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Using Variant Lipid A and Synthetic Antagonists

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DISSECTION OF ENDOTOXIN BIOLOGICAL ACTIVITIES USING VARIANT LIPID A AND SYNTHETIC ANTAGONISTS

By

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ABSTRACT

DISSECTION OF ENDOTOXIN BIOLOGICAL ACTIVITIES USING VARIANT LIPID A AND SYNTHETIC ANTAGONISTS

By

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The lipid A moiety of lipopolysaccharide is responsible for both the beneficial and detrimental effects caused by endotoxins in infected mammalian hosts. The mechanism by which lipid A exerts its effects has not been fully elucidated.

We have investigated the mechanism of endotoxin action by studying a lipid A molecule that has a variant structure from that of the classical endotoxins. The lipid A we studied is from a soil bacterium, *Rhizobium trifolii* ANU843. The lipid A of *R. trifolii* had previously been known to possess some of the classical endotoxin effects, yet it was soon discovered that this lipid A had a radically different structure. By determining the structure of the lipid A of this variant form and comparing it to a classical endotoxin, in terms of structure and conservation of biological effects, we have been able to propose a mechanism for endotoxin activity.

In studying the lipid A structure of *R. trifolii* ANU843, we used several chemical derivatization methods to determine its carbohydrate and fatty acid composition. Physical methods employed in the structural elucidation of these molecules included ^{1}H- and ^{13}C-NMR, mass spectrometry, and gas chromatography. Many separation methods such as TLC, HPLC, and gel permeation chromatography were used.

Several in vitro and in vivo assays were used to answer the question concerning conservation of endotoxic activity. Endotoxin inhibitors were also tested for their ability

to prevent the effects of lipid A, based upon electrostatic and hydrophobic interactions. The data generated by the in vitro and in vivo assays, and the structural elucidation experiments, provided insight into the mechanism of activity of bacterial endotoxins.

We propose a two-step model for the mechanism of endotoxin action. The first step involves adsorption of the LPS aggregate to the cell surface governed by charge interaction. LPS, being negatively charged, associates with a positively charged species on the cell surface. This association increases the probability of intercalation of LPS monomers into the cells' plasma membrane. This coalescence is the second step of LPS/ target cell interaction. Intercalation of lipid A decreases the fluidity of the plasma membrane and this decrease in membrane fluidity leads to cellular activation.

This work is dedicated to my grandfather, the late Joseph W. Ulch, a man who knew the value of an education.

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I would like to thank my parents, Frank and Janet Lill, my sisters, Rebecca and Jennifer, and my grandparents, Joseph and Hazel Ulch, for their support and encouragement during this endeavor.

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Thank You All,

DALE

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
B III	Bergensen's medium
BPI	bactericidal permeablizing increasing protein
C3H/HeJ	LPS hyporesponsive strain (mouse)
CSF	colony stimulating factor
DPL	diphosphoryl lipid A
EI	endotoxin inhibitor
FAB/MS	fast atom bombardment/mass spectrometry
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate isomer I
ELISA	enzyme-linked immunosorbent assay
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
HBSS	Hanks' balanced salt solution
IL-1	interleukin-1
IL-6	interleukin-6
KDO	3-deoxy-D-manno-2-octulosonic acid
LAL	*Limulus* amebocyte lysate
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MPL	monophosphoryl lipid A
NMR	nuclear magnetic resonance
PMB	polymyxin B sulfate
RaRBC	rabbit red blood cells
RBC	erythrocytes - red blood cells

R.t.-FITC	*R. trifolii* LPS - FITC conjugate
Rho	rhodamine B
Sal-FITC	*S. typhimurium* LPS - FITC conjugate
Sal-Rho	*S. typhimurium* LPS - rhodamine conjugate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TNF	tumor necrosis factor

Chapter One: Literature Review and Historical Background

Endotoxin

Life-threatening bacterial infections, that result in the death of an estimated 100,000 individuals per year in the United States alone, make septic shock the nation's 13th. leading cause of death, as estimated by the Centers for Disease Control (1). The majority of septic shock cases are caused by gram-negative bacteria. It is the endotoxin found in the outer membrane of these organisms that brings about the rapid destruction characteristic of sepsis. Sepsis claims a high number of lives because as yet, there is no specific treatment for it. Antibiotics are currently prescribed to kill and clear the bacteria from the bloodstream, yet this results in the release of more endotoxin from the dying bacterium. It is the body's natural defense against these molecules that leads to the condition known as sepsis. The original source of the problem is eliminated, but the resulting inflammatory response continues out-of-control.

Pfeiffer first discovered endotoxins in 1892 and recognized them as being different from exotoxins. Exotoxins are usually proteins which are heat labile and are inactivated by heating at 60 - 80°C. They are secreted by both gram-positive and gram-negative bacteria. Exotoxins can be converted to toxoids and be neutralized by antitoxins (3). They exert a specific and restricted biological function affecting a specific cell type or tissue.

Endotoxins are produced only in gram-negative bacteria. They are embedded in the outer leaflet of the outer membrane where they exist as high molecular weight complexes composed of polysaccharide and lipid (Figure 1). They remain attached to the cell surface and are released into their surroundings during cell lysis or during normal surface blebbing (2). Endotoxins are heat stabile and do not readily form toxoids. It is difficult to neutralize them by anti-endotoxin antibodies (1). Endotoxins affect many different cell types and tissues.

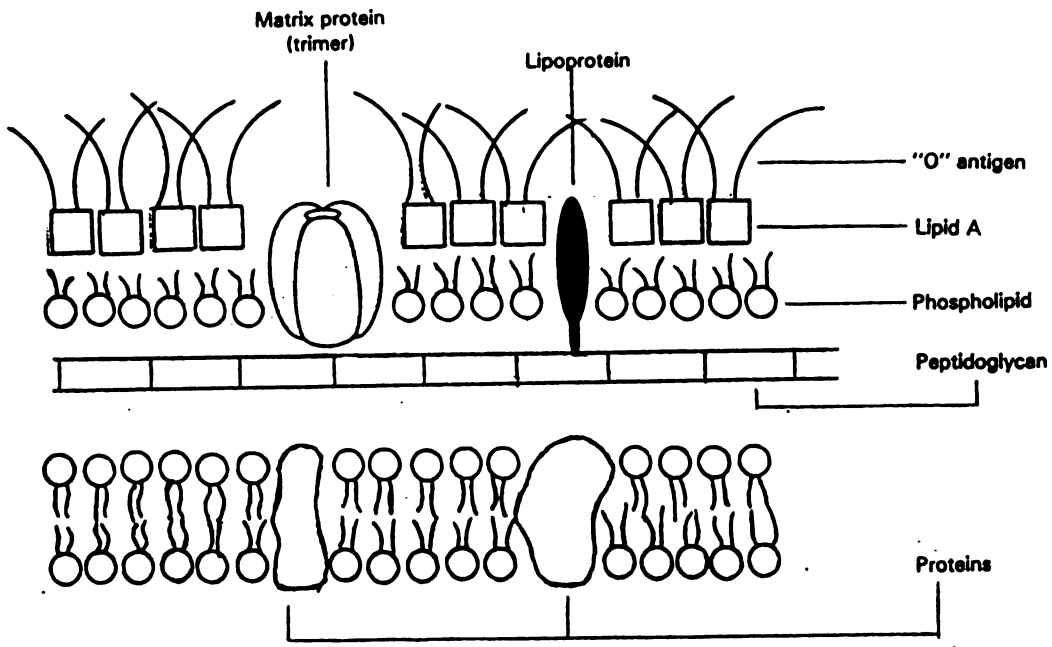


Figure 1

The structure of a gram-negative bacterial cell envelope.

Endotoxins (lipopolysaccharides, LPS) initiate a variety of biological phenomena, (Figure 2), and many review articles have been written concerning their biological activities (3,4,5,6). While some of these effects are considered beneficial (7,8,9,10,11), such as induction of immunoglobulin synthesis, B-lymphocyte proliferation and anti-tumor activity, these molecules are at the same time harmful. Endotoxins are known pyrogens, responsible for tissue necrosis, vascular damage, and can result in the death of the infected organism (12,13).

When endotoxins are released from the outer membrane, they consist mainly of lipopolysaccharide (LPS), complexed with various amounts of protein and some phospholipid (14). Purified, protein-free LPS (15) was found to exhibit many of the biological properties exhibited by unpurified endotoxin. Thus, LPS is the active moiety of bacterial endotoxin.

Lipopolysaccharides- Structure and Biological Activity

The outer membrane is an effective permeability barrier which is found in all gram-negative bacteria. It is the exterior surface of these organisms and is attached to the peptidoglycan layer through lipoprotein. The outer membrane is a lipid bilayer comprised of an inner and outer leaflet, which form a "railroad track" appearance on electron micrographs. LPS is found exclusively in the outer leaflet of the outer membrane of all gram-negative bacteria.

Gram-negative bacteria are well protected by the outer membrane when they find themselves in hostile environments. It makes them resistant to host defense mechanisms , such as lysozyme and cationic leukocyte proteins, which are successful in destroying gram-positive organisms (16,17). Enteric bacteria are normally found in the intestinal tract. Outer membranes of enterics are capable of providing protection to cells from the detergent actions of bile salts and destruction via digestive enzymes (18). The outer membrane is also able to provide resistance to many antibiotics that are effective when

BIOACTIVITIES OF LPS OR LIPID A*

Lethal toxicity	Adjuvant (immunomodulating) activity
Pyrogenicity	Increase of nonspecific resistance to infection
Preparative and provocative activity for local Shwartzman reaction	Induction of tumor necrosis
Induction of hypothermia in mice	Induction of tumor necrosis factor (TNF)
Induction of leukocytosis	Induction of interferon (IFN)
Induction of bone marrow necrosis	Induction of colony stimulating factor (CSF)
Depression of blood pressure	Induction of prostaglandin (PG) synthesis
Toxicity enhanced by BCG	Induction of tolerance to endotoxin
Toxicity enhanced by adrenalectomy	Induction of early refractory state to temperature change
Toxicity enhanced by galactosamine	
Enhanced dermal reactivity to epinephrine	Somnogenic effect[2,119]
	Analgesic effect[3]
Platelet aggregation	
Complement activation	Mitogenic activity for B lymphocytes
Hageman factor activation	Macrophage activation
Induction of plasminogen activator	Polymorphonuclear leukocyte activation
Limulus activity (activation of clotting enzyme cascade of amoebocyte lysate of horseshoe crab)	Endotherial cell activation
Embryonic bone resorption	Induction of mouse liver pyruvate kinase
Type C RNA virus release from mouse spleen cells	Inhibition of phosphoenolpyruvate carboxykinase

Modified from Westphal, O., Lüderitz, O., Galanos, C., Mayer, H., and Rietschel, E. T., *Adv. Immunopharmacol.*, 3, 13, 1986.

Figure 2

Biological phenomena initiated by lipopolysaccharides as modified from Westphal, O., Luderitz, O., Galanos, C., Mayer, H., and Rietschel, E. Th. (1986) Adv. Immunopharmacol. **3**: 13.

used against bacteria lacking an outer membrane, such as macrolides, rifamycins, and novobiocin (19). Thus the outer membrane serves as a protective barrier against hydrophobic compounds. The surfaces of gram-negative bacteria are very hydrophilic. Hydrophilicity is important in evading phagocytosis and allowing resistance to complement (18). These hydrophilic and barrier protection mechanisms intimately involve LPS. The LPS enables gram-negative bacteria to survive under hostile conditions (20).

Lipopolysaccharides (LPS) are unique molecules located in/on the outer membrane of gram-negative bacteria. LPS are amphiphilic molecules consisting of a hydrophilic region composed of varying polysaccharide chains and a hydrophobic region composed of lipid, the lipid A, (Figure 3).

The hydrophilic region is composed of the O-specific polysaccharide and the core oligosaccharide. The O-polysaccharide is very diverse in that it is composed of repeating oligosaccharide units which vary in composition between LPS isolates from different species and even strains of the same species. Each repeating unit is usually composed of four to seven carbohydrates that can be homogeneous or heterogeneous for the sugars present. The number of repeating units can vary in length from zero to forty. An LPS preparation of a given bacterial strain is always heterogeneous with respect to the O-polysaccharide chain length (unless it is a rough mutant) and this is easily demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE of LPS results in development of ladder-like structures. The ladders correspond to the presence of LPS molecules with O-polysaccharide chains differing in size by multiples of oligosaccharide repeating units. (It is the O-polysaccharide which is responsible for determining the O-serological specificity for a particular bacterial strain, such as *Salmonella, Escherichia coli* or *Shigella* (21,22).) The O-polysaccharide is also a receptor for bacteriophage and thus is useful in bacteriophage typing (23).

The core oligosaccharide is the component which links the O-specific polysaccharide

Figure 3

Schematic structure of LPS indicating the hydrophobic and hydrophipic regions.
From "Current Topics in Membranes and Transport", Vol. 17 (1982), p. 81.

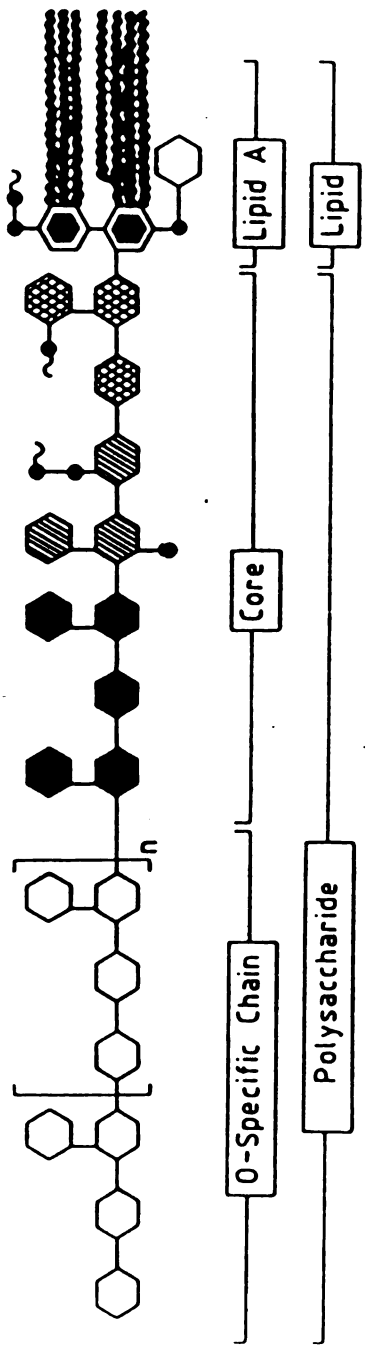

n
O-Specific Chain
Core
Lipid A
Polysaccharide
Lipid
:Monosaccharide, ● :Phosphate, ~ :Ethanolamine
:Long Chain(Hydroxy) Fatty Acid

Figure 3

(O-antigen) to the lipid A. The structure of the core is more conserved within any given genus. The core oligosaccharide and the lipid A are linked by 3-deoxy-D-manno-2-octulosonic acid (KDO). The KDO linkages are very acid labile and are preferentially hydrolyzed under mild acid conditions to allow separation of lipid A from the carbohydrates of the core and the O-antigen.

The hydrophobic portion of LPS is composed of the lipid A. The lipid A is the most conserved structure of the LPS molecule. Its structure and composition is very similar among the *Enterobacteriaceae* (22,24). Originally, the term lipid A was used to describe the hydrophobic precipitate obtained when solubilized LPS was treated with mild acid. Later this term was also used to designate the bound lipid component as it is present in LPS. Currently the term free lipid A is used for the polysaccharide-free component that is liberated during acid hydrolysis.

Much work has been devoted to the characterization of LPS. All three components, the O-polysaccharide, the core oligosaccharide, and the lipid A moiety have been extensively studied. Let us now focus on the lipid A moiety of LPS.

As was stated earlier, the endotoxic properties of gram-negative organisms are associated with the LPS. More specifically, it is the lipid A portion that is responsible for endotoxic activity, whereas the hydrophilic polysaccharide regions are immunostimulatory (24,25). This was first postulated by Westphal and Luderitz (26) in the early 1950's, when it was observed that lipid A was found to be the only component common to all LPS of the *Enterobacteriaceae* . It has been shown that free lipid A elicits the same biological effects as those exhibited by intact LPS (27,28).

Free lipid A isolated from LPS preparations of a wide variety of gram-negative organisms shows a high degree of conservation of structure. Structure of the lipid A molecule typically found in the *Enterobacteriaceae* has only recently been elucidated (29,30). This molecule is a glycolipid with a general structure which is relatively invarient from bacterium to bacterium (Figure 4).

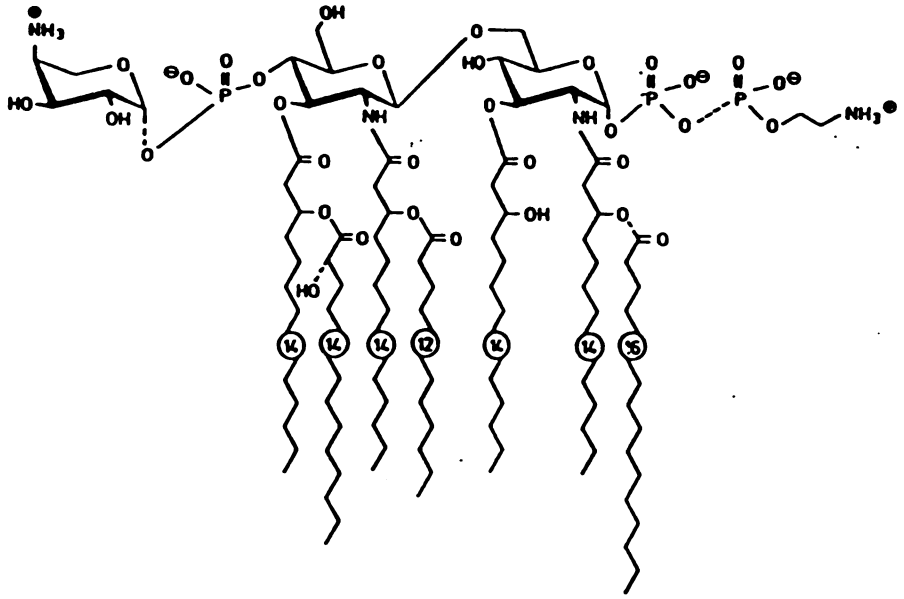


Figure 4

The structure of free lipid A from *Salmonella minnesota*.

Structure of Lipid A

Enterobacterial lipid A typically consists of a ß-1,6-linked glucosamine disaccharide backbone (31,32) bearing phosphate ester substituents at positions 1 and 4'. Linkage at the 1 position is alpha. In intact LPS the backbone is attached to KDO at the hydroxyl group of position 6'. In free lipid A the 6' position is unsubstituted. Lipid A is substituted by a variety of fatty acids at positions 2,3,2', and 3' on the disaccharide backbone. The fatty acids are usually 3-hydroxy substituted (R-configuration), with chain lengths varying from 12 to 16 carbon atoms and are attached by ester and amide linkages. Many times these 3-hydroxyl fatty acids are acylated at the 3-hydroxyl group by other fatty acids. Sometimes this is referred to as "piggy-backed" fatty acids. There is some variation among species, with relatively small variations in number, identity, and location of acyl substituents on the 3-hydroxylated fatty acid substituents. Also varying from species to species are polar substituents such as phosphate, ethanolamine, or other carbohydrate groups on the 1 and 4' phosphate substituents. Gram-negative organisms containing this lipid A structure include *Salmonella*, *Proteus*, *Escherichia coli* and *Shigella*. There are many others that are included in this grouping of "classical" lipid As (32). *Salmonella* lipid A, having been thoroughly studied, was taken as the prototype of lipid A structure, thus the term "classical" or "usual" lipid A.

The main structures present in the enteric lipid A can be found in other non-enteric, gram-negative organisms. These lipid As also exhibit endotoxic activity and contain the usual diphosphorylated ß-1,6-linked glucosamine disaccharide. They also have ester- and amide-linked fatty acids, but differ in chain length from the enterics. Bacteria such as *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Bordetella pertussis* fall into this category.

Lipid A Analogs

The biological effects of LPS have been shown to be associated with lipid A. Many

chemical and biological studies have been undertaken, aimed at determining which structural features of lipid A are responsible for the various biological effects (33,34). Great emphasis has been placed on separating beneficial from toxic effects based solely on structure. Information has been derived from the biological, chemical, and physical analysis of defined natural lipid A preparations, lipid A precursors, and chemically synthesized analogs based upon the natural isolates. Analogs were also prepared which differ from their natural counterparts based upon changes in degree of phosphorylation, acylation, and content of carbohydrate backbone. Most of this work has been performed by research groups in Germany and Japan (28,32,33,34,40,41). The following is a summary of their conclusions concerning structure and biological activity.

The conformation an endotoxin molecule adapts is based upon its structure and it is responsible for its endotoxicity. The conformation is characterized by a balance between hydrophilic and hydrophobic interactions based upon distribution of acyl and phosphoryl substituents over the glucosamine disaccharide backbone (28). This amphiphilic character allows the lipid A to be water soluble and at the same time capable of interacting with cells of the infected host. The major lipid A constituents, acyl groups, phosphoryl residues, and the glucosamine disaccharide backbone, participate in forming this stable conformation recognized by the host.

Partially deacylated LPS/lipid A is less biologically active than its native counterparts. This has been shown in studies by Munford (25,35) where removal of fatty acyl substituents resulted in decreased biological activity. Galanos et al. (33) have shown that a synthetic ß-1,6-linked glucosamine disaccharide, which carries (R)-3-hydroxytetradecanoic acid residues at positions 2,3,2' and 3' and phosphate ester at 1 and 4', exhibited LAL activity, B-cell mitogenicity, and prostaglandin synthesis in macrophage of comparable activity to lipid A precursor IVA and free lipid A. However, it lacked the ability to induce a local Schwartzmann reaction and was of moderate pyrogenicity. Rietschel et al. (28) showed that bacterial and synthetic lipid A precursors containing four

3-hydroxy 14:0 residues did not elicit the toxic effects of endotoxin, while those analogs having at least 5 acyl residues, including one 3-acyloxyacyl group were essential for eliciting toxic effects such as a local Schwartzmann reaction. In this same study it was shown that biological activity does not increase with addition of 3-acyloxyacyl groups beyond two. Rietschel concluded that additional fatty acids disarrange the endotoxic conformation, resulting in reduced biological activity.

The fatty acids of the classical lipid A range in length from 12 to 16 carbon atoms. Changing the length of these fatty acids, for instance by increasing carbon chain length, results in decreased biological activity (36,39).

In regards to phosphorylation, it was observed that the diphosphate (DPL) substituted analogs were water soluble. Monophosphoryl lipid A (MPL) was less soluble, and the analogs totally devoid of phosphate were insoluble in water. Biological activity of a homologous series of analogs increases as degree of phosphorylation increases. Rietschel concluded that phosphate rendered the lipid A soluble and thus made it available to interact with host cells.

Scientists at Ribi Immuno Chem, Hamilton, MT, have done many studies on MPL. They have found that MPL was the result of acid hydrolysis during lipid A isolation (37). MPL was missing phosphate at the C1 position of the reducing end sugar of the disaccharide backbone. It is considered a non-toxic lipid A, yet still acts as a B-cell mitogen and can stimulate interleukin 1 (IL-1) release. Thus, it still possesses immunological properties. Scientists in Freiburg and Japan (33) found the monophosphate analogs to be less toxic by a factor of 100. Whether phosphate was at the C1 or C4' positions, the toxic properties of the analogs were not greatly different, suggesting neither phosphate plays a role in toxicity. They were inclined to believe that lower toxicity in these compounds was again due to decreased solubility.

The glucosamine disaccharide backbone was found necessary for endotoxic activity. Monosaccharide lipid A precursors such as lipid X and lipid Y and chemically

synthesized monosaccharide analogs elicited immunological activities in vitro just as lipid A did. These activities included acting as mitogens and the capability to gel a *Limulus* lysate. These are activities that are also produced by other natural products such as peptidoglycan and lipoprotein (38). The monosaccharide compounds were not capable of induction of the toxic in vivo endotoxin effects such as fever production, local Schwartzmann reaction, and lethal toxicity. It appears that expression of toxic properties are caused by analogs containing the disaccharide backbone (28).

At this time there are several structural features that are believed to render lipid A endotoxic. These features are: presence of a glucosamine disaccharide backbone, phosphorylation, and the presence of acyloxyacyl substituents. Additional substitutions such as phosphorylethanolamine, non-hydroxylated fatty acids or carbohydrate substituents are not required for the biological activities of the lipid A molecule. As of yet, no one has been able to precisely relate structure with a specific biological activity. In analogs of naturally occurring lipid A, biological activities were attenuated, but not totally obliterated. The direct relationship between structure and activity is yet to emerge.

