

MICROBIOLOGY-1986

Editor: Loretta Leive

Section Peter F. Bonventre

Editors: Josephine A. Morello

Simon D. Silver

Henry C. Wu



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Contents

Section I. Medical Microbiology and Immunology

Section Editor, Peter F. Bonventre

A. Immunological Aspects of Lipopolysaccharide: Structure-Function Relationships

Introduction. DIANE M. JACOBS	3
Structures of Lipid A, Its Precursors, and Derivatives. KUNI TAKAYAMA AND NILOFER QURESHI	5
Biological Activities of Monophosphoryl Lipid A. EDGAR RIBI, JOHN CANTRELL, TIM FELDNER, KENT MYERS, AND JON PETERSON	9
Lipopolysaccharide O-Antigen Structure Controls Alternative Pathway Activation of Complement: Effects on Phagocytosis and Virulence of <i>Salmonellae</i> . LORETTA L. LEIVE AND VICTOR E. JIMENEZ-LUCHO	14
Structural Features of Lipopolysaccharide Binding to Lymphocytes. DIANE M. JACOBS	18
C3H/HeJ Mouse Strain: Role in Elucidation of Host Response to Bacterial Endotoxins. DAVID C. MORRISON	23

B. Molecular Biology of Bacterial Pathogens

Introduction. ERIC J. HANSEN	29
Genetic Studies of the M Protein of Group A <i>Streptococci</i> . JUNE R. SCOTT, SUSAN HOLINGSHEAD, KEVIN F. JONES, AND VINCENT A. FISCHETTI	30
Major Surface Proteins of the Lyme Disease <i>Borrelia</i> sp. ALAN G. BARBOUR AND TIMOTHY R. HOWE	35
Adhesin-Receptor Recognition between the Syphilis Spirochete and Fibronectin. J. B. BASEMAN, J. F. ALDERETE, L. FREEMAN-SHADE, D. D. THOMAS, AND K. M. PETERSON	39
Low-Calcium Response of Virulent <i>Yersiniae</i> . ROBERT R. BRUBAKER	43
Lipooligosaccharide and Virulence of <i>Haemophilus influenzae</i> Type b. ERIC J. HANSEN AND ALAN KIMURA	49

C. Virulence Factors of *Bordetella pertussis*

Introduction. ERIC L. HEWLETT AND ALISON A. WEISS	53
Filamentous Hemagglutinin and Fimbriae of <i>Bordetella pertussis</i> : Properties and Roles in Attachment. JAMES L. COWELL, ATSUO URISU, JIA MING ZHANG, ALASDAIR C. STEVEN, AND CHARLES R. MANCLARK	55
Adherence of <i>Bordetella pertussis</i> to Human Cilia: Implications for Disease Prevention and Therapy. ELAINE TUOMANEN	59
<i>Bordetella pertussis</i> Tracheal Cytotoxin: Damage to the Respiratory Epithelium. WILLIAM E. GOLDMAN	65
<i>Bordetella pertussis</i> Adenylate Cyclase Toxin: Structure and Possible Function in Whooping Cough and the Pertussis Vaccine. ALISON A. WEISS, GWENDOLYN A. MYERS, JOHN K. CRANE, AND ERIC L. HEWLETT	70
Pertussis Toxin: Mechanism of Action, Biological Effects, and Roles in Clinical Pertussis. ERIC L. HEWLETT AND ALISON A. WEISS	75
Conclusions. ERIC L. HEWLETT AND ALISON A. WEISS	79

D. Biology and Pathogenesis of *Chlamydiae*

Introduction. PRISCILLA B. WYRICK AND WILBERT J. NEWHALL	80
Early Phases in the Interaction between <i>Chlamydia trachomatis</i> and Eucaryotic Cells. ERIK KIHLMSTROM AND GUSTAF SODERLUND	82
Is the Intracellular Fate of <i>Chlamydia psittaci</i> Governed by a Specific Mode of Entry into Host Cells? RICHARD L. HODINKA AND PRISCILLA B. WYRICK	86

Characterization of Cloned Genes from <i>Chlamydia trachomatis</i> . LINDY PALMER AND STANLEY FALKOW	91
Cell Surface Alterations during Chlamydial Infection. CHARLES E. WILDE III, SUSAN T. KARIMI, AND RICHARD A. HAAK	96
Interferons and the Immune Response to Chlamydial Infections. GERALD I. BYRNE	99

