating its structural features and the structural determinants of various biological activities (8). Recently, several lines of chemical investigation have provided new information on (i) the chemical basis of the microheterogeneity of purified lipid A (14, 22), (ii) clarification of the site of linkage of lipid A to the 2-keto-3-deoxyoctulosonic acid component of the core polysaccharide (16–18), and (iii) precise localization and identification of hydroxylated and nonhydroxylated fatty acids (13, 19, 25). In addition, the isolation of bacterial mutants defective in the synthesis of lipid A has led to the identification of monosaccharide and disaccharide phospholipid precursors of lipid A (7, 15, 20, 21). The convergence of these new data has resulted in a revised model for the structure of lipid A (16, 19, 21), the organic synthesis of lipid A analogs according to the revised model, and the confirmation that synthetic lipid A has biological activities comparable to those of natural lipid A (6, 23). The first paper of the group which follows concerns some aspects of lipid A structure. Takayama and Qureshi describe the use of new and powerful techniques to fractionate and analyze lipid A and its precursors which aid in our understanding of both microheterogeneity and biosynthesis of lipid A. This approach will make it possible to delineate precise relationships between the structure of this complex molecule and its activities. One example of such a relationship is the work presented by Ribi et al. in which they discuss the nontoxic monophosphoryl lipid A and the structural basis for toxicity, its useful immunological characteristics as an enhancer of other immunological adjuvants, and the importance of solubility for biological activities.

The next three papers address a different structure-function relationship. The structural features of the O-antigen polysaccharides have been of immunological interest primarily as serological determinants. We are now beginning to appreciate that the polysaccharide region of LPS is also important in some LPS-mediated biological events. As Leive discusses, the O-antigen structure controls complement activation via the alternative pathway. As a consequence of such interaction, gram-negative bacteria acquire surface C3b which interacts with C3b receptors on host phagocytic cells. Variations in the saccharide composition of different *Salmonella* strains change the efficiency of both complement activation and host resistance, thus demonstrating a new role for the O antigen in the interaction between pathogen and host (7a).

Variations in O antigen exist even between LPS monomers within bacteria of a smooth strain of a member of the *Enterobacteriaceae*. Such LPS subunits can be fractionated in the presence of detergent into two major populations which differ from each other in the number of repeating units making up the O antigen (2, 11, 12). These fractions appear to differ from each other in some activities which are lipid A dependent, suggesting that the carbohydrate side chain can modulate such lipid A activities with respect to lymphocytes as binding and mitogenesis (3, 5, 24). My own paper, next, discusses my contributions to this emerging area of interest. Finally, Morrison has contributed a timely review of the use of the C3H/HeJ mouse as a tool for studying host responses to LPS, including recent research from his own laboratory.

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Structures of Lipid A, Its Precursors, and Derivatives

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Lipopolysaccharides (LPS) are located on the outer surface of the outer membrane of gram-negative bacteria (3, 13). Their structure consists of three regions: the serologically active O antigen (polysaccharide), the intermediate core (heptose-containing), and the hydrophobic inner region called lipid A (7). The lipid A anchors the LPS to the membrane. The isolated LPS have a wide range of immunological and pathophysiological activities (14), but most of these biological activities can be directly attributed to the lipid A region.

When LPS are hydrolyzed under mild acid conditions, several forms of free lipid A are liberated. Although extensive biological studies using such preparations were done and interesting results were obtained, past studies on the chemical composition were not so successful as a result of certain problems in fractionation and limitations in instrumental analysis (2). Thus, very little was known about the composition of free lipid A preparations. Our recent studies using newly developed approaches for fractionation and modern techniques of instrumental analysis now allow us to describe the precise chemical composition of such preparations (15–17, 19). From these studies, we have determined the complete structure of the lipid A moiety of the LPS obtained from the *Salmonella* strains (15, 19).

SOURCES OF LIPID A AND PRECURSORS FOR STRUCTURAL STUDIES

Several select gram-negative bacterial strains were used in the structural studies of lipid A (Table 1). They included *Escherichia coli* MN7, which provided the monosaccharide precursor called lipid X (21); *Salmonella typhimurium* i50, a 2-keto-3-deoxyoctonate-deficient mutant, which provided the disaccharide precursor designated as IVA (C. R. H. Raetz, K. Takayama, L. Anderson, I. J. Armitage, and S. M. Strain, Fed. Proc. **43**:1567, 1984); and the two deep rough mutants of *S. typhimurium* G30/C21 and *S. minnesota* R595, which provided the free lipid A containing six and seven fatty acyl groups, respectively (15–17, 19).