Unusual Lipid A

Extensive research over the years has lead to the structure elucidation of the lipid A molecule. Most of this work has been performed on the *Enterobacteriaceae* and thus this type of lipid A is termed the usual or classical lipid A. The usual lipid A consists of a glucosamine disaccharide backbone linked ß-1,6. It is phosphorylated at positions 1 and 4' and is substituted with acyl chains in positions 2,3,2', and 3'. The usual lipid A is heterogeneous in that it may vary slightly between genus and species, yet is still considered usual. These slight variances include further substitution of the phosphate groups with polar substituents, variation in fatty acid chain length, and degree of acylation of the 3-hydroxy substituents.

Lipid As with deviations in structure from the usual lipid A, in regards to what was discussed above, are termed "unusual" lipid As. Some of the structural variations

from the classical model found in unusual lipid A are listed as follows: (i) Substitution of the disaccharide backbone with additional carbohydrate substituents, many times these sugars are glycosidically attached to C4 of the reducing end glucosamine, (ii) The glucosamine disaccharide backbone is replaced by a different amino sugar, or is a monosaccharide instead of the usual disaccharide, (iii) Substitution by phosphate no longer occurs, i.e. no phosphate is present, and (iv) The (R)-3-hydroxy fatty acid substituents are replaced by the rare 3-(or 4-)oxo fatty acids, unsaturated fatty acids, or fatty acids containing a cyclopropane ring.

Most of the unusual lipid As are considered to be nontoxic. They often have reduced or are completely devoid of biological activity, as compared to the enteric lipid A. Unusual lipid A was first discovered in purple phototropic bacteria, more specifically among the *Rhodospirillaceae* family (43,44,45,46). The following is a description of just a few of these unusual lipid As.

The lipid A of *Rhodopseudomonas viridus* and *Rhodopseudomonas palustris* consists of a 2,3-diamino-2,3-dideoxy-D-glucose backbone. This lipid A backbone is a monosaccharide, not disaccharide, and was the first isolation of this carbohydrate (43). This unusual lipid A contains an amide-linked 3-hydroxytetradecanoic acid, but lacks ester-linked fatty acids and phosphate. The lipid As of these species show no cross-reactivity with *Salmonella* lipid A (47) and toxic effects such as pyrogenicity and lethal toxicity are very low compared to those of *Salmonella* (44).

Rhodospirillum tenue contains the *Salmonella* lipid A backbone, but the phosphate groups are further substituted by 4-amino-L-arabinose and D-arabinofuranose (42,48). An additional glucosamine is linked to C4 of the reducing end sugar. Its amino group is free. This lipid A cross-reacts with that of *Salmonella*, and *R. tenue* LPS exhibits moderate pyrogenicity and "cryptic" lethal toxicity. Cryptic toxicity refers to the observation that *R. tenue* LPS has low toxicity, whereas the isolated lipid A has 100-fold higher toxicity (43). The basis of this phenomenon has yet to be identified.

Rhodopseudomonas sphaeroides ATCC 17023 has been shown to possess a glucosamine disaccharide lipid A backbone and phosphate groups at C1 and C4'. *R. sphaeroides* has an unusual fatty acid composition. It possesses an amide-linked 3-oxotetradecanoic acid and 3-hydroxytetradecanoic acid, an ester-linked 3-hydroxy-decanoic acid and 7-tetradecenoic acid. Serological cross-reactivity between *R. sphaeroides* ATCC 17023 and *S. minnesota* R595 indicate structural similarity between this unusual lipid A and enterobacterial lipid A. Neither lipid A nor LPS of *R. sphaeroides* were found to be lethally toxic or pyrogenic (45).

Yet there are interesting findings concerning the unusual lipid A of *R. sphaeroides*. *R. sphaeroides* lipid A could not induce tumor necrosis factor (TNF) production in the macrophage cell line RAW264.7. However, the lipid A of *R. sphaeroides* was able to block TNF induction by an *E. coli* deep rough mutant (49). Qureshi et al. (50) have shown that this unusual lipid A does not induce IL-1, yet it does antagonize the induction of IL-1 synthesis by the same *E. coli* rough mutant, D31m4. They have shown that *R. sphaeroides* prevents in vivo TNF release caused by *E. coli* Re LPS.

Kirkland et al. (51) have shown that *R. sphaeroides* ATCC 17023 lipid A could not stimulate a murine pre-B cell line to synthesize surface immunoglobulin. Yet, it effectively blocked *E. coli* LPS-induced activation of the same cell line in a concentration-dependent manner. Their conclusions suggest *R. sphaeroides* lipid A, even though it is considered an unusual lipid A, is competing with *E. coli* LPS for some site on the pre-B cell line. This is in agreement with the previously presented data concerning cytokine release.

Until recently, most unusual lipid As displayed none or few of the classical endo-toxin activities. Due to this dilemma, they shed little light on the problem of the structural basis for endotoxin action. Few bacteria have been studied which possess an atypical or unusual lipid A. When investigations encompassing more organisms have

been undertaken, it may be shown that the usual lipid A is not so prevalent.

R. I. Hollingsworth has stated that the molecular basis of endotoxin action rests upon the realization that our response to these molecules is dictated by their complex structure (52). The primary role of LPS/lipid A, is to provide a protective barrier for the bacterium which protects it under harsh environments, not to wreck havoc with our immune system. Bacteria residing in different environments could develop the same structural organization using different chemical functionalities that are functionally equivalent. These structurally differing endotoxins may very well have the same surface topography regarding charge and geometry. They just may be capable of eliciting the same host response. More unusual lipid A molecules that possess the classical endotoxin properties need to be isolated and structurally studied, focusing on the important aspects of structure mentioned above.

Chapter Two: Literature Review of Mechanisms of Endotoxin Action

LPS/lipid A affects nearly all mammalian cells, either directly or indirectly. The precise molecular mechanisms by which this occurs are not yet known. The net effect of lipid A on a cell is to stimulate that cell to perform those functions the cell was originally intended to perform. It seems reasonable to assume that the final intracellular signal received by a cell following an encounter with LPS/lipid A is not dissimilar to the signal which the cell normally receives from its environment. However, the molecular interactions which regulate the LPS-cell encounters are ill defined, as stated above.

To initiate a cellular response, the LPS must first interact with the target cell plasma membrane. Such an interaction could be highly specific, for instance, it might require an LPS receptor. On the other hand, this recognition could be non-specific as in the interaction between the lipid A hydrophobic acyl chains and the lipid bilayer of the target cell. Either of these scenarios leads to the association of lipid A with the eukaryotic plasma membrane.

Binding of LPS to the membrane alone is not sufficient to initiate activation of all cell types, but may be only the initial step of the activation process. This was demonstrated with experiments in which LPS was bound to B-lymphocytes of normal mice and the murine LPS-hyporesponsive strain, C3H/HeJ. These experiments showed that cells from the hyporesponsive strain also bound LPS (53,54). However, a B-cell response did not occur in the C3H/HeJ strain. There are other examples of cells which definitely bind LPS, but do not appear to be activated by it. This was shown by Morrison (55) using plasma-free preparations of human platelets and granulocytes. Both seem to bind LPS from *E. coli* and *Salmonella*, but neither appear to be activated.

These data suggest that, in these cell types, the binding of LPS to the cell, by itself, is not sufficient for activation. Subsequent to the initial binding interaction, some type of perturbational event leading to the transfer of information must occur.

Information transfer could come directly, as in the translocation of LPS to the target cell's interior, or indirectly via one or more second messengers. These events would then lead to the preprogrammed cellular response (55).

Theories of the mechanism of endotoxin action fall into two major categories. The first theory is based solely upon intercalation of LPS into the lipid bilayer of the target cell. This results in a perturbation of the membrane such as a decrease in membrane fluidity. In the second theory, the signalling event is triggered by the binding of LPS/lipid A to specific membrane components which have a high affinity for lipid A. These components are thought of as "receptors" for the lipid A molecule. Let us first look at the non-specific interaction regarding intercalation of LPS into the lipid bilayer.

Hydrophobic Interactions

It has been demonstrated using chemically synthesized lipid A, that the lipid moiety of LPS is necessary for the induction of the typically observed biological activities. Hydrophobic interactions between LPS/lipid A and the phospholipids and other hydrophobic constituents on the cell membrane are known to occur. It is known that the lipid A in an LPS aggregate is not readily accessible and is buried deep inside the dense cluster. Thus, in order for lipid A to activate a cell, there must be a molecular rearrangement of LPS in order for lipid A to become accessible to the cell's plasma membrane.

There is much evidence suggesting that LPS binds and inserts into lipid bilayers. One of the first investigations into the role of lipid attachment to cells involved lecithin and cholesterol (56). Neter showed that both lecithin and cholesterol inhibited the binding of LPS to human erythrocytes (RBCs) and suggested that hydrophobic interactions were important in LPS-cell membrane interactions. In 1967, Hammerling and Westphal (57) made stearoyl derivatives of lipid-free polysaccharides that were capable of attaching to RBCs, while the native lipid-free polysaccharide could not. Springer et. al. (58) showed isolated lipid A was capable of inhibiting the attachment of

native LPS to RBCs and platelets. More recently, studies by Dijkstra et. al. (59) demonstrate the inhibition of LPS activation of macrophage following incorporation of LPS into liposomes. They observed the diminished ability of LPS to induce tumoricidal activity and TNF secretion by murine macrophage. This is consistent with a role for hydrophobic interaction between LPS and the plasma membrane in LPS-initiated cell activation.

Studies by Munford, which utilize acyloxyacyl hydrolase, a deacylating enzyme obtained from leukocytes, show a decrease in LPS-dependent cell activation once the LPS has been deacylated (35,60). Selective deacylation of the nonhydroxylated fatty acids of LPS by the above mentioned enzyme reduces toxic activities of LPS in vivo. The toxic effects of LPS decreased as the degree of LPS deacylation increased. This data is suggestive of an obligatory role for lipid A in the activation of biological responses.

Morrison et al. (61,62,63) has in vitro evidence suggesting a time and temperature dependent interaction between R-form LPS and rabbit erythrocytes (RaRBC). A brief explanation of their conclusions follows. Using polymyxin B sulfate-mediated hemolysis as a probe, they have shown that *S. minnesota* R595 LPS interacts with RaRBC in two distinguishable steps. The first step is the adsorption of LPS to the RaRBC membrane, rendering the cells sensitive to polymyxin B-initiated lysis. This is followed by a time-dependent decrease in response of the LPS-treated cells to the antibiotic. This is the result of a time-dependent rearrangement of the LPS within the lipid bilayer of the RaRBC membrane. At lower temperatures of LPS and RaRBC incubation, the decrease in antibiotic induced hemolysis is less. The increased membrane viscosity at lower temperatures allows less rapid rearrangement of LPS within the bilayer. They conclude by stating that the hydrophobic intercalation of LPS into a cell membrane is important in the LPS stimulation of responsive cells.

Membrane perturbation caused by LPS binding could result in new interactions among membrane components which lead to cell activation. Singer et al. (55,64) state this could occur by either trans effects or cis effects. Trans effects are described as

changes that occur at a localized region on the membrane's surface. They result in the transmission of a signal across the membrane. Cis effects result in the perturbation at one or a few points on the membrane surface that produce changes over the entire surface of the membrane or large areas of it. These effects can result in integral protein associations or endocytosis of membrane components, both of which are capable of generating a transmembrane signal.

Larsen et al. (65) provide more evidence in support of a lipid A dependent membrane perturbation. They examined the effect of LPS and lipid A on the fluidity of the plasma membranes of monocytes by monitoring the intensity of the fluorescence of diphenylhexatriene embedded in the monocyte plasma membranes. Preincubation of monocytes with either LPS or lipid A appeared to increase both the microviscosity of the cell membrane and the order of the lipid bilayer. These findings suggest that endotoxin becomes adsorbed onto the surface of the monocyte, presumably via attachment by the carbohydrate moiety of these molecules and the lipid A becomes directly inserted into the plasma membrane. This affects its microviscosity and crystalline order. They suggest that the initial step is reversible and displays features similar to a ligand binding an external surface receptor. However the subsequent uptake of endotoxin may not be restricted to a receptor bound molecule uptake, but involves direct intercalation of the lipid A into the plasma membrane.

Diane Jacobs has proposed a model describing the interaction of LPS with the plasma membrane as a two-step process. Her model attempts to resolve the conflicting views of whether or not a receptor is involved in LPS binding (66,67,68). These studies measured fluorescence depolarization and were conducted by using whole cells (lymphocytes) and model membranes composed of phosphatidylcholine vesicles. The first step, adherence, was shown to be ionic in nature, temperature independent, and inhibited by polycationic species such as polymyxin B. The second step, coalescence, is the insertion of lipid A into the cell membrane lipid bilayer. It is temperature dependent

and not affected by species such as polymyxin B. These studies have shown that insertion of a large segment of lipid, such as lipid A, caused a perturbation of the lipid bilayer that was evidenced by the decrease in the fluidity of the bilayer. It is possible that these LPS-induced changes in fluidity are influencing the cellular response. Therefore, Dr. Jacobs suggests that the association of LPS aggregates with the cellular surface is mediated by charge on the LPS and cell surface structures on the target cell, which are, more than likely, proteins. This association increases the probability of intercalation of LPS monomers into the target cell lipid bilayer. Intercalation of lipid A decreases the fluidity of that region of the cell membrane and this change may be the signal for cell activation.

The observations mentioned above describe mechanisms by which LPS brings about a membrane perturbation that leads to cell activation without the need for specific LPS receptors. LPS directly inserts into the hydrophobic inner region of the phospholipid bilayer of target cells. This causes the lipid A to become buried inside the bilayer while the polar carbohydrate chains protrude from the outer surface of the plasma membrane. Shands studied bacterial outer membranes and observed LPS to have such an orientation (69,70).

The details of LPS cellular activation are as yet unknown, but one would expect activation to be by a mechanism normally functioning in the cell. It seems highly unlikely that many types of mammalian cells have evolved, all possessing receptors specific for LPS. However, cells could evolve with receptors coupled to specific physiological responses. LPS in its binding is somehow able to interact with these receptors, directly or indirectly, and initiate cellular responses.

Once LPS has reacted with a membrane in a non-specific manner, the carbohydrate extending from the cell surface is in a position to react with structures present on the cell surface. These interactions may create new signals, or block signals normally generated. This type of scenario may result in a transmembrane signal that

could be translated by cells into mitogenesis, initiation of protein synthesis, or transformation (55). Evidence for the model involving lipid A facilitating LPS insertion into the membrane, followed by other LPS structures involved in specific receptor recognition is given by Cavaillon et al. (71,72). These studies suggest that KDO in the inner core is needed for the activation of monocytes to release lymphokines such as IL-1. This is not necessarily so, since other researchers, including ourselves, have found lipid A to be sufficient in stimulating macrophage.

LPS Binding Proteins

Many persons have presented evidence that cell activation is mediated by lipid A intercalating into the lipid bilayer, however some believe that LPS binds to a specific LPS receptor. The receptor is most likely a protein, and the binding event is the start of cell activation. Necessary requirements for specific LPS receptors should include: 1. Binding of LPS is specific for lipid A. 2. Binding is saturable. 3. The interaction occurs at the plasma membrane surface. 4. The interaction leads to the induction of transmembrane signalling. Several laboratories have reported the existence of LPS binding proteins which may well be candidate LPS receptors. However, let us first look at several proteins that bind LPS, but are not membrane localized.

There are a variety of host substances which have relatively high affinities for LPS and have been shown to bind LPS quite well. Mannose-binding protein is known to bind mannose and glucosamine containing structures and has been shown to bind LPS quite readily. Binding of lipid A to a variety of proteins with other known functions has also been established. These serum/plasma proteins include lysozyme, complement C1q, blood coagulation Factor XII, serum albumin, and high density lipoproteins. Binding of LPS induces the activation of serine proteases, as in the cases of C1 and Factor XII.

Lipopolysacharide binding protein (LBP) (73,74) is a protein found in trace amounts in human plasma and that of other endotoxin sensitive species. LBP is a 60 kDa glycoprotein synthesized by hepatocytes. It will rise from 0.5 ug/mL to approximately

50ug/mL within 24 hrs. after induction of an acute phase response. LBP has a binding site for lipid A and is capable of binding both smooth and rough-form LPS with a high affinity. LBP is responsible for opsonization of LPS aggregates and intact gram-negative bacteria. LBP then acts as a carrier protein that brings LPS to the surface of monocytes and macrophage. The LPS-LBP complex then binds to CD14 (75), a monocyte differention antigen, which causes the cell to become activated. It is believed that LBP may belong to a family of proteins that bind lipids and transport them through aqueous environments. Tobias et al. believe a principle function of LBP may be to increase the ability of the infected host to detect LPS early in infection and increase natural resistance in combatting the infection (76).

Bactericidal/permeability increasing protein (BPI) is a 50-60 kDa (depending on species of isolation) cationic protein found in the azurophilic granules of neutropils (77). BPI displays bactericidal activity towards several species of gram-negative bacteria. The outer membrane is the site of BPI action. BPI causes reversible alterations of the outer membrane and irreversible alterations that result in death of the bacterium. BPI acts by increasing membrane permeability to small hydrophobic molecules. Once surface bound BPI is removed from the bacterium, biosynthesis of new LPS and its transport to the outer leaflet are sufficient for repair (78). If BPI is not removed, it rapidly kills the organism.

The effects of BPI in infection may not be limited to bacterial cell death, but may also control the neutrophil response to LPS. Studies by Maara et al. demonstrate that BPI inhibits complement receptor (CR1 and CR3) up-regulation stimulated by smooth and rough-form LPS, as well as lipid A (79). Due to the range of BPI activity against these different LPS forms, and since lipid A is common to all forms, it was concluded that lipid A is the component to which BPI reacts and binds. BPI was also shown to inhibit LPS activity in the LAL assay. Neutralization of LPS by BPI was shown to occur rapidly and at low doses.

BPI and LBP have significant N-terminal sequence homology and have been shown to be cross-reactive immunologically. Both bind LPS, however they differ in their effects on LPS and gram-negative organisms, in their sites of biosynthesis, and in vivo localization (80). This has lead Tobias to conclude that LBP, BPI, and other proteins such as high density lipoprotein belong to a family of LPS binding proteins that moderate the host response to LPS, albeit in differing ways.

LPS Receptors which are Membrane Localized

Differing approaches used to characterize specific membrane components present on target cells have revealed the presence of several components, present on a number of cells, that bind LPS. These experiments have involved fractionation of cell membranes, followed by the isolation, purification, and characterization of the LPS binding substances.

One of the earliest studies identifying specific LPS receptors was carried out by Springer using human erythrocytes (81,82). A lipoglycoprotein was isolated rich in N-acetylneuraminic acid, galactose, and hexosamine. This substance was found to bind all smooth and rough-form LPS of all gram-negative bacteria tested. Although this receptor is a lipoglycoprotein, neither lipid nor carbohydrate appears to be involved in its binding activity. The peptide portion appears to be important, for the the receptor is heat labile, inactivated by aldehydes, and susceptible to proteases. Attempts to locate this lipoglycoprotein on other blood cells such as platelets and leukocytes were not successful. Springer concludes that LPS receptors on other cells may differ in nature and there may be more than one type of LPS receptor. However, they have isolated a cell surface macromolecule which specifically prevents LPS binding to the RBC, and they were the first to do so.

David Morrison and coworkers have been very active in the search for LPS binding proteins. They have synthesized and utilized a radioiodinated, photoactivatable LPS derivative to detect the specific binding of LPS to membrane components on murine

splenocytes. Splenocyte extracts were fractionated by two-dimensional PAGE and subjected to autoradiography. They found an 80 kDa LPS binding protein present on murine splenocytes. This LPS binding protein was detected on B-cells, T-cells, and splenic macrophage. It was also detected on murine B and T cell lines 70Z/3 and YAL-1. Interestingly, fractionation of membrane proteins of the C3H/HeJ endotoxin hyporesponsive mouse strain showed no difference in LPS binding proteins from those on LPS responsive mice (83,84). This glycoprotein has been found on peripheral blood mononuclear cells of a variety of species sensitive to endotoxin, humans being one such species. The 80 kDa LPS binding protein is absent from the mononuclear cells of frog and chicken, both of which are very resistant to LPS stimulation. This suggests a correlation between 80 kDa protein expression and endotoxin sensitivity. LPS binding to the 80 kDa protein is both saturable and inhibited by homologous and heterologous LPS and lipidA, but not by peptidoglycan or muramyl dipeptide (38,85,86,87).

Kirikae et al. have identified an LPS receptor on murine RBC membranes (88). Treatment of RBCs with pronase resulted in decreased amounts of ^{125}I-labeled R-form LPS bound to RBCs. This suggests that this LPS receptor is a membrane protein or glycoprotein. In this study proteins were electroeluted onto nitrocellulose and subsequently treated with LPS, followed by rabbit-anti-LPS, and goat-anti-rabbit IgG-alkaline phosphatase conjugate. This method demonstrated that LPS bound to a single 96 kDa protein.

Hampton et al. used a radioactive, chemically defined, lipid A precursor, ^{32}P-lipid IVA to demonstrate its specific binding to whole cells, RAW264.7 macrophage-like cultured cells and to immobilized proteins extracted from these cells and transferred to nitrocellulose. They found the binding to be saturable, inhibited by excess unlabeled lipid IVA, and to be proteinase K sensitive. The two major binding proteins are 31 kDa and 95 kDa in size. Fractionation studies indicate the 31 kDa protein to be enriched in the nuclear fraction and the 95 kDa protein to be enriched in the membrane fraction.

They studied other cell lines and found no binding could be detected over background in either of two types of fibroblasts, CHO-K1 and L929. The 95 kDa protein was unobservable in CHO-K1 cells. This observed lack of binding in CHO-K1 cells was explained as the cells' inability to respond to lipid IVA at all concentrations tested (88,89).

Kirkland et al. (90) also used a radioiodinated, photoactivatable derivative of LPS to label LPS receptors on 70Z/3 cells. Proteins were extracted and resolved by SDS-PAGE and viewed by autoradiography. These studies identified 18 kDa and 25 kDa proteins which bind to the lipid A region of LPS. Labeling was saturable and inhibited by excess unlabeled LPS and lipid A. These molecules are considered candidates for LPS receptors.

Mechanism of LPS Action

There is evidence for more than one specific LPS binding protein expressed on the surface of mammalian cells. However, binding may be mediated through the non-specific hydrophobic interactions between the lipid A and the plasma membrane bilayer. The relationship between these "receptors" or hydrophobic interactions and the mechanisms of cellular activation have yet to be determined.

In the field of cell biology, much information exists regarding biochemical mechanisms of signal transduction and cell activation mechanisms. Regarding endotoxins, there is no conclusive evidence concerning the mechanism of LPS mediated cell activation. Binding of LPS to a cell, by itself, is not sufficient to cause activation. To date there is evidence for lipid A activation of calcium fluxes (91), turnover of phosphatidylinositol intermediates (91), and the activation of G-binding proteins (92), yet precise mechanisms of signal transduction mediated by LPS remain to be defined. The following is a list of events suggested by Morrison (55) concerning LPS triggered cell activation.

1. The triggering event may well be intercalation of LPS into the lipid bilayer.

Intercalation results in membrane destabilization which may be the signal for cell activation. 2. The LPS may become internalized and placed adjacent to intracellular structures. This would be the result of direct translocation, pinocytosis, or endocytosis. As yet, I have not found any information as to the mechanism by which direct translocation occurs. 3. LPS may interact with specific membrane components, such as protein/glycoprotein which leads to the generation of a transmembrane signal. 4. LPS could interact with some additional external species at the cell surface. It is this species which provides the actual transmembrane signal for the cell.

Chapter Three: Literature Review of Molecular Events Resulting From the Interactions Between Endotoxin and Target Cells

The Macrophage and Cytokine Release

The cell most widely studied, which interacts with endotoxin and shows the greatest changes resulting from this interaction, is the macrophage. When treated with endotoxin, the macrophage undergoes morphological changes and begins synthesis of various proteins, collectively termed cytokines. The cytokines most studied are IL-1, IL-6, TNF, and colony stimulating factor (CSF). The cytokines are mediators of endotoxin activity by acting as second messengers carrying regulatory instructions to cells, organs, and entire systems.

Studies by Michalek et al. (93) demonstrated the importance of these cells in mediating cellular activities of endotoxins. This group used adoptive transfer to evaluate the importance of lymphoreticular cells to the effects of endotoxin on the host. Effects studied included lethality, immunogenicity, induction of interferon, and CSF induction. C3H/HeJ mice became sensitive to each of these effects after adoptive transfer of bone marrow cells from the C3H/HeN, LPS responsive mouse strain. Efficiency of transfer was found to be directly proportional to dose of X-irradiation and inversely proportional to the number of surviving host stem cells. C3H/HeN-C3H/HeJ$_X$ chimeras became sensitive to LPS for each parameter tested. C3H/HeN mice were also rendered unresponsive to LPS by adoptive transfer of C3H/HeJ bone marrow. This group suggested that lymphoreticular cells, either directly or via their soluble mediators, are essential for the expression of endotoxicity in the host. They suggest that LPS acts initially and directly to stimulate some bone marrow derived cell types. As a result of this stimulation, a variety of mediators, such as interferon, TNF, and CSF are produced. These mediators go on to induce secondary effects on the host such as dehydration, hypotension, vascular damage, et cetera. When of sufficient magnitude, these effects

become irreversible and result in death of the host. In other studies, mice which were LPS responsive, but T-cell or B-cell deficient, responded normally to the toxic effects of LPS (94,95).