Section II. Clinical Microbiology

Section Editor, Josephine A. Morello

A. DNA Probes in Clinical Diagnosis

Introduction. SYDNEY M. FINEGOLD	105
Gene Probes in the Detection and Identification of Pathogenic Bacteria. DON J. BRENNER	106
Novel Approach for Rapid and Sensitive Detection of Microorganisms: DNA Probes to rRNA. DAVID KOHNE, JAMES HOGAN, VIVIAN JONAS, ELIZABETH DEAN, AND THOMAS H. ADAMS	110
Diagnosis of Clinical Samples with Synthetic Oligonucleotide Hybridization Probes. ROBERT N. BRYAN, JERRY L. RUTH, RICHARD D. SMITH, AND JEANNE M. LE BON ..	113
Applications of DNA Probes to the Study of Human Cytomegalovirus. STEPHEN A. SPECTOR	117
Development of a DNA Probe for the Virulence Plasmid of <i>Shigella</i> spp. and Enteroinvasive <i>Escherichia coli</i> . PAMELA L. C. SMALL AND STANLEY FALKOW	121
DNA Probes for Epidemiologic Studies of Aminoglycoside-Resistant Bacteria. FRED C. TENOVER AND JAMES J. FLORDE	125
Use of DNA Probes for Diagnosis of Treponemal Diseases. PETER L. PERINE	129

B. Coagulase-Negative Staphylococci

Introduction. GORDON L. ARCHER	131
Community Structure of Coagulase-Negative Staphylococci in Humans. WESLEY E. KLOOS	132
Epidemiologic Markers in <i>Staphylococcus epidermidis</i> Infections. JOSEPH T. PARISI	139
Heterotypic Resistance among Coagulase-Negative Staphylococci. GORDON L. ARCHER, DEBORAH L. JONES, AND PHILIP E. COUDRON	145
Animal Models as Guides to Therapy of Coagulase-Negative Staphylococcal Infections. FRANKLIN D. LOWY AND NEAL H. STEIGBIGEL	150
Coagulase-Negative Staphylococci: a Pathogen for the Present. ADOLF W. KARCHMER ..	153

C. Coccidioidomycosis

Introduction. LOVELLE BEAMAN AND D. PAPPAGIANIS	158
Antigen Identification in <i>Coccidioides immitis</i> . G. T. COLE, M. E. STARR, S. H. SUN, AND T. N. KIRKLAND	159
Taxonomic and Physiologic Characteristics of <i>Coccidioides immitis</i> . B. L. ZIMMER AND DEMOSTHENES PAPPAGIANIS	165
Genetics of Resistance to Coccidioidomycosis. THEO N. KIRKLAND AND JOSHUA FIERER	169
Acquired Resistance to Coccidioidomycosis and Modulation of Host Response. REBECCA A. COX	172
Coccidioidomycosis in the Immunosuppressed Patient. DAVID L. CALHOUN AND JOHN N. GALGANI	177
Advances in Therapy of Coccidioidomycosis. DAVID A. STEVENS	179