LIPIDS X AND Y

The purified lipid X was analyzed by both positive and negative ion fast atom bombardment (FAB)-mass spectrometry (21). From these results the M_r was calculated to be 712. The dimethyl derivative of lipid X was prepared and analyzed by proton-nuclear magnetic resonance (NMR) spectroscopy. A substantial downfield shift in the signal of the proton at the 3 position of the sugar of 5.2 ppm (from the normal 3.6 to 4.1 ppm) indicated that the hydroxy group at this position is esterified. The structure of lipid X shown in Fig. 1 was established. This lipid is structurally related to lipid Y, which has an additional palmitate residue esterified to the oxygen of the amide-linked hydroxymyristate (20).

PRECURSOR LIPID A

The precursor lipid A was obtained from *S. typhimurium* i50 by an extraction procedure identical to that for lipid X (21). The extract is a complex mixture containing at least eight different structurally distinct components (Raetz et al., Fed. Proc. **43**:1567, 1984). It was initially fractionated on a DEAE-cellulose column using a linear gradient of 17 to 117 mM ammonium acetate in chloroform-methanol-water (2:3:1, vol/vol) to achieve separation according to the net charge (C. R. H. Raetz, S. Purcell, M. V. Meyer, N. Qureshi, and K. Takayama, J. Biol. Chem., in press). Fractions I, II, III, and IV were obtained, of which fractions I through III contained either aminopentose or phosphorylethanolamine or both. Fraction IV is a major component, has a net charge of −4, and is the last fraction to be eluted from the column. Fraction IV was desalted by using a two-phase chloroform-methanol-water (10:5:6, vol/vol) system and was further fractionated on a silicic acid column with the solvent system of chloroform–pyridine–methanol–88% formic acid–water (60:60:14:3:3, vol/vol). Separation was achieved according to the number of fatty acyl groups per molecule to yield fractions IVB (containing five fatty acids), IVA (containing four fatty acids), and IVC (containing three fatty acids), which are listed in the order of elution.

These samples were methylated with diazomethane and purified by high-pressure liquid chromatography as previously described (15). The highly purified tetramethyl IVA and IVB were analyzed by FAB-mass spectrometry and proton-NMR spectroscopy.

TABLE 1. Gram-negative bacterial sources of free lipid A and its precursors used in the structural studies

Bacterium	Growth temp (°C)	Lipids isolated and characterized
E. coli MN7 (*pgsA pgsB*)	42–44	Lipid X, lipid Y
S. typhimurium i50 (*kdsA*)	42–44	Precursor lipid A (fractions IVA, IVB)
S. typhimurium G30/C21 deep rough mutant (Re)	37	Monophosphoryl and diphosphoryl lipid A homologs
S. minnesota R595 deep rough mutant (Re)	37	Heptaacyl lipid A containing palmitate

Positive ion FAB-mass spectrometry of tetramethyl IVA gave an adduct ion $(M+Na)^+$ at *m/z* 1,484 and an $(M\text{-}PO_4\text{-}2CH_3)^+$ ion at *m/z* 1,336 (Fig. 2). From these results the M_r of the tetramethyl derivative was determined to be 1,461. This suggested that the molecule contains four methyl groups, two glucosamines, two phosphates, and four hydroxymyristates. An oxonium ion representing the distal unit (16) appeared at *m/z* 722, which indicated that it contains two hydroxymyristate and one dimethylphosphate groups. It follows that the reducing unit must also contain the same groups. Proton-NMR spectroscopy of this compound showed that there was a downfield shift in the signals of the protons at the 3 and 3′ positions of the sugar of 5.63 and 5.70 ppm, respectively, indicating that the hydroxyl groups at these two positions are esterified. It also showed that a phosphate group is present at the 1 position with an α anomeric configuration (S. M. Strain, I. M. Armitage, L. Anderson, K. Takayama, N. Qureshi, and C. R. H. Raetz, J. Biol. Chem., in press). The complete structure of the precursor lipid A (fraction IVA) was then determined and is shown in Fig. 1. The precursor lipid A that was isolated and only partially characterized by Rick et al. (18) and by Lehmann (10) is identical to the above-described fraction IVA.