There is strong evidence that LPS-stimulated macrophage products are the direct mediators of endotoxin induced responses. As noted earlier, macrophage produce a variety of factors including IL-1, TNF, CSF, interferon, and E-series prostaglandins in vivo in response to stimulation by LPS. Isolated macrophages from LPS-responsive mice produce a similar spectrum of soluble factors (96). When purified and injected into animals, each of these soluble factors induces one or more of the same toxic effects induced by LPS, such as fever, hypotension, shock, and death.

Compounds which affect macrophage by increasing or decreasing their state of activation result in enhanced or attenuated LPS responsiveness, respectively. Administration of anti-cytokine antibodies to normal mice who also received LPS has been shown to attenuate endotoxin effects, and even prevent death in the case of anti-TNF administration (94,97). Such studies are currently underway in human trials (98).

These findings support a role for the macrophage as a major cellular mediator of endotoxin induced effects. However, other cells are also involved in LPS phenomena. LPS stimulation of platelets has been recognized to be one of the most rapid host responses to endotoxin. The result is platelet aggregation and release of vasoactive amines (99). Endothelial cells have also been shown to be involved in these phenomena, such as in the condition known as disseminated intravascular coagulation.

Signal Transduction

It is agreed that the cytokines and other humoral factors play a most important role in the mechanism of endotoxin action. However, what are the intracellular events that result in the release of these cell products following these changes?

Nakano et al. used the C3H/HeJ mouse and its normal counterpart to look at these events. In their earlier studies, they looked at the relation between endotoxin

initiated intracellular signal transduction and the calcium-calmodulin system (100,101). They indicated that the calmodulin system relates to LPS induced IL-1 production by murine macrophage. They have since proven that cAMP and cGMP do not enhance the production of IL-1 in murine macrophage, regardless of having or not having LPS stimulation. This suggests that these cyclic nucleotides do not work as second messengers in the intracellular signal transduction for IL-1 production resulting from LPS stimulation. This same group has shown that LPS induces the phosphorylation of a distinct set of proteins located in the membrane and in the cytosol of macrophage prior to the synthesis of IL-1 and TNF by these cells. They successfully purified to homogeneity an LPS induced cytosolic phosphorylated protein in the macrophage which is interestingly lacking from the cytosol of the C3H/HeJ LPS hyporesponsive mouse strain. An LPS induced phosphorylating protein has yet to be identified (102).

Judy Spitzer studied the effects of LPS on hepatocyte signal transduction. Her studies involved inducing LPS low-responsiveness by continuous infusion of non-lethal doses of LPS into rats (i.e. endotoxin-induced tolerance). The conclusion of these experiments can be summarized as follows. 1. Endotoxemia perturbs the calcium-dependent and diacylglycerol (DAG)-activated arms of signal transduction pathways. 2. Specifically, they attributed the low responsiveness to reduction in intracellular Ca^{2+} mobilization, decrease in DAG levels, and decrease in protein phosphorylation. 3. Protein kinase C (PKC)-mediated events were implicated in the molecular mechanism of LPS action.

Ding et al. suggested that microtubules may be targets of endotoxin action on macrophage. Taxol, a plant derived anti-tumor drug, binds irreversibly to polymerized tubulin, thus preventing its depolymerization. Ding showed taxol was able to act on macrophage and mimic two effects of LPS: decrease in TNF receptors and induction of TNF release. Both actions of taxol were absent in C3H/HeJ mice. They concluded a protein associated with microtubules may be a cellular target of LPS (103).

Tanke (99) looked at GTPase involvement in sepsis. They used lipid X to induce low-responsiveness to endotoxin. Lipid X has been identified as the monosaccharide precursor in lipid A biosynthesis. Lipid X treatment was assumed to block access of LPS to target cells and thus prevent mortality. Inhibition of GTPase activity occurred, and it was concluded that a G-protein might be activated by interaction between LPS and a receptor, thus triggering signal transduction.

Cavaillon et al. (104) compared lipid A and Re-LPS in their abilities to induce IL-1 synthesis in macrophage and act as a mitogen for lymphocytes. Lipid A was mitogenic, but unable to induce IL-1 release by macrophage. They concluded that the interaction of LPS/lipid A with the cell membrane differs between different cell types.

There are several targets for LPS. It does not interact with a single organ or cell type. LPS stimulation results in the initiation of multiple metabolic pathways, simultaneously, which normally occur in the cell. Unfortunately, the metabolic processes rapidly reach a point where they run on "out-of-control". The result is the synthesis of many cytokines which are ultimately responsible for the mechanisms of endotoxin action. A conclusion concerning the nature of what LPS recognizes, or is recognized by, and how this recognition is transformed into a signal for cell activation has yet to be reached. Further experimentation is needed to determine how the above scenarios fit together. Endotoxins have been the subject of controversy for many years past and will surely be cause for discussion long into the future.

Chapter Four: *Rhizobium* Lipid A Structure

Introduction

Endotoxin literature cites three major structural features of lipopolysaccharides which are necessary for the induction of endotoxin effects. These features are the presence of a glucosamine disaccharide as the carbohydrate lipid A headgroup, phosphorylation of the disaccharide, and the presence of fatty acid substituents. No one has been able to correlate specific structural elements with a specific biological activity.

Unusual lipid A molecules have been isolated lacking the phosphorylated, glucosamine disaccharide (43,44,45,46,47,48,49,105). These molecules display attentuated or none of the typical endotoxin sequelae. They offer little to be learned concerning the structural basis for endotoxin action. It would be beneficial to have an unusual lipid A that still possesses some of the classical endotoxin consequences, but has a different lipid A structure. In this way, one could compare differences in structure and bioactivity to explain the structural basis for endotoxin action.

The LPS of the soil bacterium, *Rhizobium trifolii*, was thought to have the same lipid A structure as that of the *Enterobacteriaceae* (106). In experiments conducted by F. B. Dazzo, *R. trifolii* 0403 was isolated at different stages of growth and examined for immunochemical properties. This LPS was found to give a positive response in the LAL assay, and was capable of acting as a mitogen (107). Further chemical analysis of the fatty acids on this species by R. I. Hollingsworth et al. (108) showed the presence of 3-hydroxylated fatty acids exclusively, and an unusual 27-hydroxyoctacosanoic acid in the LPS (109). They were the first to isolate this unusual long chain fatty acid, which indicated that the structure of this lipid A was different from that of the enterics and thus could be considered unusual.

Throughout the course of this project, we have worked with LPS from strain ANU843 of the same species of *Rhizobium* . This organism infects clover and is non-

pathogenic to humans. However, LPS from *Rhizobium trifolii* was shown by Dazzo to produce a positive response in the LAL assay, as mentioned above. There are conflicting reports in the literature on the structure of the lipid A of the genus *Rhizobium* . This conflict concerns the degree of phosphorylation, fatty acid composition, and composition of the lipid A backbone (110,111,112,113). Several of the features of enteric lipid A structure which were thought to be critical to endotoxin activity are not found in rhizobial LPS (109,110,111,114).

This research project was divided into two areas. Firstly, determining the lipid A structure of *R. trifolii* ANU843. Secondly, determining which biological activities triggered by classical endotoxin are conserved in the "non-classical" endotoxin obtained from *Rhizobium*.. The data obtained from these areas will help to determine what structural features elicit a particular biological response, and develop a more encompassing view of endotoxins. It also provides insight into the mechanism of endotoxin action.

Materials and Methods:

Bacterial Cultures and LPS Isolation

Rhizobium trifolii ANU843 was grown aerobically to stationary phase in 6L of Bergensen's (BIII) broth at 29°C, with shaking at 142 rpm (115). The 6L of culture was used to innoculate 120L of medium in a B. Braun fermenter, model Biostat UE100D. The cells were grown to early stationary phase at 29°C, aerobically, with stirring at 200 rpm. The cells were harvested using a Sharples A512 centrifuge at 5100 rpm. After harvesting, the cells were again centrifuged for 30 min. at 8000 rpm.

The cell pellets were washed 3 times with 0.5M NaCl for 30 min. at room temperature and centrifuged for 30 min. at 8000 rpm. Cells were extracted 3 times using the hot phenol/water method of Westphal (116). Aqueous layers were pooled into 12,000-14,000 molecular weight cut-off dialysis tubing and dialyzed extensively against many

changes of distilled water. The volume of the dialysate was reduced to 200mL using a rotory evaporator and treated with 2.5mL of an aqueous solution of DNase I, RNase A, and $MgCl_2 \cdot 6H_2O$ to remove contaminating carbohydrate. The enzyme solution was prepared by dissolving 250mg $MgCl_2$, 25mg RNase A, and 19mg DNase I (40,000 units) in 5mL distilled water. The enzyme treated dialysate was ultracentrifuged for 4hrs. at 40,000 rpm at 4°C, using a Type 50.2 Ti fixed angle rotor. The LPS pellet was resuspended in water and centrifuged at 6500 rpm for 10 min. The supernatant liquid containing LPS was frozen, then lyophilized.

Alternatively, *R. trifolii* ANU843 was grown in BIII broth (6L) at 30°C until early stationary phase was reached (115). The cells were then centrifuged for 40 min. at 7000 rpm. The cell pellets were treated as previously described to obtain the LPS. The combined extracts were extensively dialyzed against water, the total volume reduced to 15mL, and was treated as before with enzymes. This solution was then subjected to gel permeation chromatography on a Sepharose 4B column, using 0.05M EDTA/triethyl-amine buffer at pH7.0. Fractions were assayed for carbohydrate content using the phenol -sulfuric acid assay (117). Two peaks were obtained, pooled, and dialyzed. The first eluting peak corresponded to the one obtained when the LPS was isolated by ultracentri-fugation.

Lipid A Isolation

The lipid A was cleaved from the O-polysaccharide and core oligosaccharide by hydrolysis with 1% acetic acid for 3 hrs. at 100°C. After cooling the hydrolyzed sample to room temperature, it was extracted three times with twice its volume of 5:1 chloroform -methanol. The organic layers, which were removed after centrifugation at 8000 rpm for 10 min., were pooled. The combined extracts were dried by passage through absorbant cotton , and concentrated to dryness under a stream of nitrogen.

Purification of Lipid A

The crude lipid A preparation was fractionated on a C_{18} reverse-phase column

(11 x 1.2 cm) with water (7mL fraction), 2:1 water/methanol (7mL), 2:1 methanol/water (14mL), 4:1 methanol/water (14mL), pure methanol (14mL), and pure chloroform (28mL) in that order. The pure methanol fractions were combined and subjected to preparative chromatography on C_{18} reverse-phase layers (10 x 10 cm) using 4:1 MeOH/ water as the mobile phase. Two bands which reacted positively with orcinol/sulfuric acid reagent (118) were obtained. These were scraped and eluted with 1:1 chloroform/MeOH. There were other slowly-migrating bands at and close to the origin.

Fatty Acid Analysis of Crude and Purified Lipid A Components

Lipid A components were converted to their fatty acid methyl ester derivatives by methanolysis with 5% HCl in methanol for 16 hrs. at 70°C. Methyl ester derivatives were subjected to gas chromatography (GC) analysis on a 25m Supelco SPBI capillary column or a JW DB-1 capillary column of 30m, using helium as the carrier gas and a temperature program of 150°C initial temperature, 0.00 hold time, 3.0 degree/min. rate, final temperature of 300°C, final hold time of 15 min. and a run length of 65 min. Electron impact mass spectrometry coupled to GC (GC/MS) for fatty acid analysis utilized a DB-1, 30m, capillary column, with a temperature program of 190°C initial temperature, 0.00 initial hold time, rate of 3.0 deg/min., final temperature of 300°C, final hold time of 30 min. Mass spectra were recorded on a Jeol JMA DA5000 spectrometer.

Carbohydrate Analysis of Crude and Purified Lipid A Components

Lipid A was treated with 5% HCl in methanol at 70°C for 16 hrs. After concentration to dryness, the methanolysis products were dissolved in methanol and subjected to reduction by using a sodium borohydride solution (10mg/mL). The reduction proceeded for 5 hrs. at room temperature, after which time 3M HCl was added dropwise until effervescence ceased. The oxidant was concentrated to dryness and the remaining residue was treated with 0.3mL of 2M trifluoroacetic acid for 3hrs. at 120°C. When cool, the hydrolysate was extracted with 2 times 1mL chloroform. The chloroform extracts were discarded and the aqueous layer was concentrated to dryness under

nitrogen. The aqueous residue was dissolved in methanol and reduced a second time with sodium borohydride for 1hr. 3M HCl was then added to neutralize any remaining base. The solution was concentrated to dryness and washed several times with methanol. To the residue was added 0.1mL pyridine and 0.1mL acetic anhydride. The solution was briefly sonicated and left at room temperature for 16hrs. The acetylated mixture was concentrated to dryness, then 1mL chloroform and 1mL 3M HCl were added to the residue. The chloroform layer was washed once with 1mL 0.5M NaCl, dried over anhydrous sodium sulfate, and subjected to GC analysis on a DB225 capillary column with an initial temperature of 200°C, hold time of 5.00 min., rate of 2.0 deg/min., final temperature of 230°C, final hold time of 55.00 min., and a run length of 75 min. Retention times were compared with alditol acetate derivatives of a number of sugar standards. GC/MS analysis of lipid A derivatives were analyzed on a Jeol JMA DA5000 mass spectrometer using the DB225 capillary column.

NMR and Mass Spectra

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM250 spectrometer operating at 250MHz for protons and 62.8 MHz for ^{13}C. Varian VX-NMR spectrometers operating at 300MHz and 500MHz were also used for recording ^{1}H spectra. Spectra were obtained in either deutero-chloroform containing 10% D-4 methanol, deutero-chloroform, or deuterium oxide. Chemical shifts were quoted relative to the chloroform resonances at 7.24ppm for ^{1}H and 77.00ppm for ^{13}C measurements and at 4.65ppm relative to the water resonance for ^{1}H when using deuterium oxide. Mass spectra (GC/MS) were recorded on a Jeol JMA DA5000 spectrometer. Fast atom bombardment mass spectrometry (FAB/MS) was recorded on a Jeol HX-100-HF spectrometer. In all of the FAB/MS experiments, glycerol was used as the matrix.

Phosphate Analysis

Total phosphate content was analyzed by converting organic phosphate to inorganic phosphate by the method of Lowry (119). LPS of *R. trifolii* ANU843 was

separated on a Sephadex LH-60 gel filtration column using 1:1:2 n-propanol/acetonitrile/ water as the eluting solvent. This resulted in the separation of LPS into three peaks as judged by testing of the individual fractions using the phenol-sulfuric acid assay for carbohydrate. Each fraction was screened for the presence of phosphate.

Preparation of Phenylcarbamate Derivatives of Crude Lipid A

Phenylcarbamate derivatives of the lipid A were made by dissolving 54mg of *R. trifolii* ANU843 lipid A in 7mL pyridine and 70uL phenylisocyanate. The sample was briefly sonicated and then heated at 70°C for 14hrs. It was then concentrated, dissolved in 40mL chloroform and 40mL methanol. To this was added 100uL of TFA, then the mixture was heated at 100°C for 10min. The lipid stood at room temperature for 24hrs., to ensure complete conversion of all carboxylate groups to methyl esters. The product was concentrated to a small volume and subjected to flash column chromatography using silica gel. Eluting solvents were chloroform (10mL), chloroform (30mL), chloroform (30mL), 5:1 chloroform/methanol (25mL), 5:1 chloroform/methanol (10mL), 5:2 chloroform/methanol (10mL). All fractions collected were screened for carbohydrate using the orcinol method and bands were visualized under UV light. The sample testing positive for carbohydrate was subjected to fatty acid methyl ester analysis as described previously. GC/MS was performed on the phenylcarbamate derivatized methyl esters using an initial temperature of 65°C, initial time 0.00, rate 3deg/min., final temperature of 300°C, final time of 30 min., using a 30m DB-1 capillary column.

Results and Discussion:

Phosphate Analysis

LPS submitted to gel permeation chromatography was resolved into three peaks as evidenced by carbohydrate screening using orcinol. Two of these peaks were major and the last peak was minor. The major components did not test positive for the presence of phosphate (119), however the minor peak was positive. The minor peak did not

contain the typical carbohydrate and fatty acid components of rhizobial LPS. It was concluded that this minor peak was definitely not LPS, (Figure 5). This experiment clearly showed that phosphate was absent from the LPS of *Rhizobium* .

Sulfate Analysis

Sulfate has also been reported as a polar substituent in LPS (120), and it has also been found in the LPS of *R. meliloti* (121). Several species of *Rhizobium* were grown in the lab of R. I. Hollingsworth, in the presence of ^{35}S-$MgSO_4$. Samples were screened by scintillation counting and it was found that *R. trifolii* ANU843 was completely devoid of sulfate.

Crude Lipid A ^{1}H-NMR

The ^{1}H-NMR spectrum of crude lipid A isolated from *R. trifolii* ANU843 can be described as follows, (Figure 6). There are signals at 0.80 and 1.20ppm that are characteristic of methyl and methylene signals, respectively, indicative of long chain fatty acids. Signals at 2.38ppm correlate with methylene groups adjacent to a carbonyl functionality. Farther downfield are two groups of sextets, one at 4.17ppm and the other at 4.92ppm. From multiplicity and chemical shifts, these signals correspond with methine protons attached to a carbon bearing a hydroxyl and an acyloxy function, respectively. There are unresolved resonances between 2.00 and 4.50ppm which are assigned to protons on the carbohydrate backbone of the lipid A molecule. These resonances are broad and not well resolved, for the lipid A molecules form strong aggregates and the polar groups do not have much motional freedom.

Fatty Acid Analysis

Fatty acid substituents were identified by preparing fatty acid methyl ester derivatives of the rhizobial fatty acids and comparing their retention times on a gas chromatogram with those of a known methyl ester standard and a rhizobial standard. Fatty acid methyl ester analysis by GC and GC/MS showed the presence of the following fatty acids: 3-hydroxytetradecanoic acid, 3-hydroxy-12-methyltetradecanoic acid, 3-

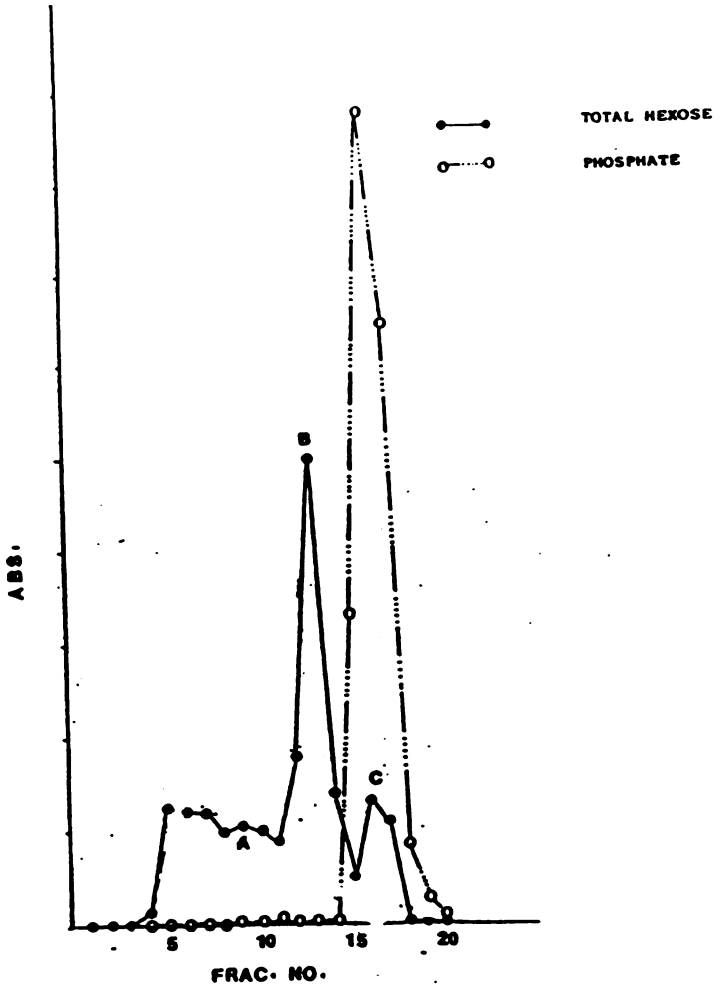


Figure 5

Gel filtration profile of *R. trifolii* ANU843 LPS on a column of Sephadex LH-60 resin. Fractions were screened for the presence of phosphate.

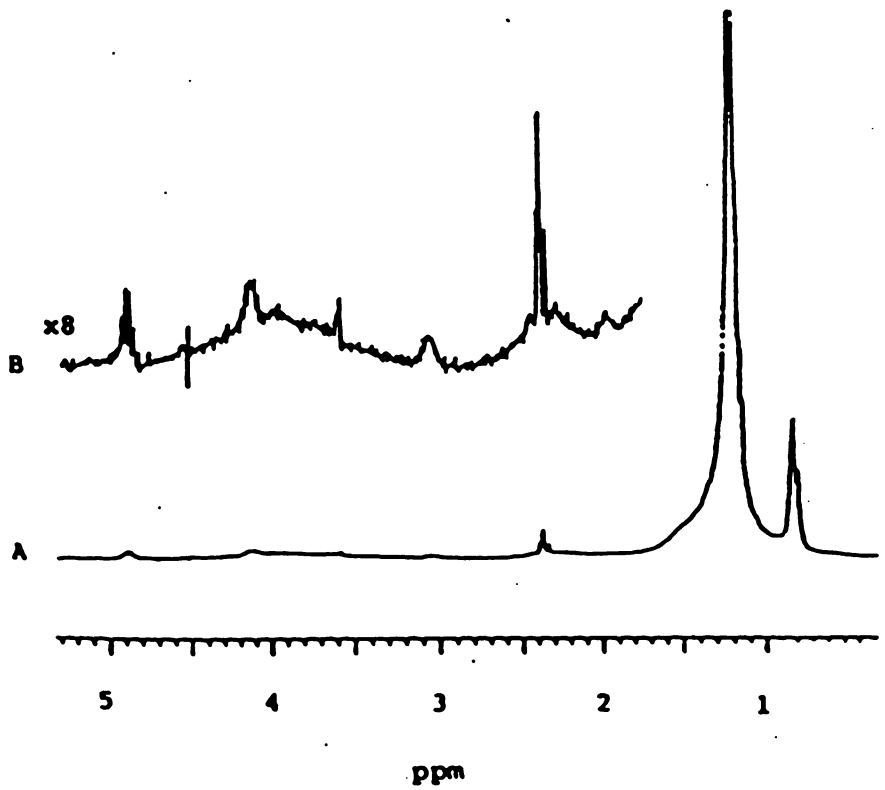


Figure 6

Proton NMR spectrum of the crude lipid A of *R. trifolii* ANU843.

hydroxyhexadecanoic acid, 3-hydroxyoctadecanoic acid, and 27-hydroxyoctacosanoic acid, (Figure 7).

We have looked at the extent of fatty acid acylation (i.e. acyloxyacyl linkages). Phenylisocyanate was used to generate carbamate esters of free hydroxyl groups present on fatty acids and the carbohydrate backbone (122). This procedure was used to determine whether or not the hydroxyl group was esterified by another acyl chain in the case of 3-hydroxy fatty acids. Fatty acid methyl esters were made of the phenyl carbamate derivatized material. Comparisons in GC retention times were made with an *R. trifolii* fatty acid methyl ester standard. Phenylcarbamate derivatives have retention times that are slightly shorter than the corresponding 3-hydroxy compound. GC/MS was also used in determination of the extent of acyl substitution of fatty acids. MS of phenylcarbamate derivatives contain fragments assignable to the phenylcarbamate group: m/z=93 aniline, m/z=119 phenylisocyanate, m/z=137 phenylcarbamic acid. Without the phenylcarbamate group, 103 is the major ion. Comparisons of the rhizobial methyl ester standard for fatty acids and that of the phenylcarbamate derivatized material are shown in Figure 8.