D. Leukocytic Rickettsiae of Humans and Animals

Introduction. IULAIMU KAKOMA, MIODRAG RISTIC, AND HERBERT WINKLER	181
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Pertinent Characteristics of Leukocytic Rickettsiae of Humans and Animals. MIODRAG RISTIC	182
Metabolism and Genetics of Rickettsiae. HERBERT H. WINKLER	188
Molecular Approach to the Study of Rickettsial Phylogeny. WILLIAM G. WEISBURG	191
Equine Monocytic Ehrlichiosis (Synonym, Potomac Horse Fever): the Disease and Its Impact. JEAN E. SESSIONS	194
Isolation and Characterization of the Causative Agent of Equine Monocytic Ehrlichiosis (Synonym, Potomac Horse Fever). CYNTHIA J. HOLLAND AND MIODRAG RISTIC	196
Ultrastructural Studies of Ehrlichial Organisms in the Organs of Ponies with Equine Monocytic Ehrlichiosis (Synonym, Potomac Horse Fever). YASUKO RIKIHISA	200
Treatment and Control of Equine Monocytic Ehrlichiosis (Synonym, Potomac Horse Fever). JONATHAN E. PALMER, CHARLES E. BENSON, AND ROBERT H. WHITLOCK	203
Sennetsu Fever: the Disease, Diagnosis, and Treatment. NOBUYOSHI TACHIBANA	205
Effect of Tetracycline Therapy on Host Defense in Mice Infected with <i>Ehrlichia senetsu</i> . DARYL J. KELLY, DAVID D. LABARRE, AND GEORGE E. LEWIS, JR.	209
Computer Simulation of Platelet Kinetics in Canine Ehrlichiosis: Model for Study of Idiopathic and Other Human Thrombocytopenias. R. D. SMITH AND I. KAKOMA	213
E. New Quinolone Antibacterial Agents	
Introduction. DAVID C. HOOPER	217
In Vitro Activity of New Quinolone Antimicrobial Agents. GEORGE M. ELIOPOULOS	219
Comparative Pharmacokinetics of the New Quinolone Agents. JINGORO SHIMADA	222
Potential Clinical Uses of the Newer Quinolone Antimicrobial Agents. DAVID C. HOOPER	226
Section III. Molecular Aspects of Protein Secretion and Membrane Assembly	
Section Editor, Henry C. Wu	
Introduction. RATHIN C. DAS AND PHILLIPS W. ROBBINS	233
A. Mechanism of Protein Insertion into Membranes	
Leader Peptidase from <i>Escherichia coli</i> . ROSS E. DALBEY, ANDREAS KUHN, AND WILLIAM WICKNER	234
Structure-Function Relationships of the Signal Sequence of <i>Escherichia coli</i> Outer Membrane Lipoprotein. STEPHEN POLLITT, SUMIKO INOUE, AND MASAYORI INOUE	238
Dual Regulation of the Allantoin Permease in <i>Saccharomyces cerevisiae</i> . TERRANCE G. COOPER, VANESSA T. CHISHOLM, HYU-JEONG CHO, AND HYANG-SOOK YOO	242
Bacteriophage Lambda Receptor Protein as Seen by Bacteriophages and Monoclonal Antibodies. CI DESAYMARD AND M. SCHWARTZ	246
Molecular Features of the Membrane Skeleton. VINCENT T. MARCHESI	250
B. Protein Processing in Prokaryotes and Lower Eucaryotes	
Genetic Analysis of the Maltose-Binding Protein Signal Peptide. J. PATRICK RYAN, JOHN D. FIKES, VYTAS A. BANKAITIS, MARTHA C. DUNCAN, AND PHILIP J. BASSFORD, JR.	254
Lipoprotein Secretion in Bacteria. HENRY C. WU AND SHIGERU HAYASHI	260
Export of Leucine-Binding Proteins and Protein Hybrids in <i>Escherichia coli</i> . BRUCE R. COPELAND, TI ZHI-SU, AND DALE L. OXENDER	266
Protein Translocation into <i>Escherichia coli</i> Membrane Vesicles. PHANG C. TAI AND LINGLING CHEN	270
The <i>Saccharomyces cerevisiae</i> KEX2 Gene, Required for Processing Prepro- α -Factor, Encodes a Calcium-Dependent Endopeptidase That Cleaves after Lys-Arg and Arg-Arg Sequences. ROBERT FULLER, ANTHONY BRAKE, AND JEREMY THORNER	273
Processing of Secreted Proteins and the Signal Peptidases of Bacilli. J. OLIVER LAMPEN, F. I. JAVIER PASTOR, AND MUSSADEQ HUSSAIN	279

C. Protein Processing in Eucaryotes

Amino-Terminal Processing of Actin. PETER A. RUBENSTEIN, KENT L. REDMAN, AND LARRY R. SOLOMON	283
Signal Recognition Particle-Mediated Translational Control of Insulin Biosynthesis and Interaction with Internal Hydrophobic Segments of the Class II Major Histocompatibility Complex-Associated γ -Chain. MICHAEL WELSH AND DONALD F. STEINER	287
Role of N-Linked Glycosylation in Intracellular Transport of Transmembrane Proteins. CAROLYN E. MACHAMER, JUN-LIN GUAN, ROBERT Z. FLORKIEWICZ, AND JOHN K. ROSE	292
Neoglycoprotein Models for the Study of Glycoprotein Processing. VICTOR J. CHEN, SAU-CHI BETTY YAN, AND FINN WOLD	297
Proline-Rich Proteins: Expressions of Salivary Multigene Families. DON M. CARLSON, DAVID K. ANN, AND HAILE MEHANSHO	303

D. Intracellular Transport and Secretion

Lysosomal Enzyme Packaging: Isolation and Characterization of Subpopulations of Coated Vesicles. NANCY L. KEDERSHA AND LEONARD H. ROME	307
<i>Saccharomyces cerevisiae</i> Mutants in the Early Stages of Protein Glycosylation. KURT W. RUNGE AND PHILLIPS W. ROBBINS	312
Invertase Forms Octamers during Secretion. PAMELA C. ESMON, BRENT E. ESMON, AND RANDY W. SCHEKMAN	317
Targeting of Carboxypeptidase Y to the <i>Saccharomyces cerevisiae</i> Vacuole. VYTAS A. BANKAITIS AND SCOTT D. EMR	320

E. Applied Secretion Research

Culture Medium Effects on Periplasmic Secretion of Human Growth Hormone by <i>Escherichia coli</i> . JUDY Y.-H. CHANG, RONG-CHANG PAI, WILLIAM F. BENNETT, RODNEY G. KECK, AND BARRY R. BOCHNER	324
Engineering <i>Saccharomyces cerevisiae</i> for the Efficient Secretion of Heterologous Proteins. GRANT A. BITTER	330