Positive ion FAB-mass spectrometry and proton-NMR spectroscopy of tetramethyl IVB indicated that the structure is identical to that of IVA except for the additional presence of a palmitate in an acyloxyacyl linkage on the amide-linked hydroxymyristate at the 2 position (Strain et al., in press).

LIPID A FROM *S. TYPHIMURIUM*

The diphosphoryl lipid A, TLC-3, obtained from *S. typhimurium* G30/C21 was analyzed by positive ion FAB-mass spectrometry (16, 17, 19). The molecular formula and M_r of the major component were established to be $C_{94}H_{178}N_2O_{25}P_2$ and 1,797 (as the free acid), respectively. Similar analysis of the monophosphoryl lipid A, TLC-3, showed that the distal unit contains two hydroxymyristate, one myristate, and one laurate resi-

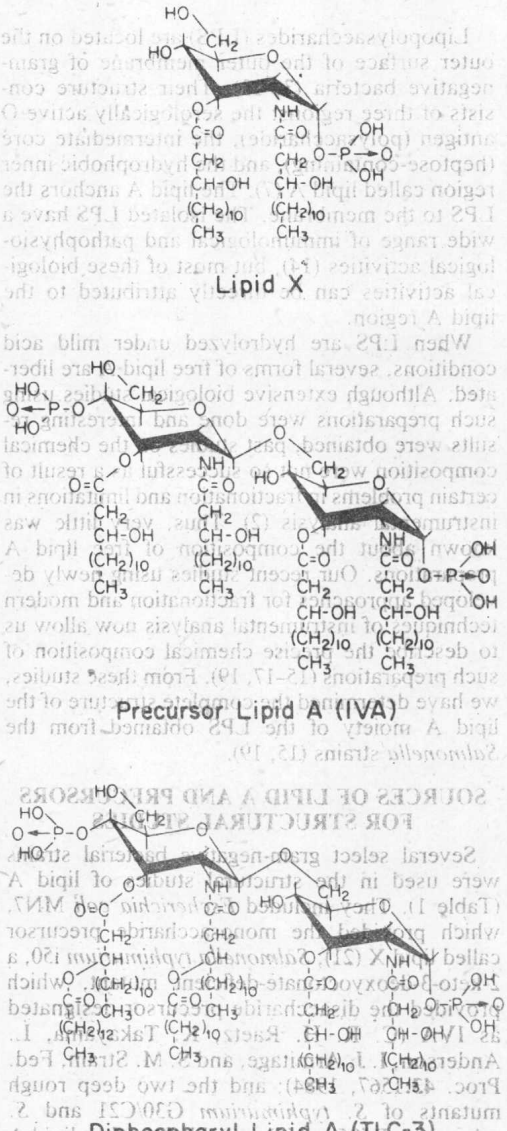

FIG. 1. Established structures of the monosaccharide precursor (lipid X), the disaccharide precursor (fraction IVA), and the free or complete diphosphoryl lipid A. In the native LPS, the polysaccharide is attached to the lipid A through the 2-keto-3-deoxyoctonate unit at the 6′ position.

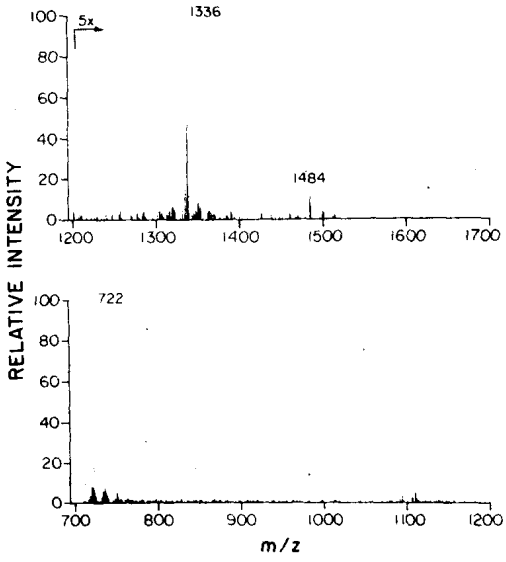

FIG. 2. Partial positive ion FAB-mass spectrum of the high-pressure liquid chromatography-purified tetramethyl derivative of a precursor lipid A (fraction IVA) (K. Takayama, N. Qureshi, K. Hyver, and C. R. H. Raetz, unpublished data).

dues, whereas the reducing-end unit contains two hydroxymyristates.