Based upon comparison of GC retention times with a known standard and in ion fragmentation patterns from GC/MS, we concluded the following concerning *R. trifolii* ANU843 lipid A fatty acids: (i) The 27-hydroxyoctacosanoic acid is always linked at the 27-hydroxyl position, (ii) 3-hydroxytetradecanoic acid is found in the form of a free 3-hydroxyl group, and in a form substituted at the 3-hydroxyl position. GC analysis intensities indicated that the free hydroxyl is the predominant form (Figure 9), and (iii) 3-hydroxy-12-methyltetradecanoic acid is free at the 3-hydroxy position as evidenced by a change in GC retention time and the presence of characteristic ion fragments in GC/MS. We believe the 3-hydroxyhexadecanoic acid and the 3-hydroxyoctadecanoic acid are free at the 3-hydroxyl positions. Both were modified by phenylisocyanate, however the mass spectra lacked the characteristic ions of phenylcarbamate derivatives and indicated that

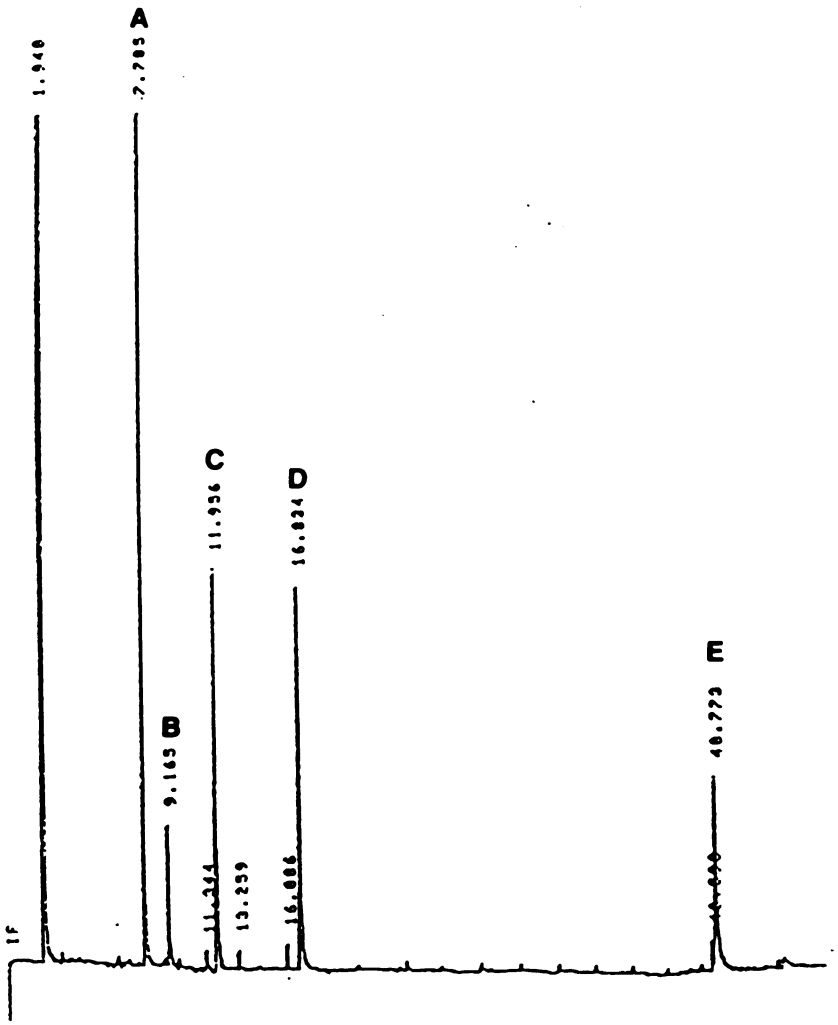


Figure 7

GC profile of fatty acid methyl esters derived from *R. trifolii*. (A) 3-hydroxytetradecanoic acid, (B) 3-hydroxy-12-methyltetradecanoic acid, (C) 3-hydroxyhexadecanoic acid, (D) 3-hydroxyoctadecanoic acid, (E) 27-hydroxyoctacosanoic acid.

Figure 8

8A: GC profile of fatty acid methyl esters derived from *R. trifolii* ANU843.

8B: GC profile of *R. trifolii* ANU843 fatty acid methyl esters previously derivatized with phenylcarbamate. Lower case letters are indicative of the corresponding phenylcarbamate ester.

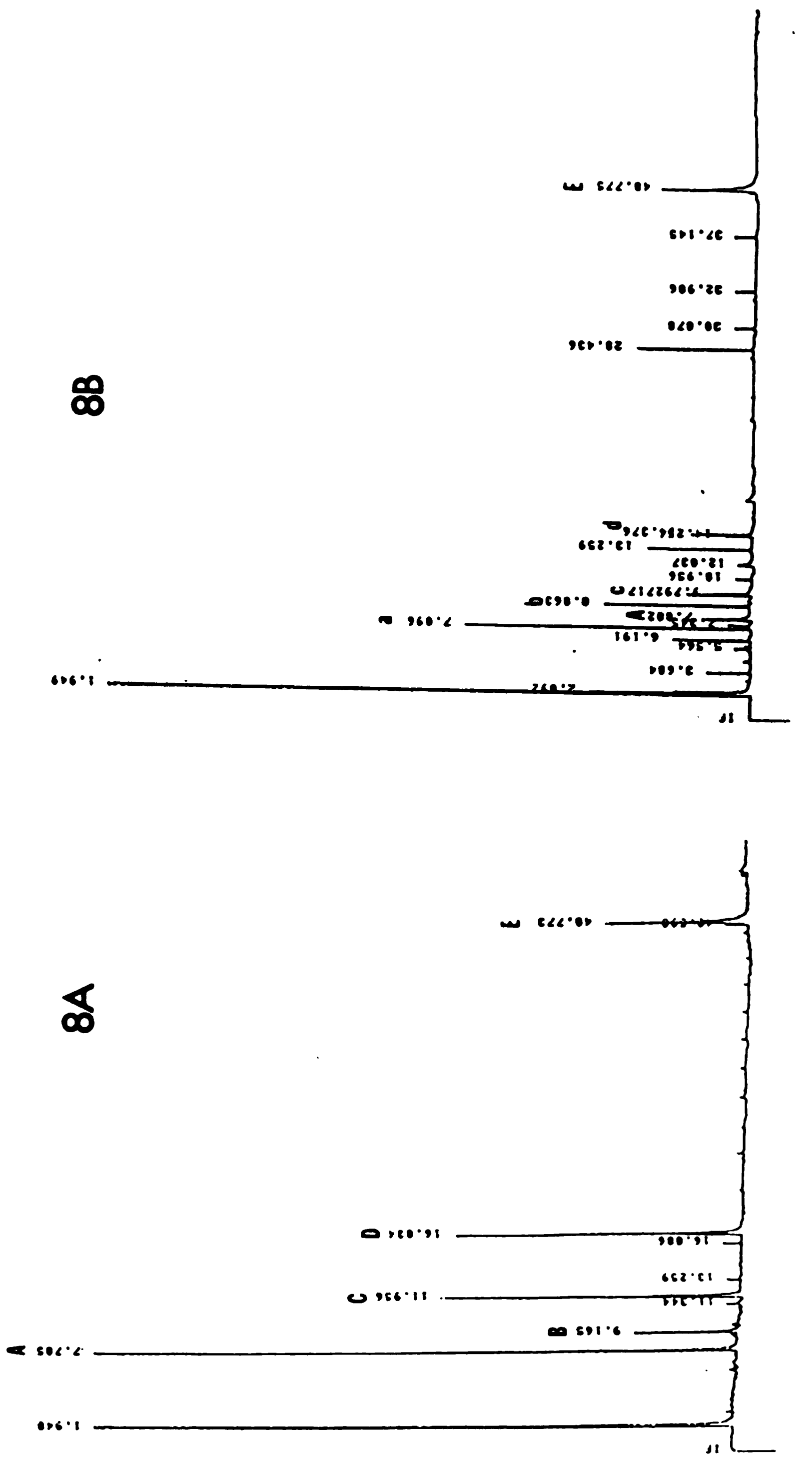
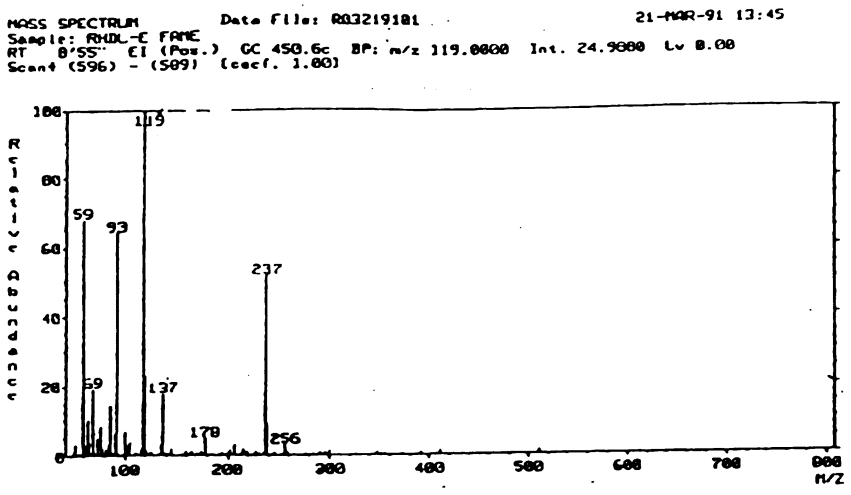
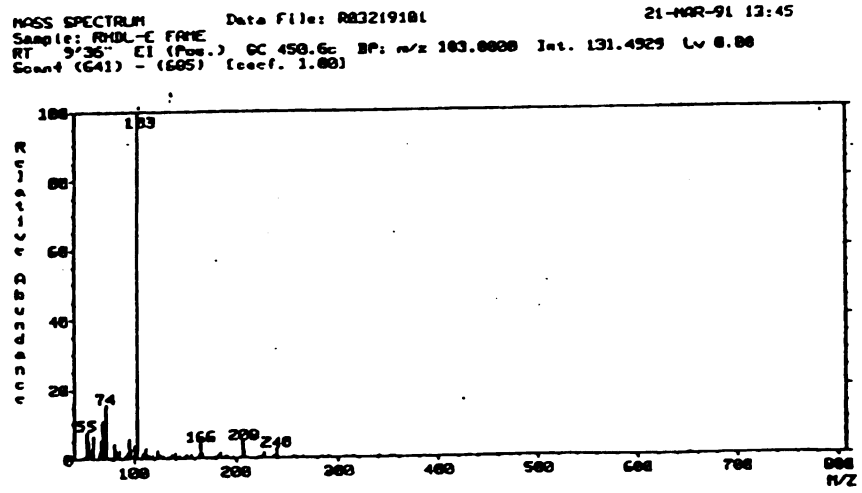


Figure 8



(9A)



(9B)

Figure 9

Electron impact mass spectra of both the (9A) phenylcarbamate derivatized 3-hydroxytetradecanoic acid, and (9B) 3-hydroxytetradecanoic acid, indicating it is linked at its 3-hydroxyl position.

they were converted to unsaturated acids by an elimination process.

Carbohydrate Analysis of *R. trifolii* Lipid A

Carbohydrates were identified as their alditol acetate derivatives by comparison of their retention times on the gas chromatograph with those of known alditol acetates and by analysis of their mass spectra. GC/MS of alditol acetates of lipid A without a carboxylic acid prereduction step, following conversion to the methyl ester, gave no desirable peaks. When lipid A was prereduced with sodium borohydride before hydrolysis and conversion to the alditols, two peaks were visible on the GC tracings. One of the peaks corresponded to glucosamine and the other one to galactose, (Figure 10). Since the glycosyl components could not be visualized as alditol acetate derivatives without prereduction, it was evident that they occurred as uronic acids. This was confirmed by deuterium labelling with sodium borodeuteride during prereduction before conversion to alditol acetates. The lipid A of *R. trifolii* contained galacturonic acid and comparitively large amounts of glucosamine. The relative composition of the crude lipid A preparation was 1:1 galacturonic acid to total amino sugar.

We wanted to examine the carbohydrate backbone of *R. trifolii* lipid A. The carbohydrate resonances were indistinguishable in ^{1}H-NMR spectroscopy because of the degree of aggregate formation. Several attempts were made to remove the fatty acid components from crude lipid A in order to liberate the free carbohydrate backbone. Saponification using 1N KOH in n-butanol was not successful and led only to partial saponification. No release of the 27-hydroxyoctacosanoic acid was observed. The 3-hydroxytetradecanoic acid, 3-hydroxy-12-methyltetradecanoic acid, 3-hydroxyhexadecanoic acid, and 3-hydroxyoctadecanoic acid were released, indicating that they are O-linked and confirming the belief that 27-hydroxyoctacosanoic acid is N-linked and more difficult to release. The GC tracings of the free fatty acids are shown in Figure 11. This suggests that the lipid A molecules formed aggregates in which the polar head group was inaccessible to nucleophiles. The aggregates were strong enough to prevent a very polar

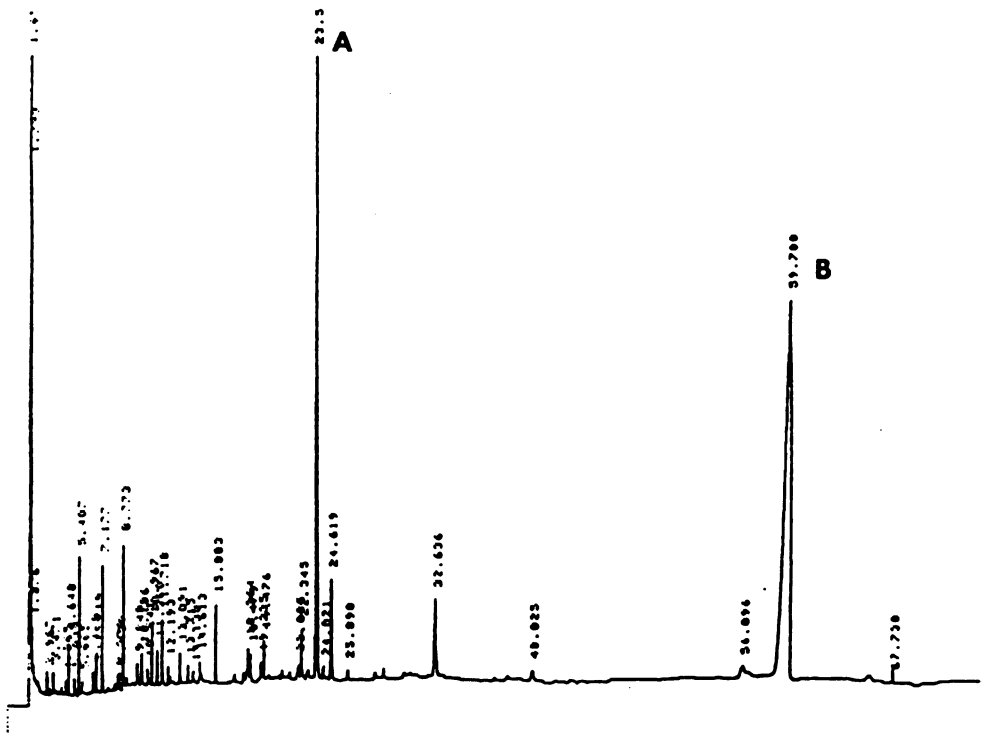


Figure 10

GC analysis of the alditol acetate derivatives of carbohydrates present in the crude lipid A of *R. trifolii* ANU843. Peaks A and B correspond to the alditol acetate derivatives of galactose and glucosamine, respectively.

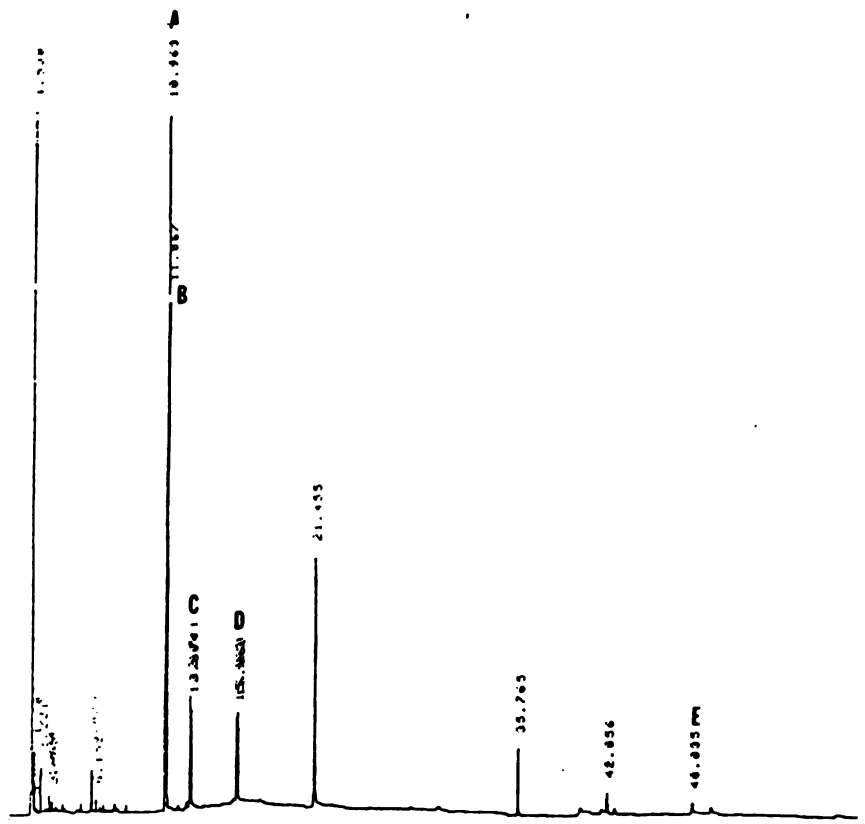


Figure 11

GC tracings of fatty acid methyl esters of fatty acid components liberated during KOH saponification. (Letters represent the same fatty acids as in Figure 7.)

nucleophile such as hydroxide anion from getting past the strong hydrophobic interactions of the fatty acyl groups attached to the lipid A head group.

We tried removal of fatty acyl groups with hydrazine. Hydrazine is not as polar as hydroxide anion, but it is a much better nucleophile. Hydrazine has also been effective in the cleavage of fatty acyl groups (123). We had hoped that hydrazine, being less polar, would bypass the strong hydrophobic interactions of the acyl groups and get near the carbohydrate head group to cleave the acyl moieties. We found hydrazine to be just as ineffective as hydroxide anion in deacylating rhizobial lipid A.

We tried a more non-polar reagent, tertiary butyl ammonium hydroxide, believing that the counterion would be non-polar enough to bypass the negative interactions of the hydrophobic acyl chains. Again, we report that tertiary butyl ammonium hydroxide was not effective in promoting saponification.

Crude lipid A was treated with methyl sulfonyl anion and methyl iodide, in an attempt to cleave ester/amide bonds and methylate the carbohydrate backbone to give linkage information. This procedure gave essentially the same results as KOH treatment. On this occasion, the free fatty acids were isolated directly as fatty acid methyl esters. ^{1}H-NMR spectra looked essentially the same as crude lipid A as far as carbohydrate resonances were concerned. The GC profile of fatty acids liberated as methyl esters is shown in Figure 12. Again, the 27-hydroxyoctacosanoic acid was missing, indicating the difficulty in which to remove it.

We have tried obtaining NMR spectra at higher temperatures (50°C) and in different solvents such as d-DMSO and d-pyridine. This enhanced the carbohydrate signals somewhat, but they were still broad since the long chain fatty acids were still present. The methods used on the "typical" lipid A were not effective on our lipid A molecules. *R. trifolii* ANU843 lipid A molecules seem to have very strong hydrophobic attractions for each other, thus causing the formation of very stable aggregates. It is the unique 27-hydroxyoctacosanoic acid that is the cause of these effects.

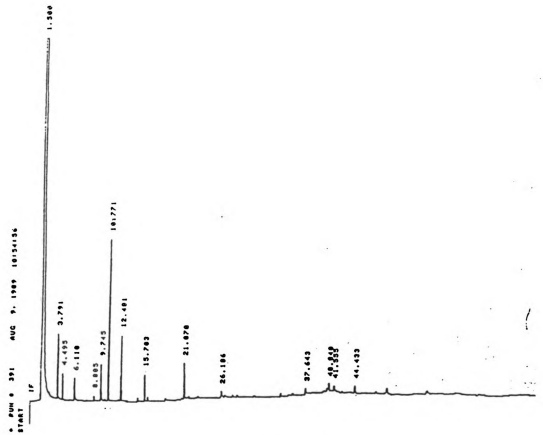


Figure 12

GC profile of *R. trifolii* ANU843 fatty acid methyl esters derived by treating crude lipid A with methyl sulfonyl anion and methyl iodide. Note the absence of 27-hydroxyoctacosanoic acid, indicating its amide linkage.

This aggregation problem has also made it difficult to obtain mass measurements of the rhizobial lipid A using fast atom bombardment mass spectrometry. Rhizobial samples have been submitted many times for mass analysis, and yet nobody was able to obtain a spectrum. This was again attributed to aggregate formation of lipid As, making it difficult for the sample to leave the matrix on the probe. The only person to successfully obtain a lipid A FAB/MS spectrum was Dr. R. I. Hollingsworth, and this data will be presented later.

Information regarding the anomeric position of the rhizobial lipid A was obtained from GC and GC/MS analysis of alditol acetates prereduced with sodium borodeuteride. The GC/MS fragmentation pattern for prereduced galacturonic acid indicated that the reducing end is tied-up as a glycoside. The ion fragments are two units apart for all major ions, indicating that one end of the molecule was labeled with deuterium. The fragmentation patterns for glucosamine showed that some of this carbohydrate was free, while some of the carbohydrate was found in glycosidic linkage at the anomeric position. This was evidenced by the presence of isotope peaks of equal intensity for the smaller mass ions. During the prereduction step using sodium borodeuteride, any free anomeric position would have been reduced to the alditol and thus contain one deuterium. This made the ion fragments from this end one unit greater than those molecules that were glycosidically linked and did not pick up a deuterium at this point. Furthermore, the ion fragments were identical to those of the glucosamine standard. Since the glucosamine standard was not subjected to a prereduction, and contains two protons at position C1, this corresponded to a glucosamine that was linked at its glycosidic position (Figure 13).

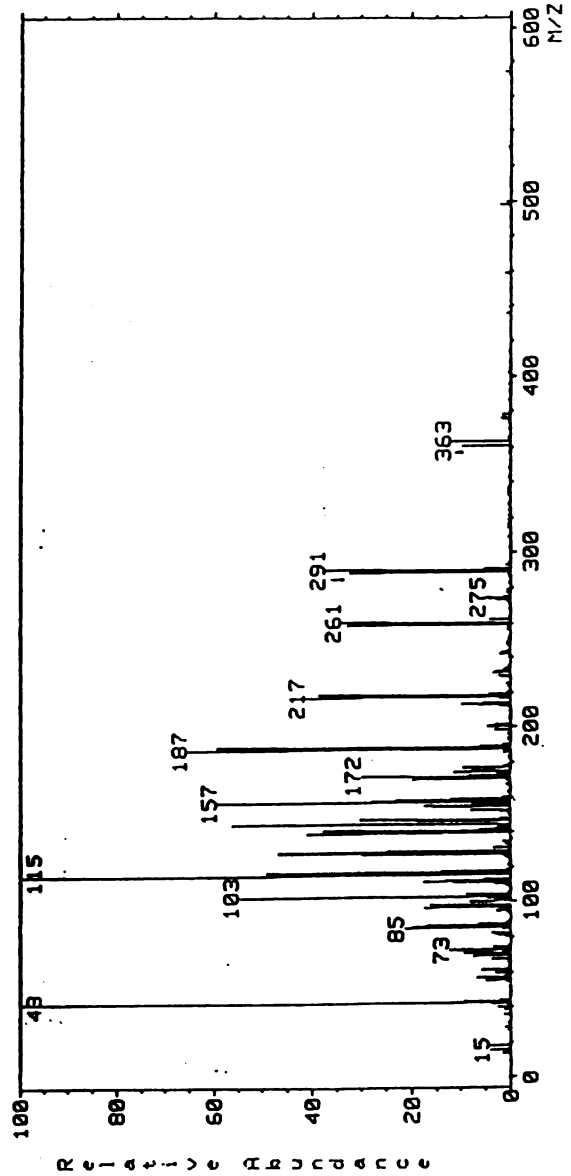
Our structural information suggests that *R. trifolii* lipid A carbohydrates are free and glycosidically linked at the anomeric carbon. Close examination of the ^{1}H-NMR spectrum for the lipid A that was treated with phenylisocyanate indicated that ethanolamine was present as a substituent at the anomeric site (Figure 14). ^{1}H-NMR

Figure 13

13(A) GC/MS fragmentation pattern for prereduced galacturonic acid. The ion fragments are two units apart for all major ions, indicating one end of the molecule was labeled with deuterium, and therefore the reducing end is tied-up as a glycoside.

13(B) GC/MS fragmentation pattern of glucosamine. The ion fragmentation pattern shows some of the carbohydrate to be free, as evidenced by the presence of isotope peaks of equal intensity for the smaller mass ions. Some carbohydrate was found in glycosidic linkage, as evidenced by identity of peaks with those of the glucosamine standard not containing deuterium.