Section IV. Molecular Biology of Archaeobacteria

Section Editor, Simon D. Silver

Introduction. JOHN N. REEVE	337
Molecular Biology of Archaeobacteria. PAUL T. HAMILTON AND JOHN N. REEVE	338
Archaeobacterial tRNA Genes: Structure and Intron Processing. CHARLES J. DANIELS, SUSAN E. DOUGLAS, ANDREW H. Z. MCKEE, AND W. FORD DOOLITTLE	349
Structure and Expression of rRNA Genes in Archaeobacteria. PATRICK P. DENNIS, JOHN CHANT, AND IVY HUI	356
Bacterio-Opson Gene Expression in <i>Halobacterium halobium</i> . MARY C. BETLACH, DIANE LEONG, FELICITAS PFEIFER, AND HERBERT W. BOYER	363
Unique Antibiotic Sensitivity of Protein Synthesis in Archaeobacteria and the Possible Structural Basis. HEIDI HUMMEL, MICHAEL JARSCH, AND AUGUST BÖCK	370
Author Index	375
Subject Index	376

Section I.
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-deoxyoctulonic 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 monosac-

charide 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 bombard-

ment (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.

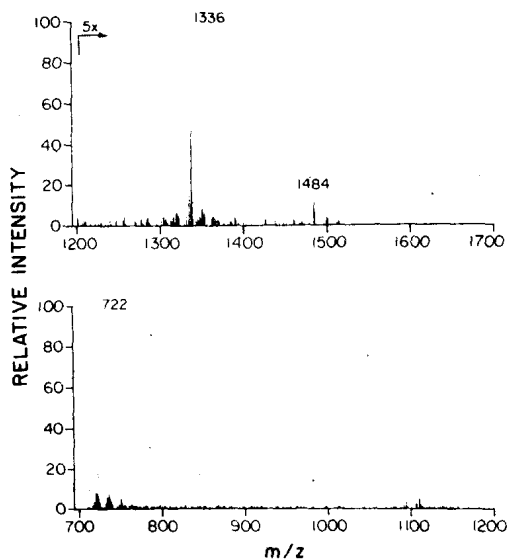


FIG. 2. Partial positive ion FAB-mass spectrum of the high-pressure liquid chromatography-purified tetramethyl derivative of a precursor lipid A (fraction IVA) (K. Takayama, N. Qureshi, K. Hyver, and C. R. H. Raetz, unpublished data).

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

The purified monophosphoryl lipid A, TLC-3 fraction, was converted to the dimethylpentamethylsilyl 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 con-

tains a glucosamine disaccharide with a $\beta(1\rightarrow6)$ 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 glycerophospholipids 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.

ACKNOWLEDGMENTS

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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

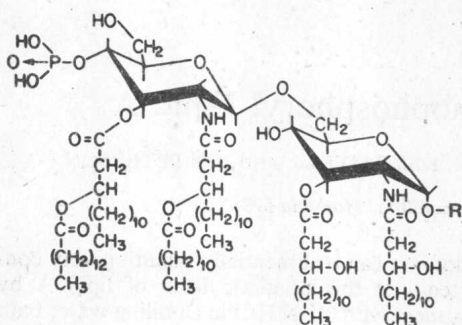
Ironically, the realization that endotoxin can be detoxified by acid hydrolysis came about as a result of efforts to obtain a highly toxic lipid A. By use of the procedure of Rosner et al. (9), endotoxin isolated from a heptoseless Re mutant strain of *Salmonella typhimurium* was subjected to mild acid 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 Schwartzman 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 $\mu\text{g}/\text{m}^2$ (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

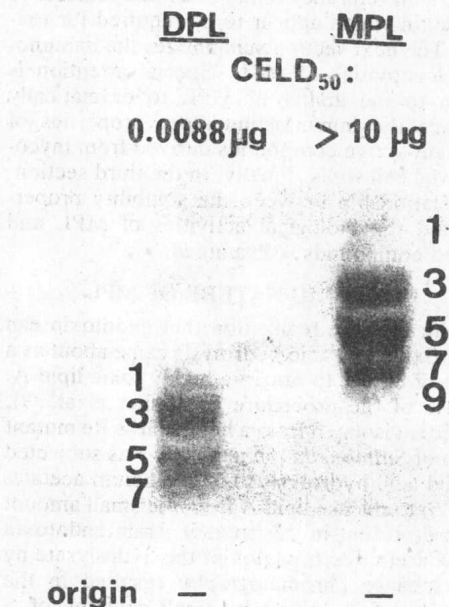


FIG. 2. TLC of toxic DPL and nontoxic MPL. A silica gel TLC plate was developed in chloroform-methanol-water-ammonium hydroxide (50:25:4:2) and then visualized with phosphomolybdic acid spray. (CELD_{50} , 50% lethal dose for chicken embryo.)