The purified monophosphoryl lipid A, TLC-3 fraction, was converted to the dimethylpentatrimethylsilyl derivative and analyzed by proton-NMR spectroscopy at 400 MHz (19). The results indicated that the 3 and 3′ positions on the sugar rings were acylated. From the combined FAB-mass spectrometry and proton-NMR spectroscopy as well as the chemical degradation studies, the complete structure of the free diphosphoryl lipid A with the highest degree of acylation (six fatty acids) was determined (Fig. 1).

As shown in Fig. 1, we have established a structural relationship between the monosaccharide precursor (lipid X), the disaccharide precursor (fraction IVA), and the free or complete lipid A (diphosphoryl lipid A, TLC-3). Thus, lipid X can serve as a precursor for either a reducing or a nonreducing unit of fraction IVA. The addition of laurate and myristate in acyloxyacyl linkage to the distal unit of the precursor disaccharide yields the complete lipid A.

HEPTAACYL MONOPHOSPHORYL LIPID A FROM *S. MINNESOTA*

Prior to our investigation, studies done by others specifically on the LPS obtained from *S. minnesota* R595 had established the following: (i) Gmeiner et al. (5) showed that lipid A contains a glucosamine disaccharide with a β(1′→6) linkage and a phosphate group occupies the 4′ position, (ii) Batley et al. (1) showed that the reducing-end sugar has an α configuration, and (iii) Wollenweber et al. (22) showed that their unfractionated lipid A preparation contains lauroxymyristate and palmitoxymyristate in amide linkages. In our study (15), monophosphoryl lipid A obtained from the LPS of the heptoseless mutant *S. minnesota* R595 was fractionated on a silicic acid column to yield the heptaacyl, hexaacyl, and pentaacyl lipid A. Each of these was methylated with diazomethane to yield the dimethyl derivative and purified to homogeneity by reverse-phase high-pressure liquid chromatography.

Positive ion FAB-mass spectrometry of purified dimethyl heptaacyl monophosphoryl lipid A allowed us to establish the molecular formula and M_r of $C_{112}H_{211}N_2O_{23}P$ and 1,983, respectively. It also showed that the distal sugar unit contained one dimethyl phosphate, two hydroxymyristates, one laurate, and one myristate, while the reducing sugar unit contained two hydroxymyristates and one palmitate. By utilizing two-dimensional NMR spectroscopy, we found that the 3 and 3′ positions were occupied by ester groups. FAB-mass spectrometry of the hexaacyl and pentaacyl lipid A showed that these structures were identical to the previously designated TLC-3 and TLC-5 fractions, respectively, from *S. typhimurium* (16, 17). On the basis of these results, the structure of a diphosphoryl lipid A from R595 would be identical to that of the diphosphoryl lipid A, TLC-3, shown in Fig. 1, except for the additional presence of a palmitate in an acyloxyacyl linkage to the hydroxymyristate located at the 2 position.

It is interesting to note that the unique forms of lipid A-containing palmitate residue exist at the monosaccharide (lipid Y), disaccharide (fraction IVB), and complete lipid A (heptaacyl) levels. The significance of this observation is not clear.

CONFIRMATION OF STRUCTURE OF LIPID A BY ORGANIC SYNTHESIS

Shiba, Kusumoto, and co-workers organically synthesized lipid X (12), precursor lipid A (IVA) (11), and the diphosphoryl lipid A, TLC-3 (6) (structure as shown in Fig. 1). These synthetic lipid As and precursors were then tested extensively for biological activities, and the results showed that they were essentially identical to the corresponding glycophospholipids obtained from natural sources (4, 8). This is significant, since previous examination of a series of synthetic lipid A with the "incorrect" structures gave biological activities that were generally much lower than the LPS or the lipid A from

natural sources (9). Thus, it can now be stated that the correct structure of the free diphosphoryl lipid A (containing six fatty acids) from *S. typhimurium*, as shown in Fig. 1, has been confirmed by organic synthesis.