13A



13B

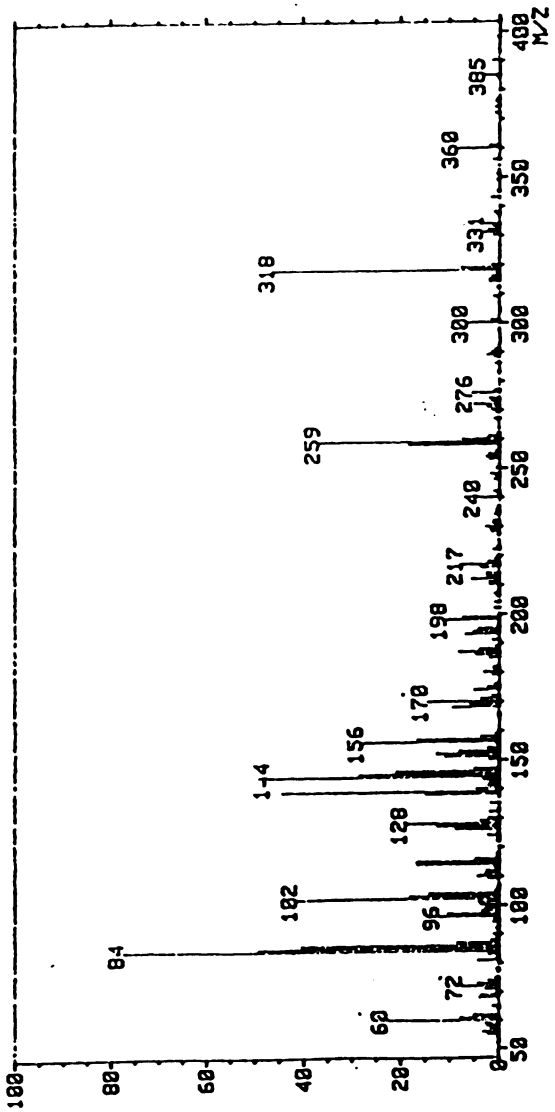


Figure 13

Figure 14

^{1}H-NMR spectrum for *R. trifolii* ANU843 lipid A treated with phenylisocyanate, indicating the presence of ethanolamine at the anomeric site.

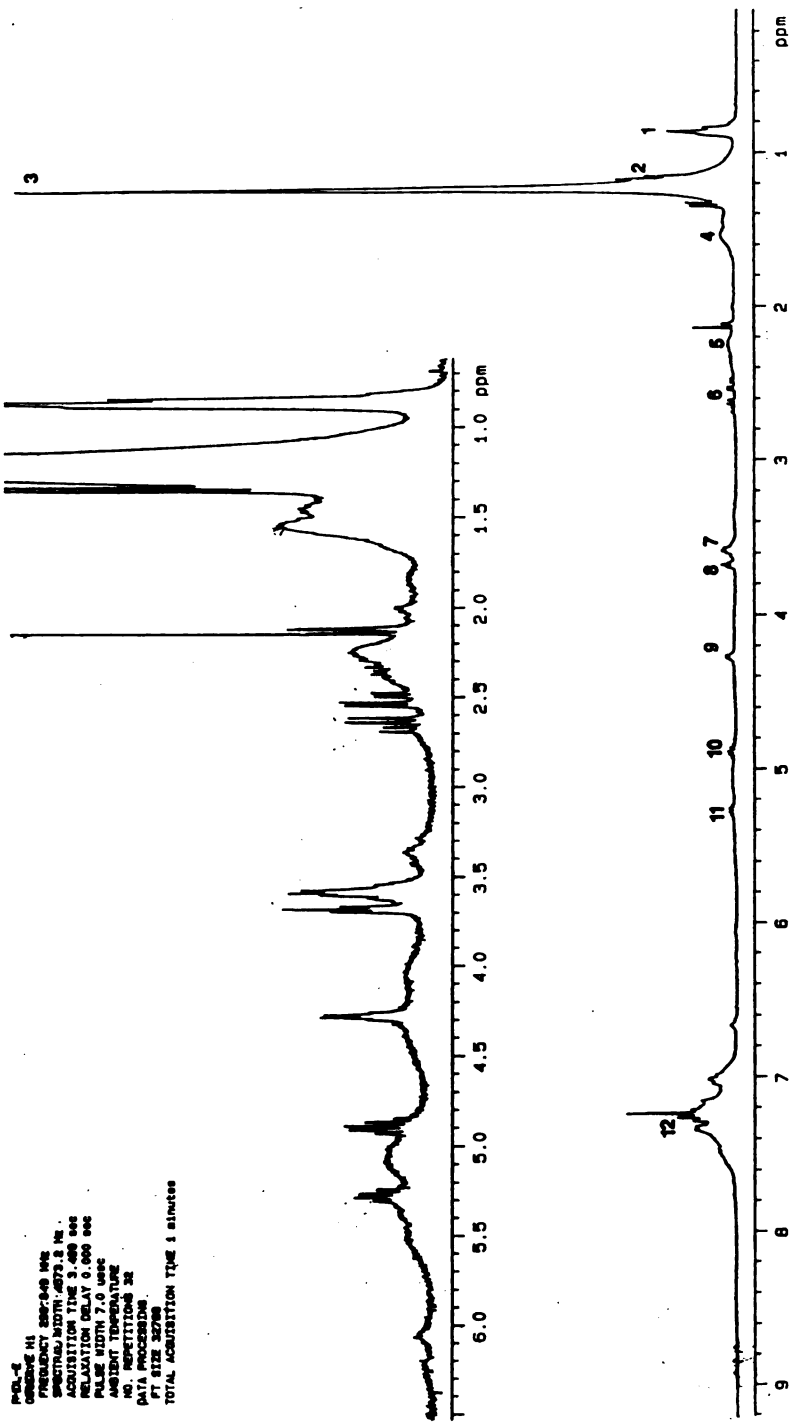

PDL-4
CDCl3CHI
FREQUENCY 300.548 MHz
SPECTRAL WIDTH: 4873.8 Hz
ACQUISITION TIME 3.400 sec
RELAXATION DELAY 0.000 sec
PULSE WIDTH 7.0 usec
AMBIENT TEMPERATURE
NO. REPETITIONS 32
DATA PROCESSED.
FT SIZE 32768
TOTAL ACQUISITION TIME 1 minutes
ppm
1 CH3-CH2-CH2-
2 CH2-CO-
3 CH2 of long chain fatty acids
4 CH2-CH2-CH2
5 -CH2-CO- 27-hydroxyoctacosanoic acid
6 -CHOCH2-CO
7 -OCH3
8 -NHCH2-CH2-O-
9 -OCH2-CH2-NH-
10 -CHOCH2
11 -CHOCH2-CO-
12 ring protons phenylcarbamate

Figure 14

signals correspond to: signals at 1.9ppm (CH_3 on the end of fatty acyl chains), 1.0 - 1.4ppm (CH_2 of acyl chains), 1.0ppm (CH_3-CHO-CH_2 for C28), 1.5ppm (CH_2-CH_2-CHO), 2.3ppm (CH_2-CO for C28), 2.4-2.7ppm (CHO-CH_2-CO), 3.5-3.6ppm (OCH_3), 3.6-3.7ppm (NH-CH_2-CH_2-O), 4.2-4.4ppm (O-CH_2-CH_2-NH), 4.8-4.9ppm (OCH-CH_3) 5.2-5.4ppm (OCH-CH_2-COO), 7.0-7.8ppm (ring ^{1}Hs of phenylcarbamate). Selective proton decoupling of the above peaks gave coupling patterns that agree with the above peak assignments.

In order to prove the presence of ethanolamine at the anomeric position, we attempted to synthesize a structural analog from glucose containing ethanolamine functionalized as a phenylcarbamate derivative at the anomeric position. In this way we could compare the chemical shifts of NMR signals of the synthesized product with that of rhizobial lipid A. Unfortunately, we were not successful in this endeavor.

Studies on purified lipid A components of crude lipid A were much more rewarding. Crude lipid A was fractionated on a C_{18} reverse-phase column by HPLC. Fractions were eluted from the column with water, 2:1 water/methanol, 2:1 methanol/ water, pure methanol, and pure chloroform. The pure methanol fractions were combined, subjected to preparative TLC on C_{18} reverse-phase plates, and two bands reacting positively with orcinol spray were scraped and eluted with 1:1 chloroform/methanol.

The presence of carbohydrate and fatty acyl components were seen in the ^{1}H-NMR spectrum of the methanol fraction from C_{18} reverse-phase separation of crude lipid A, (Figure 15). Resonances between 0.7 and 0.9ppm, 1.1 and 1.3ppm, 2.1 and 2.5ppm, and 3.2 and 5.3ppm correspond to methyl resonances of fatty acyl components, methylene groups of fatty acyl groups, methylene groups next to carbonyl functions, and glycosyl resonances and resonances due to protons attached to other hydroxylated carbons, respectively. The presence of resonances for only one glycosyl residue support a monosaccharide component in the methanol fraction of the lipid A components obtained from HPLC separation. GC/MS analysis of the fatty acid methyl esters liberated

Figure 15

Proton NMR spectrum of the methanol fraction obtained from C_{18} reverse-phase chromatography of *R. trifolii* ANU843 crude lipid A. Fatty acid proton resonances occur between 0.7 and 2.5 ppm. Carbohydrate resonances are between 3.2 and 5.3 ppm.

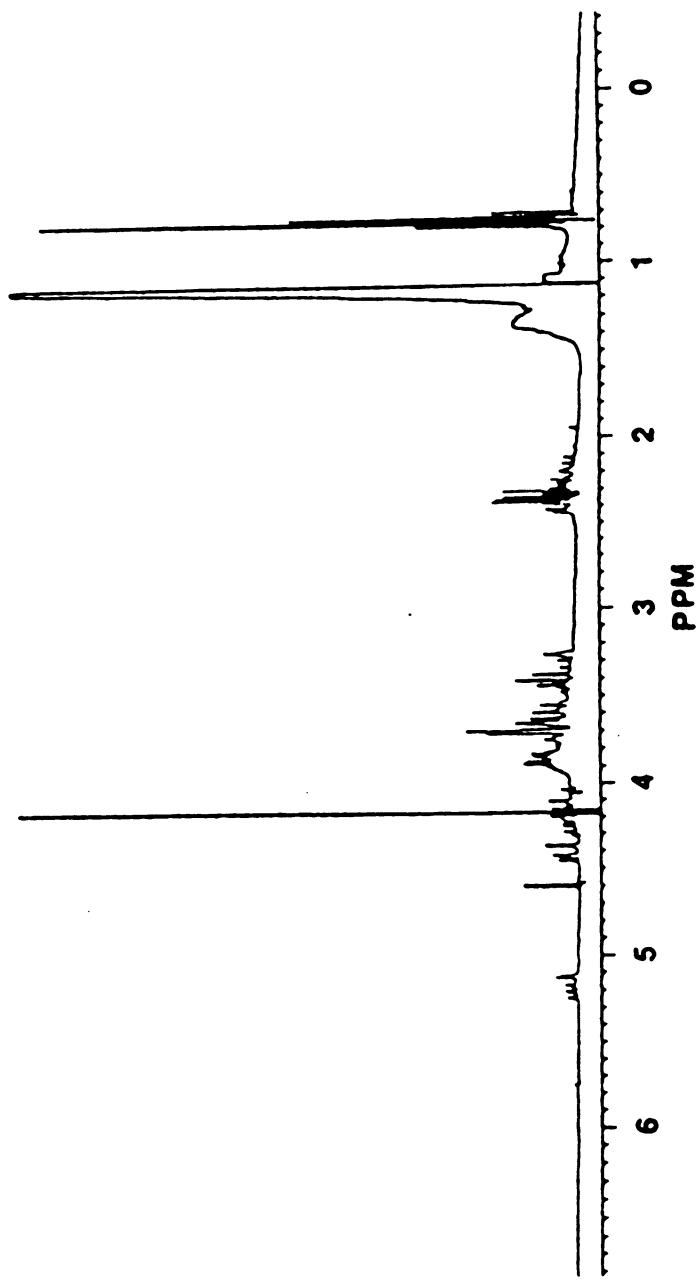


Figure 15

by acid catalyzed methanolysis indicated that the major fatty acyl components of the methanol fraction were 27-hydroxyoctacosanoic acid and the 3-hydroxytetradecanoic acid.

^{13}C-NMR analysis supported the presence of a monosaccharide component in the methanol fraction, (Figure 16). Resonances were seen at 99.99 and 97.96ppm, corresponding to ß and α carbon resonances of a reducing glycosyl residue. No other glycosyl carbon resonances were observed. A signal at 54.10ppm was assigned to a carbon atom attached to nitrogen. This indicated the presence of a single amino sugar and not a glucosamine disaccharide.

As was previously mentioned, TLC of the methanol fraction yielded two major components. The ^{1}H-NMR spectrum of the faster moving band appeared very similar to the parent compound, (Figure 17). A downfield triplet present in the spectrum of the parent compound is missing in the spectrum of this fraction. This proton could be assigned to an axial ring proton. Its chemical shift may be due to acylation at the carbon this proton was attached to. Absence of this resonance in the faster migrating TLC band was indicative that this molecule lacked an acyl residue. Fatty acid analysis indicated that 27-hydroxyoctacosanoic acid was the only fatty acid present. Pseudo-molecular ions at m/z=616 and m/z=638 ($[M+H]^+$ and $[M+Na]^+$) were observed in the FAB/MS of the faster moving TLC component, (Figure 18). This was indicative of a molecular weight of 615.

The second TLC component was the major band obtained. In the ^{1}H-NMR spectrum, the downfield triplet was present as in the parent compound, indicating the presence of an O-linked acyl residue, (Figure 19). GC analysis of the alditol acetates in this fraction showed glucosamine after prereduction. This indicated the presence of the uronic acid of glucosamine in the lipid A. This sugar residue correlated with the FAB/MS data of this second TLC component. The spectrum contained ions at m/z=842 and m/z=864. This corresponded to $[M+H]^+$ and $[M+Na]^+$ to give a molecule with a

Figure 16

^{13}C-NMR spectrum of the methanol fraction from C_{18} reverse-phase chromatography. Signals at 99.99 and 97.96 ppm are due to ß- and α-forms of the anomeric carbon of the glycosyl component. The signals in the 60-80 ppm region confirm the presence of one glycosyl component. There is one carbon signal at 54.1 ppm attributable to a carbon bearing nitrogen.

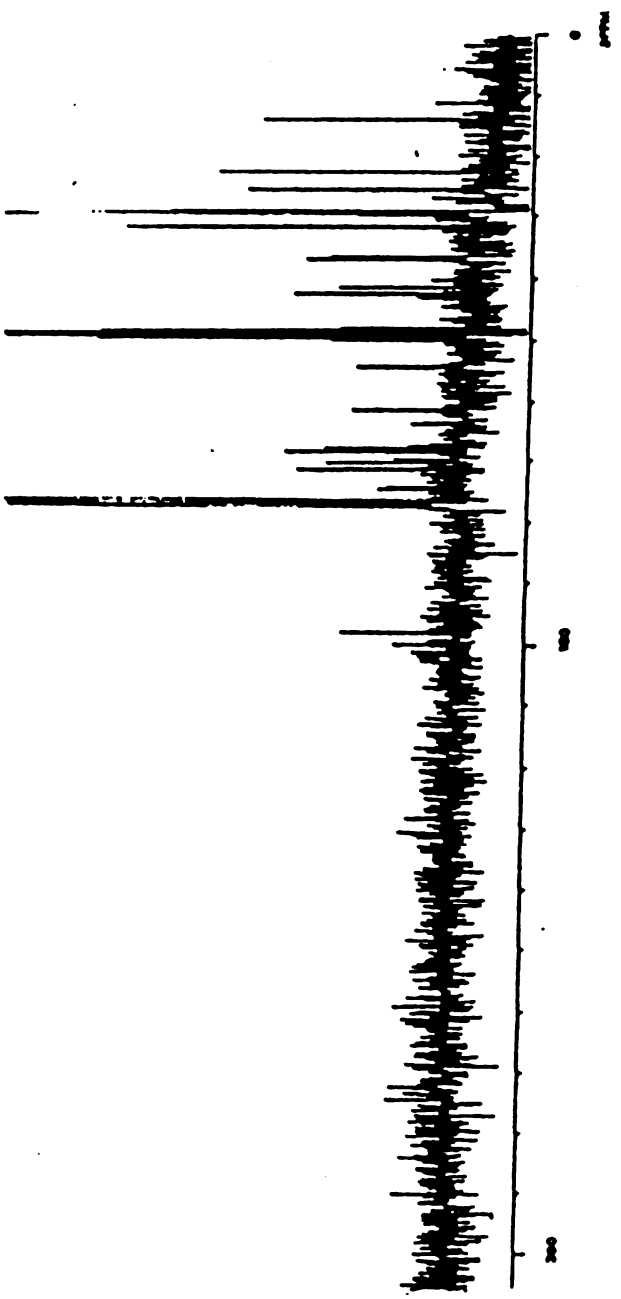


Figure 16

Figure 17

Proton NMR spectrum of the faster moving band on preparative TLC analysis of lipid A. The triplet at 5.21 ppm present in the parent compound is missing, indicating the lack of acyl substitution. Intense signals between 3.1 and 3.4 ppm are due to traces of methanol and other hydroxylated species such as moisture.

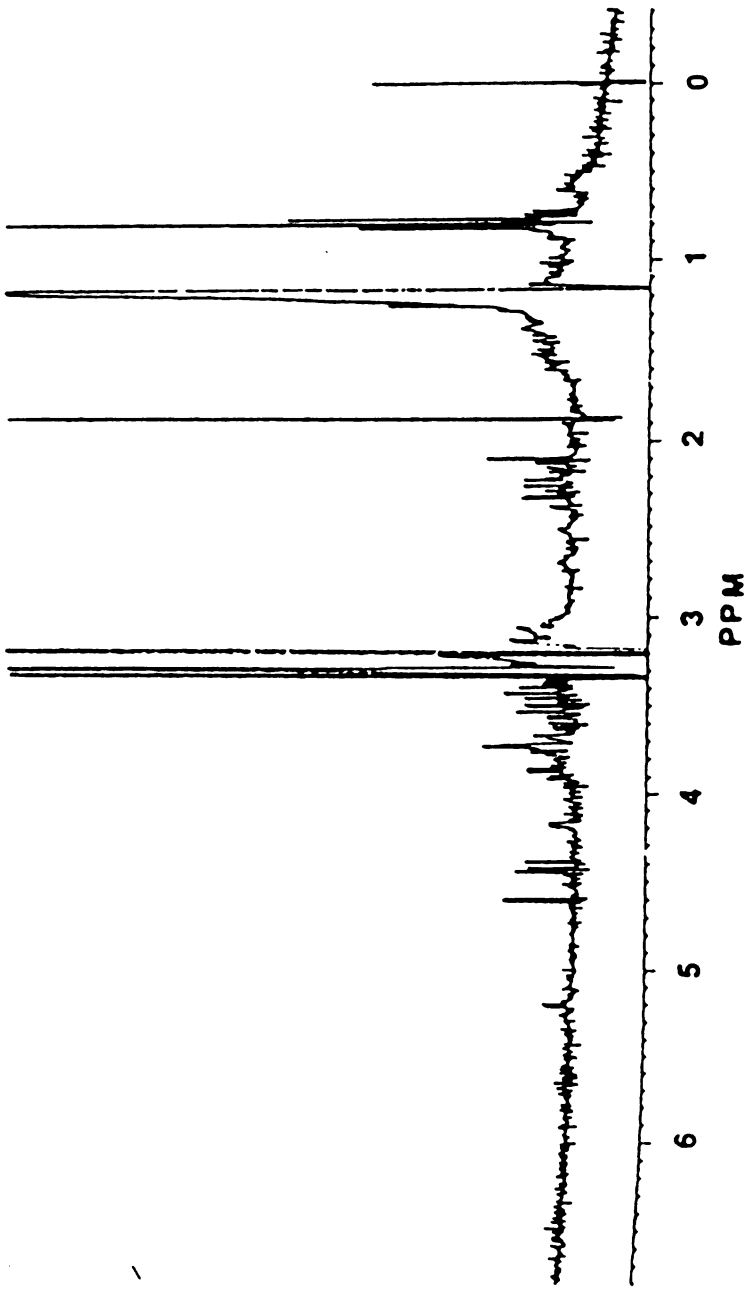


Figure 17

Figure 18

Fast atom bombardment mass spectrum of the faster moving TLC band. The pseudomolecular ions appear at m/z=616 ($[M+H]^+$) and m/z=638 ($[M+Na]^+$).

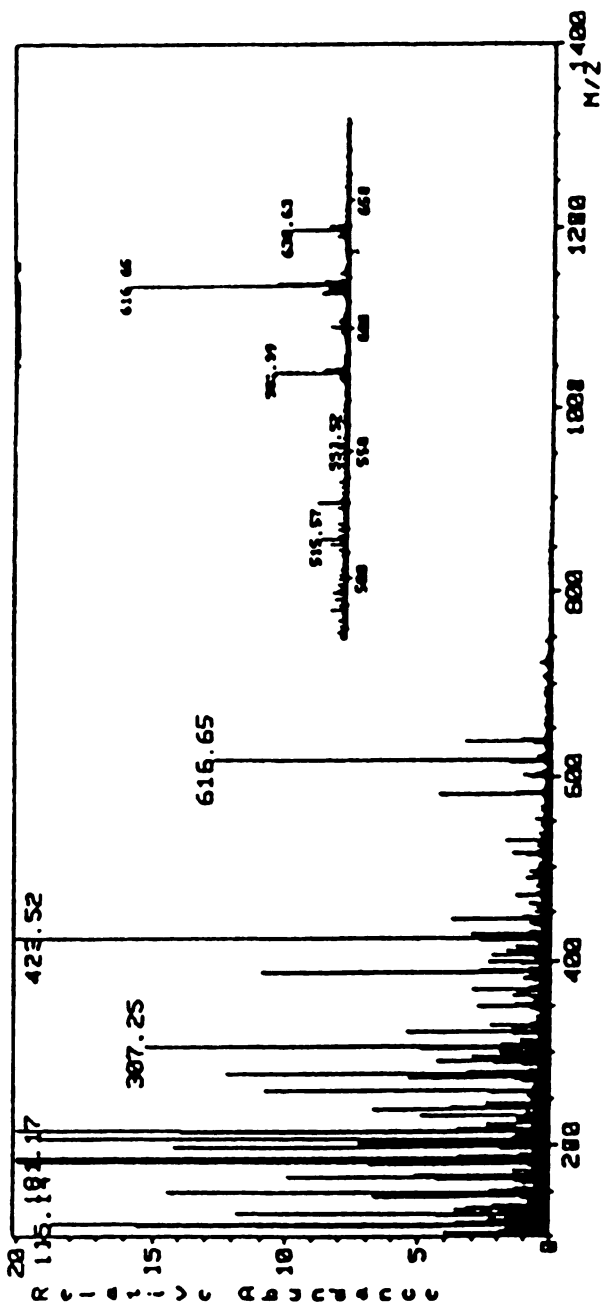


Figure 18

Figure 19

Proton NMR spectrum of the slower moving band of preparative TLC. Note the presence of a triplet at 5.22 ppm, indicative of acylation.

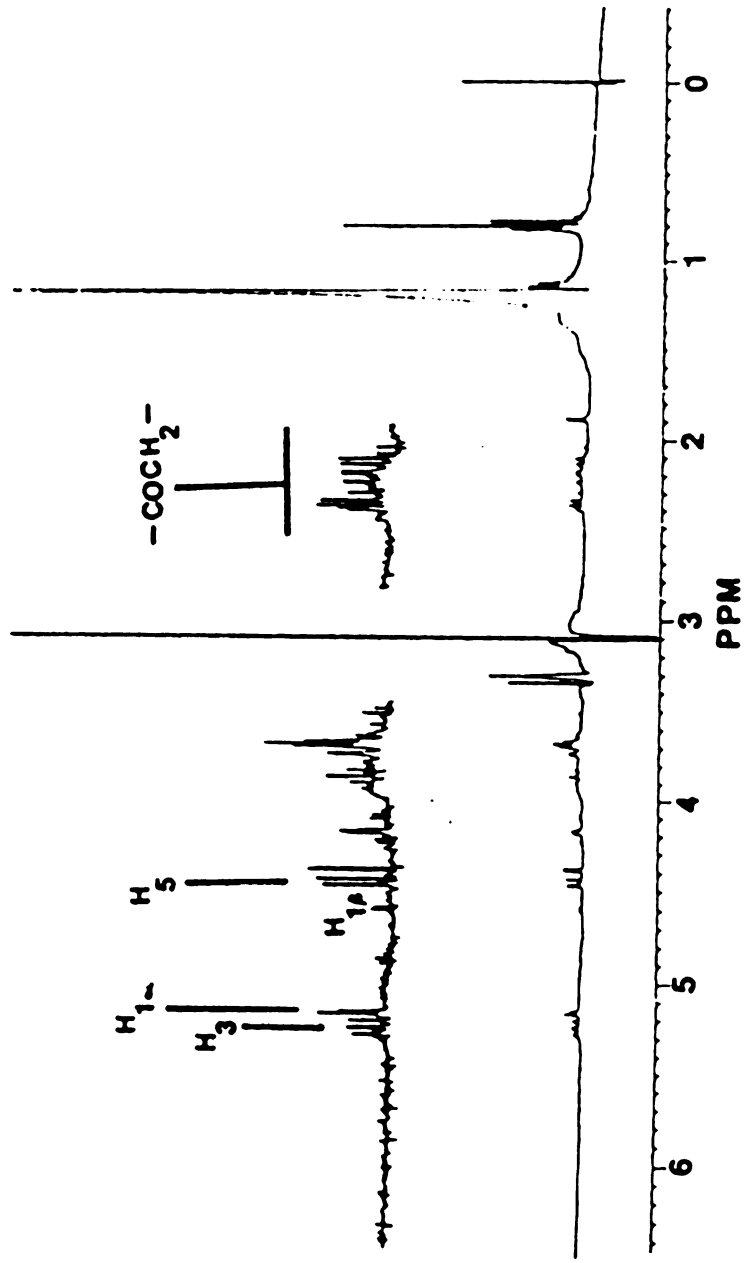


Figure 19

Figure 20

Fast atom bombardment mass spectrum of the slower migrating TLC band. Pseudomolecular ions appear at m/z=842 and m/z=864.

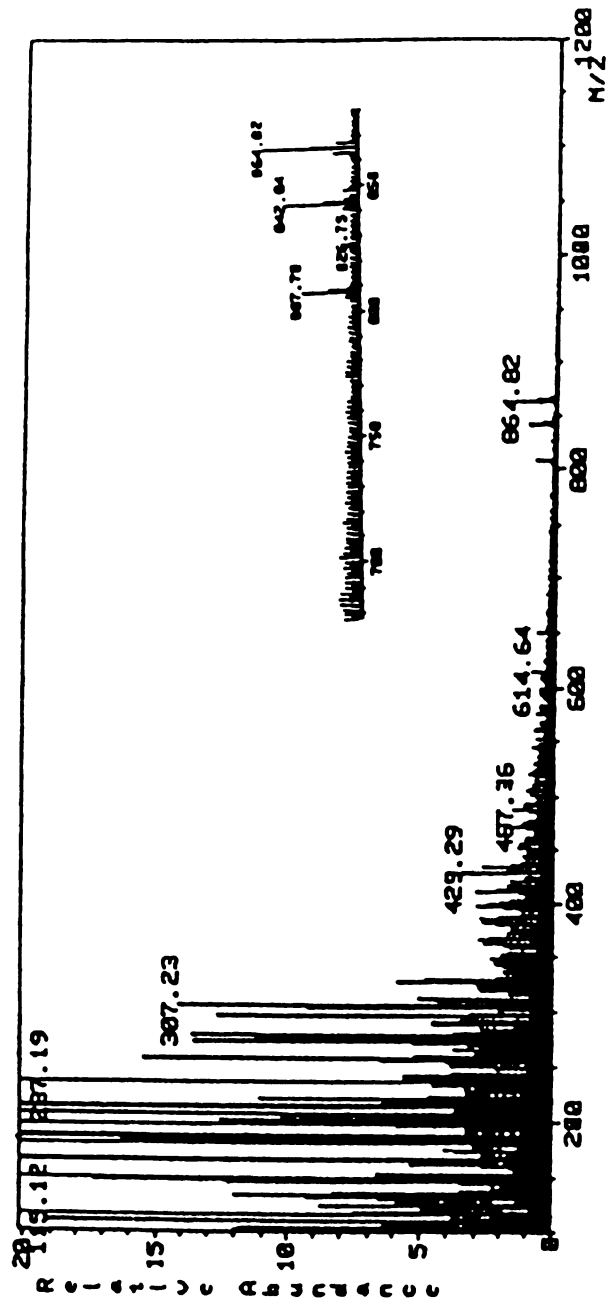

Relative Abundance
m/z
307.23
429.29
487.35
614.64
864.82
847.79
842.84
825.13
841.82
200
400
600
800
1000
1200
5
10
15
20

Figure 20

molecular weight of 841, (Figure 20). This corresponded to the molecular weight of the first TLC component after substitution by a 3-hydroxytetradecanoyl residue. Mild methanolysis of the second TLC fraction liberated a 3-hydroxytetradecanoyl residue, thus confirming that an amide linkage binds the 27-hydroxyoctacosanoyl residue to the carbohydrate backbone of *R. trifolii* ANU843 lipid A.

FAB/MS showed that neither of these molecules are phosphorylated. This agreed with our results of the absence of phosphate in *R. trifolii* ANU843 LPS and that of other literature citings (110,111).

Conclusions Regarding *R. trifolii* ANU843 Lipid A Structure

The lipid A component of endotoxin is responsible for the diverse biological effects brought on by this molecule. Whether lipid A acts directly or indirectly remains to be determined. Lipid As extensively studied from enteric organisms such as *Salmonella* and *E. coli* and non-enteric genuses such as *Pseudomonas* and *Vibrio* all possess the same basic structure. Lipid As with varient structures are considered non-toxic and do not induce biological activity or induce attenuated effects.

Free lipid A obtained from chemical treatment of LPS contains several components which vary in structure due to natural heterogeneity and also because of degradation. Hence, the classical lipid A will typically contain monophosphorylated and diphosphorylated backbone variations of head group substitution by polar substituents, heterogeneity of chain length and variation in degree of and site of substitution by fatty acids. These factors make purification of pure homogeneous species very difficult and a synthetic route to pure species is very often preferred (34,124,125).

Rhizobial LPS was previously shown to elicit some of the classical endotoxin activities such as elicitation of positive LAL and Schwartzmann reaction responses and the ability to act as a mitogen. At the time, it was assumed that *Rhizobium* lipid A had the same structure as enteric lipid A, based upon its ability to bring about these classical endotoxin effects.

One of the major questions addressed by this research was : Does the classical lipid A structure apply to the genus *Rhizobium* ? We have previously mentioned conflicting reports in the literature addressing this issue. Data obtained from our study showed that the usual lipid A model does not apply to the lipid A of *Rhizobium trifolii* ANU843.

Work in this lab on crude lipid A has confirmed earlier observations that *R. trifolii* ANU843 LPS contains an unusual, branched fatty acid as well as another unusual fatty acid component, 27-hydroxyoctacosanoic acid (109). Gas chromatography of fatty acid methyl esters of the LPS of *R. trifolii* clearly shows that this fatty acid is a major component of the lipid A of this organism. Neither of these fatty acids had been reported before in gram-negative bacteria, thus making the lipid A of *R. trifolii* unusual in its structure. This finding has been confirmed by Urbanik-Sypniewska et. al., who found this same fatty acid in *Rhizobium meliloti* LPS (126). The long chain fatty acid has also been found in other nitrogen-fixing organisms (127).

The lipid A we obtained from *R. trifolii* ANU843 contains several components when analyzed by TLC. We have isolated and completely deduced the structure of a 2-amino-2-deoxy-2-N-(27-hydroxyoctacosanoyl)-3-O-(3-hydroxytetradecanoyl)-gluco-hexuronic acid and its de-O-acylation product from the free lipid A of *R. trifolii* ANU843 (114). These components were found in the methanol fraction from C_{18} reverse-phase chromatography of the crude lipid A and are known to be minor components, especially since they lack the other 3-hydroxy fatty acid components, (Figure 21).

Other evidence of these structures being minor components comes from the phenylcarbamate derivatization of fatty acids. In this experiment the 27-hydroxyocta-cosanoic acid was shown to be linked to another fatty acid at its 27-hydroxyl position. There was no evidence of the free 27-hydroxyoctacosanoic acid as was isolated in the minor lipid A components fully characterized. In experiments utilizing a sodium boro-

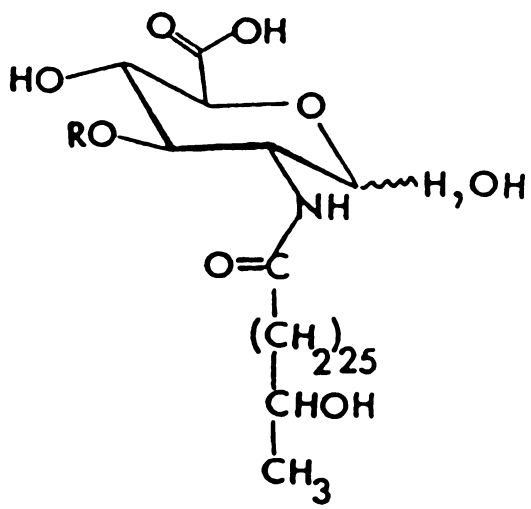


Figure 21

Structure of 2-amino-2-deoxy-2-N-(27-hydroxyoctacosanoyl)-3-O-(3-hydroxytetradecanoyl)-gluco-hexuronic acid and its de-O-acylation product when R=CH_3-$(CH_2)_{10}$-CHOH-CH_2-CO; R=H, respectively.

deuteride prereduction step to determine the status of the anomeric carbon of the rhizobial lipid A carbohydrates, no evidence of the uronic acid of glucosamine was found.

After prereduction of the crude lipid A of *R. trifolii* ANU843, followed by hydrolysis, reduction, acetylation, and GC/MS analysis, we found another major carbohydrate component of the lipid A to be galacturonic acid as a major constituent of the lipid A head group (52). Since then, another group has also found galacturonic acid to be a major constituent of rhizobial lipid A (127). Prereduction using sodium borodeuteride showed that glucosamine was also a major component of the amino sugar found in *R. trifolii* . The composition of our crude lipid A preparation is approximately 1:1 galacturonic acid to amino sugar.

I propose that one of the major lipid A structures found in *R. trifolii* consists of a disaccharide composed of glucosamine as the reducing end sugar, linked glycosidically to the anomeric position of galacturonic acid. This is based upon the following results. When crude rhizobial lipid A is not prereduced before derivatization to the alditol acetate, nothing is seen by GC analysis. Uronic acids are known to be difficult to cleave under acidic conditions. Thus, glucosamine was not liberated during acid cleavage and remained bound to galacturonic acid. This type of molecule would not be derivatized properly and if it were, would not be easily eluted from the GC column. When the uronic acid was converted to the methyl ester and then prereduced, both galactose and glucosamine alditol acetates were liberated. Remember, experimental evidence showed galacturonic acid to be linked at its C1 position, and therefore it cannot be at the reducing end. Glucosamine was shown to be linked at the reducing end in the same experiment. We also know that ethanolamine was shown to be a substituent in NMR experiments. The ethanolamine most likely is linked to the reducing sugar, glucosamine, at position C1. This substituent would have been hydrolyzed during the derivatization procedure for alditol acetates and would not have shown up in GC/MS analysis. This scenario also applies to galacturonic acid-galacturonic acid-ethanolamine.

Another possibility for a rhizobial head group is a disaccharide of galacturonic acid and glucosamine, but this time they are linked through both anomeric positions, as are glucose and fructose in sucrose. Again, absence of prereduction would result in no loss of glucosamine. When prereduction does occur, one would liberate the alditols of galacturonic acid and glucosamine. Again, this fits the model of galacturonic acid and glucosamine being linked at their anomeric positions. One might also have galacturonic acid linked to galacturonic acid through their anomeric positions.

There is also evidence of glucosamine being free at the anomeric position. This could be explained by galacturonic acid-glucosamine, free at the reducing end. We do not have a glucosamine disaccharide nor a glucosamine-galacturonic acid. Without prereduction one would still liberate the alditol acetate of glucosamine. This did not happen, so they cannot be present.

R. trifolii LPS was found to be completely devoid of phosphate. This result is in agreement with the findings of Zevenheuzen et al. (111), who studied the hexosamine content of *Rhizobium* LPS. They found a low hexosamine content of rhizobial LPS as compared with corresponding values for enteric LPS. They suggested that the lipid A portion of *Rhizobium* is essentially different from that of the *Enterobacteriaceae* and that it must have a different backbone. They also concur with the results of Humphrey and Vincent (110), who found a low phosphorous content in rhizobial LPS and furthermore suggested that the small amount of phosphorous present was due to contamination by phospholipids and nucleic acids. All of these phosphorylation results are at variance with the model of the typical lipid A.

All of our results differ with those obtained by the group of Russa et al. (112). This group of investigators studied two wild type and two mutant strains of *R. trifolii* and concluded that the lipid A of *Rhizobium* contains glucosamine disaccharide, phosphate, and no unusual fatty acids. Their study relied heavily upon chromatographic comparisons of their lipid A sample with enteric lipid A samples. Their approach does

not allow for possibly finding structurally different lipid As if the differences do not significantly contribute to chromatographic or electrophoretic mobility of the molecule.

As stated previously, *R. trifolii* lipid A was found to elicit some of the classical endotoxin phenomena such as LAL response, Schwartzmann reaction, and the ability to cause B-cell proliferation. However, we now know that *R. trifolii* has a radically different structure from that of the classical endotoxin with respect to carbohydrate content, fatty acid content, and degree of phosphorylation. How does this lipid A varient form relate to the classical endotoxins?

Structural analogs of endotoxin have shown that the lengths of fatty acyl chains are important in rendering the molecules toxic. It has long been accepted that the acyl chains may vary in length between 12 and 18 carbons in length. The presence of a 28-carbon long fatty acid is inconsistent with the traditional endotoxin model. Rhizobial lipid A molecules, being membrane derived, must contribute to the architecture of the outer membrane of these organisms. We propose the 27-hydroxyoctacosanoic acid normally spans the outer membrane of *Rhizobium* in a trans-membrane orientation. This is consistent with what is known about the ultrastructure of these organisms (128). It has been shown to be impossible to effectively freeze fracture the outer membrane of these organisms (52). R. I. Hollingsworth had previously demonstrated that the 27-hydroxyl group in 27-hydroxyoctacosanoic acid was esterified by another 3-hydroxy fatty acid (109). Our phenylcarbamate derivatization also supports this, as it showed the 27-hydroxyl position to be esterified. An arrangement where a 3-hydroxytetradecanoyl residue esterifies the 27-position of this long chain fatty acid would give a structure where two fatty acid chains meet in the middle of the membrane and one chain completely traverses it, (Figure 22).

This unusual arrangement of fatty acids in the membrane of *Rhizobium* has been proposed as an obligatory requirement for their viability in their natural environment. This structure may pack very well into the membrane and serve as a permeability barrier

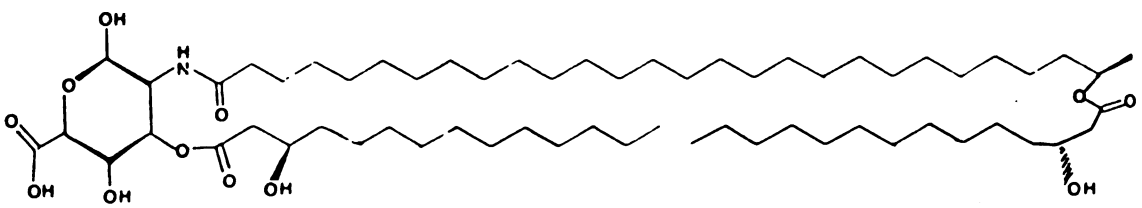


Figure 22

Structure of a possible lipid A component or substructure from *R. trifolii* ANU843 LPS. Notice the two shorter acyl chains meet in the middle of the long chain, transmembrane fatty acid.

to oxygen while nitrogen fixation occurs. From our chemical analyses, we know that these molecules form very stable aggregates. The usual procedures to chemically modify endotoxins do not work on these molecules. The length of the hydrophobic region of the molecule displayed in Figure 22 is 37 Angstroms, exactly the dimensions of the hydrophobic region of a typical membrane bilayer. A structural requirement necessary for a transmembrane component would be presence of polarity at both ends. This requirement is met by the structure shown in Figure 22.

Rhizobial lipid A completely violates the "law" that endotoxins must possess phosphate substituents. When comparing the rhizobial and enteric lipid As however, there is a unifying feature of negative charge. Both the classical lipid A molecule and the rhizobial lipid A have charged backbones. LPS serves to protect gram-negative organisms from their environment. If the matrix required is a negatively charged mass, cross-linked by divalent cations, then the bacterium will use those building materials more readily available to it. A soil bacterium might choose to use a carbon source as its charged element, such as carboxylate, since phosphate would be more importantly used for nucleic acid biosynthesis. A bacterium which exists in the digestive tract has an unlimited source of phosphate from plant and animal materials. There is a greater chance that phosphate would be used as the charged element, in this case. The final product synthesized by both organisms could function in the same manner. An external receptor system on target cells may have difficulty distinguishing between the two.

This has led us to propose a model for the interaction of LPS with target cells. It is the same model as that proposed by Diane Jacobs (67) and Larsen et al. (65). This is not the model that is most widely accepted by the endotoxin community.

On the cell there are mediators/receptors (probably proteins) which recognize certain structural features of the LPS complex. Not every LPS aggregate from varying bacteria will be identical, but the mediators will recognize certain structural features of the LPS complex. The target cell will react to a three-dimensional matrix of charge,

which can be factored out of both the rhizobial and enteric aggregates.

After recognition, the lipid A aggregates fuse with the target cell plasma membrane. Analogs having greater than 18 carbon atoms or less than 12 atoms, have been shown to induce little or no activity. This does not appear to be the case for *R. trifolii* LPS. For the analogs, stable integration into the bilayer is not possible since the chains are too long for one leaflet and too short to span the membrane or get stuck in the middle of a leaflet since they are too short for it. They create disorder which leads to improper packing and a marked increase in fluidity. This is not true for the rhizobial lipid A, for it has been shown that its fatty acids successfully cross the membrane, based upon computer analyses.

We are looking at charge, packing, and overall conformation of the LPS/lipid A molecule, instead of just which sugars are present, or how long the fatty acid chains are. We have studied how these molecules interact with mammalian cells and what these radically different structural features do to the ability of rhizobial LPS to behave as a typical endotoxin molecule. The biological aspects of this project are discussed in the following chapter.

Chapter Five: Biological Responses Induced by *Rhizobium trifolii* ANU843 LPS

Introduction

It is known that LPS is capable of causing a wide variety of biological effects in mammalian systems. Some of these effects are beneficial such as proliferation of B-cells, immunoglobulin synthesis, and tumor necrosis (7,8,9,10,11). On the other hand, these molecules are potent toxins capable of fever induction, vascular damage, and causing death of the infected host (12,13). Molecules with the classical endotoxin structure, as previously described in this text, are able to bring about all of these effects, both good and detrimental. Those molecules having varient lipid A structures are considered non-toxic or less toxic compared to their enteric counterparts.

LPS from *R. trifolii* , as shown by Dazzo et al., exhibited some of the classical endotoxin activities (52,106,107). We now know that this lipid A has a radically different structure from that of the enterics, and this was described in the previous section of the text. We set out to determine which of the classical endotoxin responses are conserved by the LPS of *R. trifolii* . The data obtained provide insight into the mechanism of LPS action.

In order for LPS to exert its effect upon mammalian systems, it must successfully interact with the plasma membrane of its target cell. One set of experiments comparing enteric and rhizobial LPS involved the binding of fluorescently labeled LPS to various eukaryotic cell lines and examining the extent of this binding.

We followed the theory which suggests that LPS associates with cells in a manner that is indicative of a two-step process (66,67). The first step involves association of the LPS aggregate with the cell surface as mediated by electrostatic interaction. LPS, being negatively charged, associates with positively charged species on the cell surface. This association increases the thermodynamic probability of intercalation of LPS monomers into the cell's plasma membrane. This coalescence is considered to be the second step.

Intercalation of the lipid A will decrease the fluidity of that region of the plasma membrane, and this decrease in membrane fluidity leads to cellular activation. Increase in microviscosity of the lipid bilayer, as caused by LPS, has been demonstrated by Larsen et al. and Price et al. (65,68). Both groups monitored the intensity of polarized light emitted by diphenylhexatriene, a probe embedded in the membrane of mononuclear cells previously treated with LPS/lipid A. From their experiments they determined that pretreatment with LPS/lipid A increased the apparent microviscosity of the lipid bilayer which resulted in increased order within the membrane.

All of our binding studies were performed at low temperature to ensure observation of the initial binding step followed by coalescence and not just fusion of lipids. We chose to label several cell lines to show that LPS is capable of binding cells that are not just of bone marrow origin. We chose cell line RAW264.7 because it has previously been used in LPS activation studies. CHO-K1 is a Chinese hamster ovary cell line chosen because it was previously shown by Raetz (89) not to bind LPS and thus provided evidence suggestive of the presence of an LPS receptor. BCL1clone5B1b was chosen because it is a transformed cell line that was supposedly responsive to endotoxins and could be used in studies that look at immunoglobulin secretion and cell proliferation.

In this section we will present data involving biological responses brought about by *R. trifolii* LPS/lipid A, or the lack thereof. Several in vitro and in vivo assays were used to answer the question concerning conservation of endotoxic activity. In vitro assays included the induction of IgM, IgG, IgA, TNF, IL-1, and IL-6 synthesis and secretion, besides the extent to which LPS binds varying cell lines. The *Limulus* Amebocyte Lysate assay was also used as a screen for endotoxicity. The ability of *R. trifolii* to act as a mitogen was also tested. In vivo testing included the ability to generate fever and lethal toxicity testing.

There have been several instances where polycationic molecules have been used to block the activity of endotoxin (142). Polymyxin B sulfate and polylysine are two

examples of molecules with net positive charge that are capable of interacting with LPS electrostatically and preventing it from binding to cells such as B-lymphocytes (66). Molecules having a net negative charge, such as dextran sulfate or heparin, are also capable of blocking LPS activity by interacting with positively charged species on cell surfaces, thus preventing LPS from doing so. Besides electrostatic forces, hydrophobic interactions are also important. For example, polymyxin B nonapeptide, a derivative of polymyxin B lacking the hydrophobic tail, inhibits LPS binding to a lesser extent than polymyxin B. Endotoxin inhibitors were synthesized to interact with LPS both electrostatically and hydrophobically. These molecules were tested for their ability to prevent binding of LPS to macrophage-like cells and for their ability to prevent a positive response in the LAL assay. The data generated by all of the above in vitro and in vivo experiments provides insight into the mechanism of bacterial endotoxins.

Materials and Methods:

LPS Isolation

The same procedures used to grow *R. trifolii* ANU843 and isolate its LPS were used as those described in the Materials and Methods section of Chapter 4. *Salmonella typhimurium* LPS was purchased from Sigma Chemical Co. (St. Louis, MO). Both LPS types were converted to their triethylammonium salt forms by passage over a Sepharose 4B size exclusion column (129,130) using 0.05M formate buffer, pH adjusted by addition of triethylamine.

USP XXII Pyrogen Assay

This assay involved measuring the rise in temperature of rabbits following the intravenous injection of an endotoxin test solution. *S. typhimurium* LPS was injected at a dose which equaled 0.05 μg/kg body weight. *R. trifolii* ANU843 was injected at a dose of 0.05 μg/kg and 1.0 μg/kg body weight. Both LPS types were dissolved in pyrogen-free diluent and were injected into an ear vein of each of three rabbits in volumes of 1.0 mL. Rectal temperature-measuring probes, remaining inserted throughout the testing period , were used to record body temperature at 1, 2, and 3 hrs. subsequent to injection.

Lethal Toxicity Testing

Healthy, previously unused albino mice, weighing 17-23 grams, were used. Water and food were provided ad libitum. The endotoxin solution and a corresponding negative control were injected in groups of five mice each. All LPS samples were dissolved in pyrogen-free phosphate buffered saline and administered intraperitoneally by injecting 0.5 mL of material into each mouse. *R. trifolii* was administered at concentrations of 1000, 500, 100, and 50 μg/animal. *S. typhimurium* LPS was administered at the same concentrations as rhizobial LPS. After injection, the animals were observed immediately and at 0.25, 4, 24, 48, and 72 hrs. After 72 hrs., the animals were weighed.

Limulus Amebocyte Lysate Assay

R. trifolii LPS was screened for its ability to be recognized as an endotoxin by utilizing the *Limulus* amebocyte lysate assay. We purchased the Whittaker Bioproducts QCL-1000 Quantitative Chromogenic LAL , Whittaker Bioproducts, Inc. (Walkersville, MD). This was a chromogenic assay which relies upon the ability of endotoxin to activate an enzyme, a serine protease, found in the lysate, which will then cleave p-nitro-aniline conjugated to a peptide substrate. This resulted in chromophore release. The amount of chromophore released is proportional to the amount of endotoxin present. The *R. trifolii* ANU843 was tested in parallel with *E. coli* 0111:B4 endotoxin, the reference standard. The assay was performed following the test kit directions.

B-Lymphocyte Proliferation

R. trifolii 0403 was grown to early stationary phase (K90) and to exponential phase (K50). LPS was extracted and tested for its ability to act as a mitogen with splenocytes isolated from LPS-responsive mice, C3H/St and from LPS-hyporesponsive mice, C3H/HeJ. Measurement of tritium-labeled thymidine uptake was used as a means of visualizing B-cell proliferation. Assays were performed in the presence and absence of fetal bovine seum (FBS). Tests were run in parallel with LPS from two different strains of *Salmonella* LPS. The data from these experiments was kindly provided by F. B. Dazzo, with the actual experiment performed in the lab of Dr. D. M. Jacobs.

Alternatively, proliferation assays were performed using a transformed, B-cell leukemia cell line, BCL1clone5B1b, purchased from American Type Culture Collection (ATCC), using the procedure described in "Selected Methods in Cellular Immunology" (131). Cells were prepared to a final concentration of 2.5×10^5 cells/mL in RPMI-1640 medium, 15% FBS. 100 μL of the cell suspension was added to wells in a 96-well microtiter plate. 10 μL of LPS solutions of *R. trifolii* ANU843 and *S. typhimurium* were added to the appropriate wells to give final concentrations ranging from 4.1-16.4 μg/mL.