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Biological Activities of Monophosphoryl Lipid A

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Endotoxin has for many years been a double-edged sword, capable of such beneficial effects as tumor regression, yet also causing a number of unfavorable and potentially lethal side effects. A long-standing goal has been to find some way of attenuating the toxic nature of endotoxin without destroying or diminishing its immunostimulatory properties. This goal was finally realized when it was discovered that acid hydrolysis of endotoxin from heptoseless mutant strains of gram-negative bacteria releases a nontoxic form of lipid A, referred to as monophosphoryl lipid A (MPL) (8, 11). MPL is nontoxic, but is still a potent immunostimulator; it therefore represents the beneficial side of endotoxin's double-edged character (3).

The three sections of this paper review some of the work which has been done to characterize and explain the biological activities of MPL in relation to those of the parent endotoxin. The first section focuses on the nontoxic nature of MPL, with reference to the structural features in endotoxin which appear to be required for toxicity. The next section summarizes the immunological activities of MPL. Special attention is drawn to the ability of MPL to dramatically enhance the immunostimulatory properties of adjuvant-active compounds derived from mycobacterial cell walls. Finally, in the third section, the relationship between the solubility properties and the biological activities of MPL and related compounds is examined.

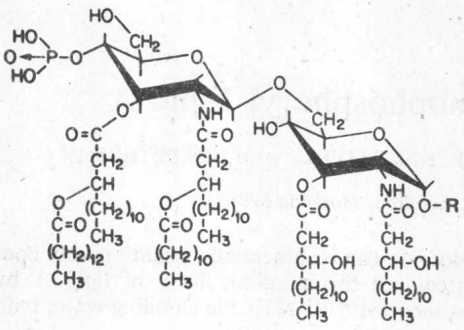
NONTOXIC NATURE OF MPL

Ironically, the realization that endotoxin can be detoxified by acid hydrolysis came about as a result of efforts to obtain a highly toxic lipid A. By use of the procedure of Rosner et al. (9), endotoxin isolated from a heptoseless Re mutant strain of *Salmonella typhimurium* was subjected to mild acid hydrolysis (0.02 M sodium acetate, pH 4.5) to release lipid A from the small amount of core present in this mutant strain endotoxin (12). Careful fractionation of the hydrolysate by ion-exchange chromatography resulted in the isolation of a relatively small amount of a nontoxic form of lipid A, which still possessed the ability of endotoxin to cause tumor regression when combined with trehalose dimycolate (TDM) plus a peptidic component. Subsequent work showed that both lipid A and Re mutant endotoxin can be essentially quantitatively converted into this nontoxic form of lipid A by treatment with 0.1 N HCl in a boiling-water bath (6).

Chemical and physical techniques have been used to show that the nontoxic form of lipid A which is released by acid hydrolysis of Re mutant endotoxin is identical to toxic lipid A (referred to here as diphosphoryl lipid A, or DPL), except that it lacks a phosphate group at the C-1 position of the reducing-end glucosamine (Fig. 1) (4, 5, 11). This nontoxic form of lipid A is therefore called monophosphoryl lipid A, or MPL. It is now apparent that the toxicity of the lipid A released by acid hydrolysis of endotoxins from smooth and rough strains of bacteria is determined by the relative amounts of (toxic) DPL and (nontoxic) MPL (8; E. Ribi, J. L. Cantrell, K. Takayama, H. O. Ribi, K. R. Myers, and N. Qureshi *in Proceedings of the International Symposium on Biomedical Science, The Biology of Endotoxins*, in press).

More detailed structural studies on DPL and MPL were initially frustrated by the heterogeneous nature of these compounds. This heterogeneity is evident from the thin-layer chromatography (TLC) patterns of DPL and MPL (Fig. 2). As reported by Takayama and Qureshi (this volume), methods have been developed which allow both DPL and MPL to be fractionated into the individual components which are visible as TLC bands in Fig. 2. The individual components, referred to in terms of the order in which they appear in the TLC pattern (i.e., TLC 1, 3, 5, 7, and 9), were isolated, purified, and then structurally characterized by using a variety of chemical and spectroscopic techniques (4, 5, 8).