10 μL of medium was added to negative control wells. The cells plus LPS were incubated for 48 hrs. at 37°C and at a 5% CO_2 atmosphere. ^{3}H-thymidine (ICN Radiochemicals, Division of ICN Biomedicals, Inc., 6.7Ci/mmol, 248GBq/mmol, 1.0mCi, 1.0 mL sterile aqueous solution) was diluted 1:10, and 10 μL of diluted ^{3}H-thymidine was added per well. Cells were pulsed 16 hrs., after which time they were harvested onto filters, washed with distilled water, and then lysed with 10% TCA, followed by further water washes. The filters were dried, cut, and placed into scintillation vials. 10 mL of scintillation cocktail was added to the filters, and each vial was kept in the dark, in the cold, for 45 min. Vials were counted on the ^{3}H-channel of an LKB Wallac 1209 Rackbeta liquid scintillation counter for 2 min. (LKB Wallac, Finland).

Proliferation assays using the BCL1clone5B1b cell line were also performed with the following modifications: (i) We used a more dilute cell suspension, 2.5×10^4 cells/mL, (ii) ^{3}H-thymidine was added after 24 hrs. incubation of LPS with cells, then pulsed, followed by harvesting, (iii) ^{3}H-thymidine was added at the same time as LPS addition, followed by the usual workup, (iv) ^{3}H-thymidine was diluted 1:5 with cold thymidine and then further diluted 1:9 with RPMI-1640 medium. This was then added to the cells incubating with LPS, and (v) The cell medium, RPMI-1640, was prepared without 2-mercaptoethanol and FBS, and without only FBS. The assay was performed as originally described.

Immunoglobulin Secretion

Cell line BCL1clone5B1b, a B-cell leukemia which is LPS responsive (132), was stimulated with LPS from *S. typhimurium* and *R. trifolii* ANU843. The media in which the cells grew were assayed for the secretion of IgM using the ELISA (enzyme linked immunosorbant assay) technique. IgG and IgA secretion were also investigated. BCL1 clone5B1b was resuspended to a final concentration of approximately 3.0×10^4 cells/mL. One milliliter of this suspension was placed into each well. Into each well was also

placed 100 μL of each LPS solution, or RPMI-1640 for the negative control. Final LPS concentrations ranged from 8-80 μg/mL of LPS. The cells were allowed to continue growing for 5 days at 37°C in 5% CO_2 atmosphere, at which time 250 μL of the medium was removed for IgM screening. The removed medium was centrifuged and 200 μL of the supernatant was removed and frozen until assayed. The cells were allowed to continue growing for a total of 7 days, at which time 250 μL medium was removed for IgG and IgA screening. Again, the medium was centrifuged and the supernatants were removed and frozen until tested. Alternatively, the BCL1clone5B1b cell line was allowed to grow in the presence of LPS for 24, 48, and 72 hrs., at which times media was removed, centrifuged, and frozen until ready for use.

All supernatants were assayed for immunoglobulin release in the following manner. The appropriate standard (100 μL) and 100 μL of the supernatants diluted 1:10 with 1% FBS/PBS/0.02% NaN_3 were added to 96-well plates, previously coated with GAMIg (goat-anti-mouse IgG, IgM, IgA) at a final concentration of 0.015 mg/mL. Plates were covered with parafilm and incubated 1.5 hrs. at 37°C. The plates were next washed 3 times with 0.05% Tween/PBS, followed by 4 washes of distilled water. Plates were patted dry. To each well was added 100 μL of the appropriate alkaline phosphatase conjugated antibody, diluted in 1% FBS/PBS/NaN_3. We incubated the plates at 37°C for 1.5 hrs., covered with foil. All steps hereon were light sensitive. Again, the rinsing step was repeated. The phosphatase substrate, Sigma 104 Phosphatase Substrate (1 mg/mL), was added to all wells and the plate was again incubated at 37°C for 1 hr. 100 μL of 1M NaOH was added per well to stop the enzyme reaction. The plate was then read at 405nm and absorbances were recorded. Amount of antibody secreted was calculated from the standard curve.

Production of TNF

R. trifolii ANU843 lipid A was sent to XOMA Corporation, Berkeley, CA, to test its ability to cause macrophage to release TNF. The assay in which rhizobial lipid A was

tested was run in parallel with *E. coli* J5 (Rc) lipid A, obtained from Ribi ImmunoChem Research, Inc., Hamilton, MT. Recombinant human TNF-å (20 U/ng) and rabbit anti-human TNF-å polyclonal antibody were purchased from Genzyme (Cambridge, MA). Freshly drawn blood from healthy human donors was collected into Vacutainer tubes containing anticoagulant (ACD, Benton Dickinson, Rutherford, NJ). Aliquots of blood (225μL) were mixed with 20 μL of RPMI medium containing varying concentrations of lipid A. The mixture was incubated at 37°C for 4-6 hrs. and the reaction was stopped by the addition of 750 μL of RPMI, followed by centrifugation at 500g for 7 min. Super-natants were assayed for TNF levels using the Biokine ELISA test ,T Cell Sciences, (Cambridge, MA).

Release of Interleukins, IL-1,IL-6, and IL-8 by *Rhizobium* LPS

R. trifolii ANU843 lipid A was sent to XOMA Corporation, Berkeley, CA, to test for its ability to bring about production and secretion of IL-1 and IL-6 by macrophage. Results are forthcoming from XOMA Corp. A measure of the ability of *R. trifolii* ANU843 lipid A to induce IL-8 secretion was run in parallel with that of *E. coli* lipid A at XOMA Corp. Freshly drawn blood from healthy human donors was collected as described for the TNF assay reported in this section of text. Aliquots of blood were mixed with 20 μL of RPMI medium containing varying concentrations of lipid A. The mixture was incubated at 37°C for 4-6 hrs. and the reaction was stopped by the addition of 750 μL of RPMI, followed by centrifugation at 500g for 7 min. Supernatant liquids were assayed for IL-8 levels using the Quantikine Human IL-8 Immunoassay (ELISA), R & D Systems, Inc., (Minneapolis, MN).

Cell Lines Used in LPS Binding Assays

We have made a comparative study of the binding of rhizobial LPS and enteric LPS to several eukaryotic cell lines. RAW264.7 is a murine, Abelson leukemia virus transformed macrophage. RAW264.7 was grown in Dulbecco's modified Eagle's medium, 90%, FBS 10%. Subcultures were prepared by scraping and resuspending in

fresh DMEM, then aspirated and dispensed into new flasks.

CHO-K1 is a Chinese hamster ovary cell line, *Cricetulus griscus*. CHO-K1 was grown in Ham's F-12 medium, 90%, FBS 10%. These cells were subcultured by rinsing the cell sheet twice with fresh trypsin (0.25%) solution. The trypsin was removed and the cells were incubated at 37°C for 5-10 min., until the cells detached. Fresh culture medium was added, and the cells were aspirated, then dispersed into new flasks.

BCL1clone5B1b is a murine B-cell leukemia lymphoma cell line. Cells were cultured in RPMI-1640 medium with 2.0 mM L-glutamine and 0.05mM 2-mercapto-ethanol, 85%, FBS 15%. Subcultures were prepared by scraping and resuspending the dislodged cells in fresh RPMI-1640. Aliquots were aspirated and discharged into new flasks.

All three cell lines were purchased from the American Type Culture Collection (ATCC). They were purchased under the ATCC No.: TIB 71, CCL 61, and TIB 197, which correspond to the RAW264.7, CHO-K1, and BCL1clone5B1b cell lines, respectively.

Conjugation of Fluorescent Dyes to LPS

R. trifolii ANU843 and *S. typhimurium* were chemically linked through their hydroxyl groups to the fluorescent dyes, fluorescein isothiocyanate isomer I (FITC) and rhodamine B (Rho), so that LPS binding to cells could be visualized using fluorescent microscopy. LPS (15-25 mg, depending on which LPS type was used) was sonicated briefly in 1.5 mL pyridine until the LPS went into solution. 4-dimethylaminopyridine (5 times the weight of LPS) was added. This was followed by the addition of FITC or Rho (1/5th. the weight of LPS). The solutions were covered in foil, sonicated, and left to set overnight at room temperature. The LPS-dye conjugates were placed on a rotary evaporator to remove pyridine. They were then placed on an LH-20, lipophilic gel permeation column, and eluted with 1:1 water/methanol, collecting 80 drops/fraction.

Elution of FITC-LPS conjugates resulted in the resolution of 2 peaks containing FITC. 50 μL of each fraction was screened for the presence of carbohydrate using 5% aqueous phenol/H_2SO_4. Only the first peak contained dye plus carbohydrate and these fractions were pooled, frozen, and lyophilized. The second peak did not contain carbohydrate and was discarded as only containing free FITC. Elution of the Rho-LPS conjugates gave two peaks containing dye. The fractions containing rhodamine were screened for carbohydrate by using the phenol/H_2SO_4 reagent. Only those fractions containing both dye and sugar were pooled, frozen, and lyophilized. This time, much free dye remained on the column and the free rhodamine was eluted off the column using pure methanol.

Labeling of Cell Lines with LPS-Dye Conjugates

Pre-cleaned coverslips were placed into 6-well plates and cells suspended in medium were added at concentrations ranging from $3x10^5$ to $1x10^6$ cells/well. Cells were allowed to grow and adhere overnight, after which time they were washed in a balanced salt solution and then treated with LPS-dye. Cells were labeled with the LPS-dye by placing the coverslips face-down onto 10 μL of the LPS-dye solution, ranging from 5.7 to 5.9 μg/mL, (that had been previously cooled at 6°-8°C) for 5 hrs. Labeling was performed in the cold at 6°-8°C. In some instances the cells were pretreated with a 10mM solution of sodium azide before labeling with LPS-dye.

Fixation of Cells Labeled with LPS-Dye

The following steps were done at 6°-8°C, being careful to keep the cells out of the light as much as possible. The coverslips with the adherent cells now fluorescently labeled were transferred to clean, 6-well plates, and washed once with 4.0 mL of balanced salt solution and twice with 4.0 mL NaN_3 (10mM) solution. The cells were fixed for 10 min. in 4.0 mL 3.7% formaldehyde in HBSS. They were washed once in 4.0 mL of distilled water and then mounted onto slides in 1 drop of glycerol (Difco FA mounting fluid, pH 9.0). The coverslips were sealed using clear nail polish.

Fluorescent Microscopy

Slides were viewed using a Leitz fluorescent microscope. Cells were viewed as phase contrast images and fluorescent images. Images were magnified 128x.

Confocal Microscopy

Slides were viewed using a Zeiss 10 Laser Scanning Confocal Microscope. Magnification was set at 2800x. Three-dimensional imaging utilized the scanning confocal microscope plus a Silicon Graphics 4D-30 personal Iris computer. Software was VoxelView E by Vital Images (Fairfield, IA).

Results

The LPS of a closely related strain, *R. trifolii* 0403, which we know to have the same lipid A composition as that of *R. trifolii* ANU843, was studied under the assumption that it had the same lipid A structure as the *Enterobacteriaceae* (106). In this study, the LPS of this strain was shown to give a positive response in the LAL assay (data not shown). It was also capable of acting as a splenic B-cell mitogen with the same activity per μg as standard *S. typhimurium* LPS preparations (Table 1) in the absense of serum and in LPS responder and hypo-responsive mouse strains. *R. trifolii* 0403 also tested positive for the local Schwartzmann reaction. This was an in vivo assay involving the formation of lesions after intravenous injection of LPS at the site of a previous intra-dermal injection of the same LPS. Hemorrhage and/or lesions appearing at the site of original injection at approximately 4 hrs. after intravenous injection are indicative of a positive reaction (133). The amount of *R. trifolii* 0403 LPS (on a per weight basis) needed to elicit a response of equal magnitude to the enterics, was much greater than the amount of *Salmonella* LPS necessary to induce the same response (134).

R. trifolii ANU843 possesses structural features radically different from that of classical endotoxin molecules. The question asked was, "What do the radically different structural features of this LPS molecule do to its ability to behave like the typical endotoxins?"

LAL Activity

The LAL assay is an endotoxin-induced coagulation reaction of the blood of the horseshoe crab, *Limulus polyphemus* (135,136). It is a rapid method that is widely used in the detection of endotoxin. We utilized a chromogenic assay in which *R. trifolii* ANU843 was tested in parallel with *E. coli* 0111:B4, the reference endotoxin standard. The same magnitude of response, per mg, was obtained for the LPS of both organisms, even though *R. trifolii* LPS is lacking in phosphate, and has a different carbohydrate and fatty acid composition. In fact, the LPS of strain ANU843 demonstrated a slightly higher

Table 1

The mitogenic response of *R. trifolii* LPS, harvested at different growth periods, as measured by ^{3}H-thymidine uptake. Experiments were performed by D. M. Jacobs.
K90=*R. trifolii* 0403 grown to early stationary phase.
K50=*R. trifolii* 0403 grown to exponential phase.
K90 and K50 are both mitogenic, however K90 is more so than K50.

Table 1

Cells	dose, ug	LPS K90	LPS K50	LPS St #1	LPS St#2
		cpm			
Serum-free medium					
C3H/St	0.05	1173	700	1033	1340
	0.5	3756	2147	2260	2694
	5.0	3168	1610	3500	3179
bkg = 833 cpm					
C3H/HeJ	0.05	127	115	129	148
	0.5	297	151	567	287
	5.0	511	140	438	609
bkg =300					
5% FBS					
C3H/St	0.05	195	155	903	920
	0.5	527	221	1803	2138
	5.0	2243	542	4177	4306
bkg = 304					
C3H/HeJ	0.05	68	51	35	45
	0.5	104	52	184	70
	5.0	239	53	162	235
bkg = 350					

St #1, St#2 = *Salmonella* LPS
C3H/St = LPS-responsive mouse strain
C3H/HeJ = LPS-hyporesponsive mouse strain

activity per mg compared to the standard *E. coli* 0111:B4 LPS. Results are shown in Figure 23.

Proliferation Assays

As stated previously, the LPS of *R. trifolii* 0403 was capable of inducing a mitogenic response of equal intensity, as measured by ^{3}H-thymidine uptake, to that of *Salmonella* LPS (Table 1). Since it is known that *R. trifolii* 0403 and *R. trifolii* ANU843 possess identical lipid A structures, and knowing the lipid A is responsible for induction of biological response, we concluded that *R. trifolii* ANU843 LPS would also be capable of causing B-cells to proliferate.

We attempted to show this with *R. trifolii* ANU843 LPS by using the transformed cell line, BCL1clone5B1b. ATCC recommended BCL1clone5B1b as being useful for studying the mechanisms and biochemistry of cell surface receptors and the mechanisms underlying signalling of B-cells to differentiate and replicate, as the cell line is hyper-responsive to LPS (137,138). Again, we were measuring uptake of ^{3}H-thymidine as an indicator of proliferation in response to LPS. Both *R. trifolii* ANU843 and *S. typhimurium* LPS were tested in parallel.

Cell line BCL1clone5B1b was not well suited for studying proliferation. Since it is a transformed cell line, these cells divided too rapidly on their own, at a rate which was independent of the presence or concentration of LPS. We tried modifying the experimental conditions to accomodate for rapid proliferation as described in the Materials and Methods section. None of these modifications helped to give an accurate account of the effects of LPS upon B-cell proliferation with this cell line.

Looking back on the literature cited concerning BCL_1 cells, the original leukemia cells from which BCL1clone5B1b was derived, these tumor cells were originally isolated from the animals and used as a primary culture. Obviously, sometime during the conversion from BCL_1 to the final BCL1clone5B1b, the cells have lost their ability to respond to LPS supranormally in vitro.

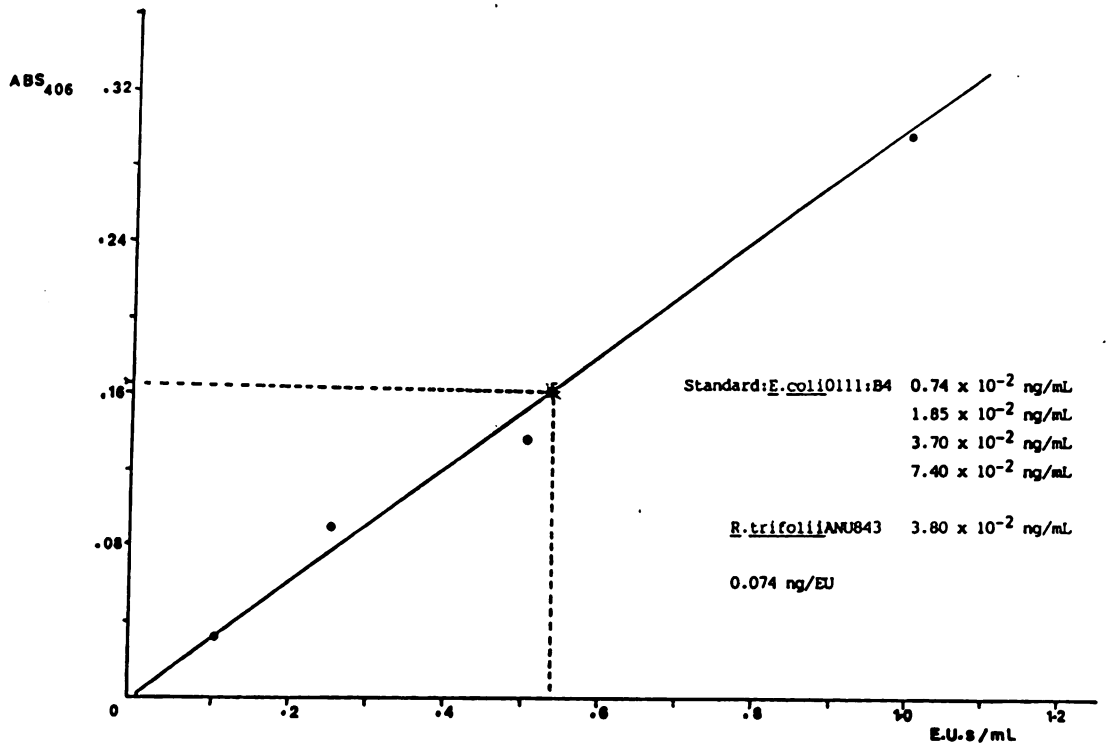


Figure 23

Standard curve of absorbance versus concentration in endotoxin units (E.U.s)/mL for *E. coli* 0111:B4 LPS. The point connected by the broken line corresponds to the average of a triplicate ANU843 LPS sample at 3.8×10^{-2} ng/mL. The third point on the standard curve is at a similar weight/mL concentration and has a similar value for endotoxin units/mL. Absorbance is proportional to the amount of endotoxin present. These are the results of the LAL assay.

Immunoglobulin Secretion

One of the beneficial effects of LPS is its ability to stimulate lymphocytes to synthesize immunoglobulin. BCL1clone5B1b had previously been shown to synthesize and secrete IgM in response to LPS (132). We looked at the ability of *R. trifolii* ANU843 LPS and *S. typhimurium* LPS to stimulate these cells to secrete IgM. We tested over a final concentration range of 8-80 μg/mL of LPS.

Originally the cells were left to incubate with LPS for 5 days before the medium was harvested for IgM. It appears that the longer the cells are allowed to grow, the more apt they are to secrete IgM. Negative control cells which did not receive LPS secreted IgM at relatively high levels. The cells stimulated with *Salmonella* LPS gave higher readings for IgM than did the negative control cells, however there was no difference in IgM secretion levels between the negative control cells and those treated with rhizobial LPS. The BCL1clone5B1b cell line has IgM bound to its surface. The supernatants were not contaminated by fragments from the cell membranes, because the medium was centrifuged and the supernatants were removed for testing. Therefore, it appeared that the cells themselves were secreting IgM without stimulus. We decided to harvest the medium after 72, 48, and 24 hrs. Again the cells secreted IgM, but to a lesser extent as the incubation time was reduced. At 72, 48, and 24 hrs. there was stimulation of the B-cells to secrete IgM by *Salmonella* LPS. The longer the cells were allowed to incubate, the lower the difference between background and stimulation. The cells treated with LPS for 24 hrs. gave the greatest difference between background IgM and *Salmonella* induced IgM secretion. *R. trifolii* LPS did not induce IgM secretion under any condition. The results of LPS stimulation of B-cells, for 24hrs., are shown in Figure 24.

We also looked at the ability of both LPS types to stimulate release of IgG and IgA by BCL1clone5B1b. These cultures were allowed to incubate for 7 days at which time the medium was harvested and assayed for the presence of IgG and IgA. Neither *S.*

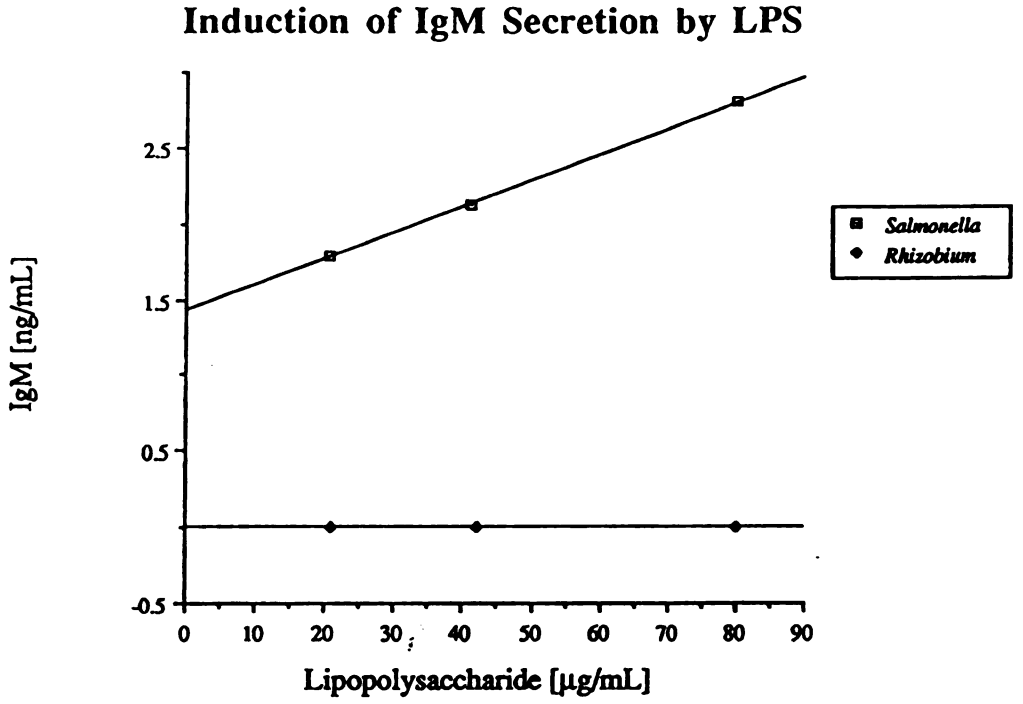


Figure 24

BCL1clone5B1b lymphocytes were treated with LPS. *Salmonella* LPS was capable of inducing IgM secretion whereas *Rhizobium* LPS was not.

typhimurium nor *R. trifolii* LPS were capable of inducing IgG or IgA secretion by this cell line at the concentrations tested (data not shown).

It is interesting to note that proliferation of B-cells and stimulation to secrete antibody are not necessarily coupled. Remember, both *Salmonella* and *Rhizobium* LPSs were able to cause B-cell proliferation, however only the *Salmonella* LPS was able to bring about IgM secretion.

TNF Production

R. trifolii ANU843 lipid A was sent to XOMA Corporation, Berkeley, CA, to test its ability to cause macrophage to release TNF. *R. trifolii* was tested in parallel with the lipid A of *E. coli* J5. The lipid A of *R. trifolii* ANU843 was able to induce TNF production, However it did so at a concentration much greater than that of *E. coli* lipid A (Figure 25). For example, 200 ng/mL of rhizobial lipid A was needed to give the same response as that produced by less than 0.5 ng/mL of *E. coli* lipid A. We conclude that *R. trifolii* can induce TNF production, however it is not very efficient in doing so, as compared to the classical lipid A.

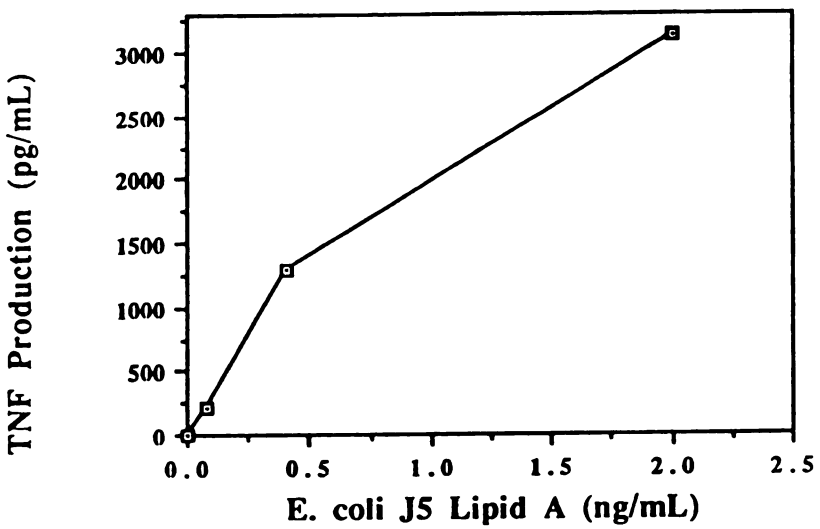
Interleukin Production

A sample of *R. trifolii* ANU843 lipid A was also tested for its ability to induce macrophage to release IL-1 and IL-6. These assays were performed at XOMA Corp. The results of these assays are forthcoming. Assays measuring the release of IL-8 from macrophage in response to rhizobial and enteric lipid As were performed at XOMA Corp. These results showed that macrophage responded to *R. trifolii* lipid A in the same manner as they did in the TNF assay. Again, rhizobial lipid A was less effective in the synthesis of IL-8 as compared to its enteric counterparts (Figure 26).