These studies revealed that the components in the TLC patterns for both DPL and MPL differ only in terms of the number of fatty acyl groups which are attached to the diglucosamine backbone (Table 1). The most polar component in the TLC pattern, TLC 9, contains three fatty acyl groups, while TLC 7 contains four, TLC 5 contains five, and so forth. Thus, DPL and MPL are actually composed of a homologous series of components, with only the number of fatty acyl groups varying from homolog to homolog. Furthermore, as shown in Table 1, there is an exact correspondence between the homologs of DPL and MPL (e.g., DPL TLC 3 corresponds to MPL



R= PO_3H_2 Diphosphoryl Lipid A , Mol. Wt. 1797 amu

R= H Monophosphoryl Lipid A , Mol. Wt. 1717 amu

FIG. 1. Complete structure of TLC 3 homologs of DPL and MPL. The two compounds are identical except for the phosphate group at the C-1 position of DPL. amu, Atomic mass units.

TLC 3, etc.). This is reflected by the fact that each homolog in the MPL series is 80 atomic mass units, or the mass of one phosphate group, lighter than the corresponding homolog in the DPL series.

The change in the toxic properties of endotoxin and DPL brought about by removal of the reducing-end phosphate is striking (11). For example, intravenous administration of doses as low as 1 to 10 μg of endotoxin were lethal for rabbits, while doses of up to 15,000 μg of MPL were tolerated. Similar results have been obtained with guinea pigs, dogs, and horses. MPL is also 1,000 times less pyrogenic and 200 times less reactive in the dermal Shwartzman test than either DPL or the parent endotoxin when tested in rabbits (10). It is important to note that the susceptibility of humans to endotoxin is estimated to be similar to that of rabbits. In this regard, a phase I drug trial has shown that MPL can be safely administered clinically in doses of 100 $\mu g/m^2$ (13).

An important property of endotoxin is its ability to dramatically enhance the immunostimulatory properties of mycobacterial cell wall components such as TDM and muramyl dipeptide. For example, such combinations are effective in causing tumor regression and in conferring resistance to subsequent rechallenge. Unfortunately, the toxicity of endotoxin is also amplified in these combinations. This is illustrated in the first part of Table 2, where the effectiveness of various combinations of endotoxin, cell wall skeleton or muramyl dipeptide, and TDM in causing regression of transplantable line-10 tumors in guinea pigs is reported (8). Animals in the group which received endotoxin + muramyl dipeptide + TDM became lethargic for 24 h, and 14% of them died. In contrast to this, animals in the test groups which received MPL instead of endotoxin showed no signs of any adverse reactions. Of key importance is the fact that such combinations were still very effective in causing tumor regression (see the paragraph on Antitumor activity in the next section).

Recent work has established other structural requirements for toxicity besides the presence of a reducing-end phosphate group. Thus, the following structural features appear to be necessary: a diglucosamine backbone, two phosphates, two amide-linked β-hydroxy fatty acids, and at least one fatty acid which is ester linked to a β-hydroxy fatty acid (2, 10, 11). For example, the absence of an esterified β-hydroxy fatty acid in an otherwise normal DPL would result in a nontoxic molecule. This is actually observed in the case of a precursor of lipid A, which lacks any esterified β-hydroxy fatty acids and is nontoxic (2, 10, 11).

IMMUNOLOGICAL PROPERTIES OF MPL

MPL in Water

MPL can be dissolved in water containing between 0.05 and 0.2% triethylamine to yield solutions of vesicular aggregates (see the last

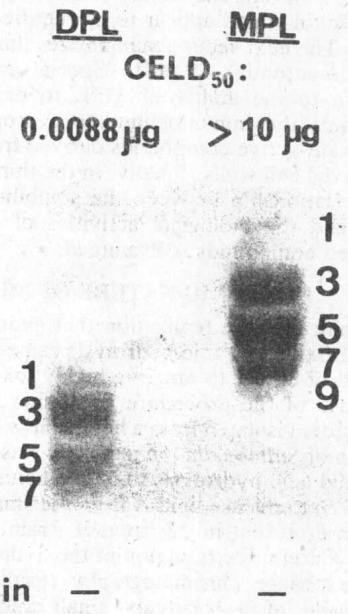


FIG. 2. TLC of toxic DPL and nontoxic MPL. A silica gel TLC plate was developed in chloroform-methanol-water-ammonium hydroxide (50:25:4:2) and then visualized with phosphomolybdic acid spray. ($CELD_{50}$, 50% lethal dose for chicken embryo.)