Pyrogenicity

Endotoxins are known pyrogens (139,140). Samples of *R. trifolii* ANU843 LPS and *S. typhiurium* LPS were sent to the Biological Test Center, Irvine, CA, to facilitate a comparison of their abilities to act as pyrogens. *S. typhimurium* LPS, injected into

TNF Production



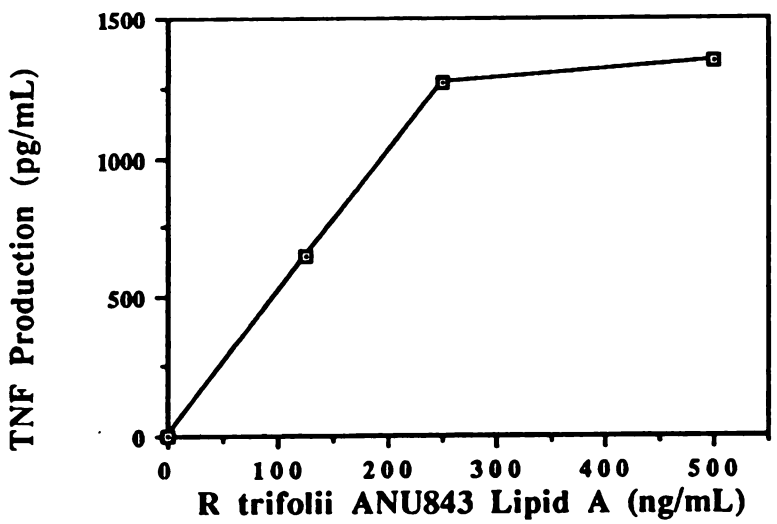


Figure 25

Comparison of TNF production induced by *R. trifolii* ANU843 lipid A and *E. coli* J5 lipid A.

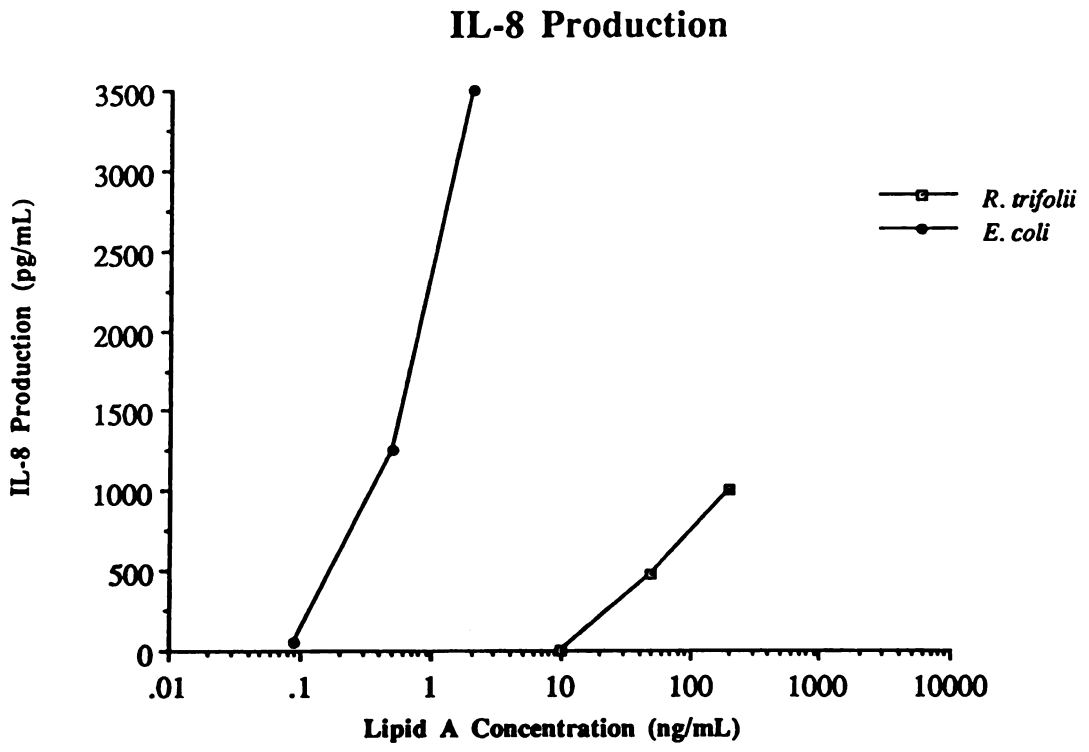


Figure 26

Comparison of IL-8 production induced by *R. trifolii* ANU843 lipid A and *E. coli* lipid A.

rabbits at a concentration of 0.05 μg/kg body weight, induced fever in 3 out of 3 rabbits, and was considered pyrogenic at this concentration. *R. trifolii* ANU843 was non-pyrogenic when injected at the same dose that gave a positive response with *Salmonella* LPS (Table 2). When the concentration of *R. trifolii* was raised to 1.0 μg/kg, it was capable of fever production in 3 out of 3 rabbits. This was a dose proportional to the amount of MPL or lipid X (on a weight basis) necessary to cause fever. Remember, both MPL and lipid X are non-toxic. It was concluded that the LPS of *R. trifolii* was non-pyrogenic.

It should be noted that the LAL assay is more frequently being used instead of the in vivo test for pyrogens. Companies that market the kit claim it is as effective in screening for endotoxins as is the pyrogen assay. Our in vivo pyrogen results showed there was a difference between the classical LPS and the varient LPS in their abilities to be endotoxic, whereas the results of the LAL assay demonstrated there was no difference between the classical and varient LPSs. Obviously the two assays involve different mechanisms of action.

Toxicity

The Biological Test Center, Irvine, CA, tested for the toxic effects of both *R. trifolii* ANU843 LPS and *S. typhimurium* LPS in mice. The results are tabulated in Table 3. *Salmonella* LPS was lethally toxic in 100% of the animals into which it was injected, at the higher concentration (1000 μg), and 40% lethal at half this concentration. The other 60% of mice receiving the lower concentration were adversely affected as evidenced by lethargy and weight loss. *R. trifolii* LPS induced lethargy in all animals at both concentrations tested, however this state was transitory and these mice showed no significant change in body weight. We concluded *R. trifolii* LPS was non-toxic compared to the enteric LPS.

LPS Binding

In order for LPS to induce its biological effects it must first interact with the target

Table 2

USP XXII pyrogen assay comparing fever induction between *R. trifolii* ANU843 LPS and *S. typhimurium* LPS. A result is considered positive if one rabbit in three shows a temperature 0.6°C or greater than its control temperature, or if the sum of the three rabbit temperature rises exceeds 1.4°C. In either case, a repeat test is done on five additional rabbits. If not more than three of the total of eight rabbits have individual increases of 0.6°C or more, and if the sum of the eight increases does not exceed 3.7°C, the material under examination meets the requirements for absence of pyrogens.

Table 2

USP XXII Pyrogen Assay

S. typhimurium LPS (0.05 μg/kg)

Temperature = degrees C

Rabbit	Wt. (kg)	Control	1st. Hour	2nd. Hour	3rd. Hour	Max. Rise
202-B	2.63	39.62	40.80	40.53	39.87	1.18
428-V	3.26	39.13	40.26	40.04	39.64	1.13
324-D	2.74	39.30	39.80	39.88	39.56	0.58

Total Rise : 2.89 °C

R. trifolii LPS (0.05 μg/kg)

Rabbit	Wt. (kg)	Control	1st. Hour	2nd. Hour	3rd. Hour	Max. Rise
193-X	1.91	39.07	39.37	39.28	39.09	0.30
204-Y	2.59	39.04	40.17	39.63	39.11	1.13
222-Z	2.50	38.73	38.86	38.78	38.65	0.13

Total Rise : 1.56 °C

Rabbit	Wt. (kg)	Control	1st. Hour	2nd. Hour	3rd. Hour	Max. Rise
236-D	2.64	38.96	39.35	39.22	39.14	0.39
241-F	2.49	39.05	39.20	39.11	38.95	0.15
242-C	2.43	39.34	39.62	39.46	39.27	0.28
253-W	2.63	38.96	39.40	39.16	39.00	0.44
281-W	2.25	39.32	39.82	39.62	39.43	0.50

Total Rise : 1.76 °C

\+ 1.56 °C

3.32 °C

R. trifolii LPS (1.0 μg/kg)

Rabbit	Wt. (kg)	Control	1st. Hour	2nd. Hour	3rd. Hour	Max. Rise
128-D	2.37	39.42	40.15	40.13	40.05	0.73
174-Z	2.15	39.47	40.66	40.48	40.36	1.19
190-A	2.24	39.07	39.85	39.59	39.52	0.78

Total Rise : 2.70 °C

Table 3

Lethal toxicity in mice comparing *R. trifolii* ANU843 LPS with *S. typhimurium* LPS.

Table 3

Test Sample	Animal #	Weight	Observations 0	.25	4	24	48	72	Weight
RHDL-A	1	19g	0	0	d	T	-	-	
	2	21g	0	0	d	T	-	-	
	3	20g	0	0	d	T	-	-	
	4	22g	0	0	d	T	-	-	
	5	19g	0	0	d	T	-	-	
RHDL-B	1	21g	0	0	d	T	-	-	
	2	20g	0	0	d	T	-	-	
	3	20g	0	0	d	d	d	d	14g
	4	19g	0	0	d	d	d	d	15g
	5	17g	0	0	d	d	d	d	14g
RHDL-C	1	22g	0	0	d	d	0	0	21g
	2	20g	0	0	d	d	0	0	20g
	3	20g	0	0	d	d	0	0	20g
	4	18g	0	0	d	d	0	0	19g
	5	17g	0	0	d	d	0	0	16g
RHDL-D	1	21g	0	0	d	0	0	0	21g
	2	21g	0	0	d	0	0	0	23g
	3	21g	0	0	d	0	0	0	23g
	4	22g	0	0	d	0	0	0	24g
	5	20g	0	0	d	0	0	0	22g

d=lethargy T=death Observations are in hours.

RHDL-A *S. typhimurium* LPS 1000μg/animal

RHDL-B *S. typhimurium* LPS 500μg/animal

RHDL-C *R. trifolii* LPS 1000μg/animal

RHDL-D *R. trifolii* LPS 500μg/animal

cell. The following experiments were done to make a comparative study of the binding of rhizobial LPS and enteric LPS to eukaryotic cells. We wanted to determine if both LPS types bound to a macrophage cell line, that had previously been shown to bind LPS (59,89), to the same extent. We determined whether binding was specific, if one LPS could inhibit binding of the other. We looked at the extent to which both LPS types could interact with cell lines that did not originate from bone marrow. Lastly, we studied the effects of molecules which we synthesized to block LPS activities, such as binding to target cells. These molecules were termed endotoxin inhibitors (EIs) and their effects will be discussed last.

We have made a comparative study of the binding of rhizobial LPS and enteric LPS to a macrophage-like cell line, RAW264.7. As stated in the Materials and Methods, the LPSs of *S. typhimurium* and *R. trifolii* were chemically linked to fluorescein isothiocyanate (FITC) and rhodamine B (Rho), so that binding could be visualized using fluorescence microscopy. The lowest concentrations at which the LPS-dye could be visually detected were: *R. trifolii* LPS-FITC, 5.87 mg/mL (R.t.-FITC), *Salmonella* LPS-Rho, 5.82 mg/mL (Sal-Rho), and *Salmonella* LPS-FITC, 5.47 mg/mL (Sal-FITC). Concentrations at one tenth these working concentrations could no longer be detected visually on the Leitz microscope.

Since RAW264.7 is an adherent cell line, the best results were obtained when the cells were grown directly on coverslips. The RAW264.7 cells on coverslips were labeled with the LPS-dye by placing the coverslips face-down onto 10 μL of the LPS-dye solution (that had been cooled to 6°C) for 5 hrs., a time which had been determined to be optimum for binding. The Sal-FITC and Sal-Rho could be visualized after 15 min., but the R.t.-FITC was faintly visible at 2 hrs., and best seen at 5 hrs., so we opted to do all labeling for 5 hrs. to be consistent. Labeling was performed at 6° - 8°C. We envision that LPS binds to eukaryotic cells in a two-stage process. The first stage is an electrostatic interaction between the negatively charged LPS and the positive charge on

the cells' surface (possibly a protein). This is followed in stage 2 by intercalation of the lipid A long chain fatty acids into the plasma membrane. We thought that at low temperatures we would detect only the electrostatic component. Low temperature should also prevent uptake by endocytosis/phagocytosis, as this process is less effective at temperatures below 18°C. After allowing contact between LPS-dye and the cell, the cells were treated with a 10mM solution of sodium azide to prevent further active metabolic processing resulting in dye uptake. The results of labeling were the same whether the cells were treated with azide before adding LPS-dye conjugate or after labeling. Both Sal-FITC and Sal-Rho were capable of crossing the plasma membrane and getting into the interior of the cell. R.t.-FITC was detected only at the cell surface. Further proof of this was provided by confocal microscopy. It was shown that cells labeled with Sal-dye conjugate fluoresced at the cell surface, as well as in the cell's interior, whereas R.t.-FITC was found only at the cell surface, or just inside the plasma membrane. Results are shown in Figures 27 and 28.

We know that the *Salmonella* LPS did not disrupt the plasma membrane, making it leaky so that more Sal-dye conjugate gets inside the cells. RAW264.7 cells labeled with free LPS for 0, 3, and 7 hrs. were treated with trypan-blue dye and cell viability was determined in different fields based upon the ability of cells to exclude dye. It was found that these macrophage-like cells had 90% viability or greater in some instances.

From the initial binding studies we concluded that *Salmonella* and *Rhizobium* LPSs interact with RAW264.7 cells differently. *Salmonella* LPS was capable of crossing the plasma membrane and was found in the cells' interior. *R. trifolii* ANU843 LPS was found to bind only at the cell surface, as evidenced by normal fluorescence and confocal fluorescence microscopy.

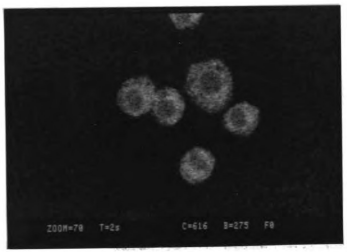
Previous literature utilizing radiolabeled LPS (89,141) has shown that unlabeled LPS can reduce the extent to which labeled LPS binds. This would be suggestive of the presence of an LPS-receptor on the target cell. We have performed experiments utilizing

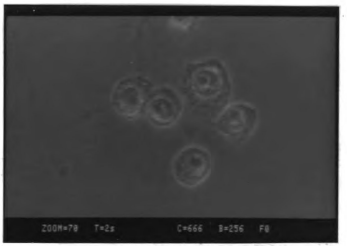
Figure 27(A)

Phase contrast and confocal microscopy of RAW264.7 cells labeled with *Salmonella* LPS-Rhodamine conjugate.

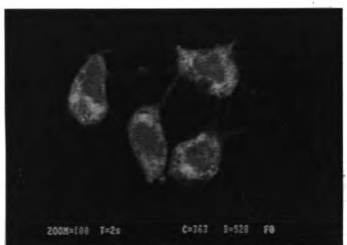
Figure 27(B)

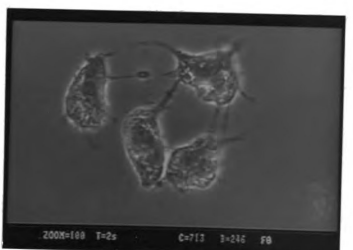
Phase contrast and confocal microscopy of CHO-K1 cells labeled with *Salmomella* LPS-Rhodamine conjugate.





27A



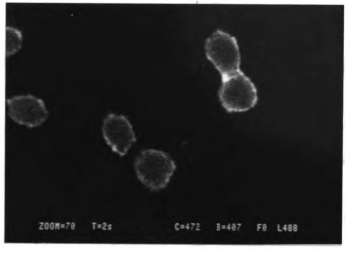


27B

Figure 27

Figure 28

Phase contrast and confocal microscopy of RAW264.7 cells labeled with *R. trifolii* LPS-FITC conjugate.



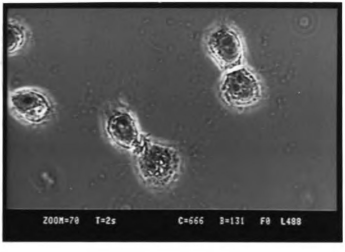


Figure 28

unlabeled *Salmonella* and *Rhizobium* LPSs in attempts to determine if one LPS can prevent the binding of the other type. RAW264.7 cells preincubated with 10 μL of unlabeled rhizobial LPS (LPS stock concentration was 5 and 20 mg/mL) for 5 hrs. After this time the cells were placed in 10 μL of Sal-Rho for an additional 5 hrs. Fluorescent microscopy showed that rhizobial LPS had no effect on binding of Sal-Rho. The cells still bound Sal-Rho as they had before. In another instance, cells were preincubated with unlabeled *Salmonella* LPS (5 and 20 mg/mL) for 5 hrs., followed by 5 hrs. incubation with either R.t.-FITC or Sal-Rho. In both cases and at either concentration the results were the same. The unlabeled *Salmonella* LPS had no effect on binding of Sal-Rho or R.t.-FITC. The R.t.-FITC was still found to bind the cells' surfaces. This suggests the *Salmonella* LPS does not disrupt the plasma membrane since the R.t.-FITC stays at the surface and does not move to the cells' interior. The Sal-Rho bound to cells after pretreatment with unlabeled *Salmonella* LPS and the dye was again found throughout the cells.

Binding studies using Sal-Rho and R.t.-FITC were carried out with two other cell lines under the same conditions. CHO-K1 and BCL1clone5B1b were Chinese hamster ovary and mouse B-cell leukemia lymphoma cell lines, respectively. As with the RAW264.7 cell line, Sal-Rho moved into the interior of these cells, whereas R.t.-FITC bound each cell type only at the surface (Figure 27). Experiments using unlabeled *Salmonella* LPS followed by labeled *Salmonella* and *Rhizobium* LPSs had the same results as those of the RAW264.7 cell line.

The confocal microscope was used to generate a 3-dimensional image of Sal-Rho, specifically where it was localized inside the cell (Figure 29). Results indicated that the dye was localized in vesicles which were distributed throughout the cell, but tended to appear in higher frequency near the nucleus (in the perinuclear region). The dye was definitely excluded from the nucleus. Location of dye was the same in both the macrophage and Chinese hamster ovary cell lines. We did not look at the B-cell

Figure 29

3-dimensional imaging, shown in various orientations, of fluorescence localization in a RAW264.7 cell. The Sal-Rho is localized in vesicles throughout the cell, but appears at higher frequency in the perinuclear region.

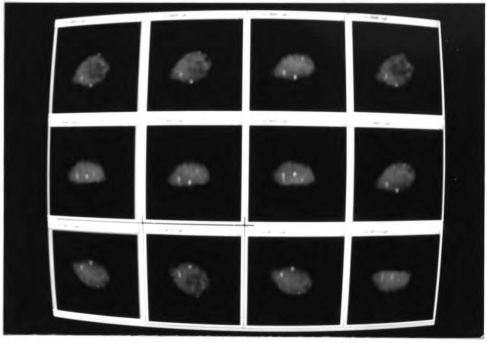


Figure 29

leukemia cell line.

Considering that the Sal-Rho was capable of moving into the cells' interior, and R.t.-FITC was not, we set out to determine if the *Salmonella* LPS was modified in any way. Such a modification may have involved cleavage of the LPS into smaller units, which would allow its entry into the cells' interior. We labeled the macrophage with Sal-Rho as usual, then extracted the LPS-dye conjugate out of the cells. We had hoped to resolve this LPS on a size exclusion column and then compare its elution profile with that of intact Sal-Rho conjugate. Unfortunately the small amount of LPS-dye conjugate used in labeling could not be detected in collected fractions.

Endotoxin Inhibitors (EIs) in the LAL Assay

Researchers in our lab have synthesized several molecules which have a net positive charge, hydrophobic moieties, or a combination of these two. The molecules are referred to as endotoxin inhibitors (EIs), and are denoted as EI-1, EI-2, EI-3, and EI-4. Their structures are given in Figure 30. The EIs were designed to interact with LPS both electrostatically and hydrophobically, and in doing so should block endotoxin activity.

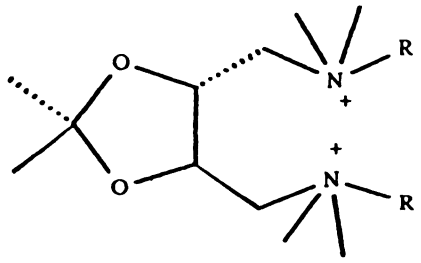
The extent to which these molecules function to inhibit endotoxin activity was measured by their ability to prevent a positive reaction in the LAL assay. EI-1, EI-2, EI-3, and EI-4 were tested at various concentrations in the presence of a fixed amount of *E. coli* endotoxin. The results of Figure 31 show EI-1 and EI-2 to inhibit at concentrations similar to that of polymyxin B. EI-4 will inhibit at higher concentrations. EI-3 does not inhibit at all. In fact, its values suggest enhancement of LAL activity. This was explained by the presence of yellow coloring in EI-3 that could not be removed by decolorization. The normal presence of color in EI-3 absorbed in the wavelength range of 402-410 nm, where the LAL assay is measured. What we actually saw were false positive values in the case of EI-3.

Effects of EIs on RAW264.7 Cell Growth

A sample of EI-1 was sent to XOMA Corp. to test its ability to prevent TNF

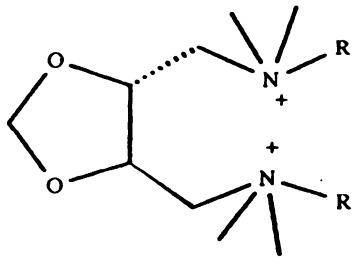
Figure 30

The structures of the endotoxin inhibitors and polymyxin B sulfate are shown. The number 7 used in EI-3 and EI-4 indicates that 7 sugar residues are linked together in these cyclodextrin structures.

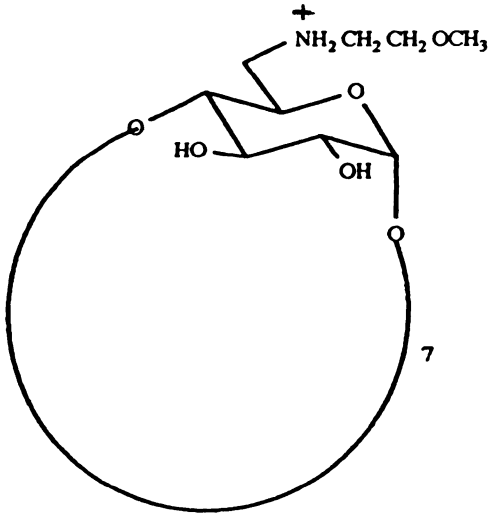


EI - 1

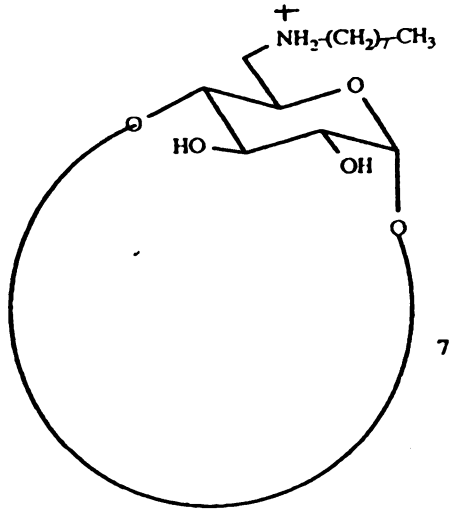
R = alkyl



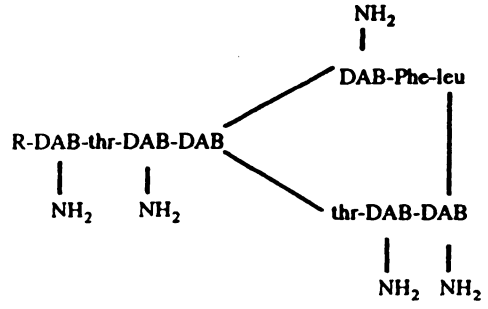
EI - 2



EI - 3



EI - 4



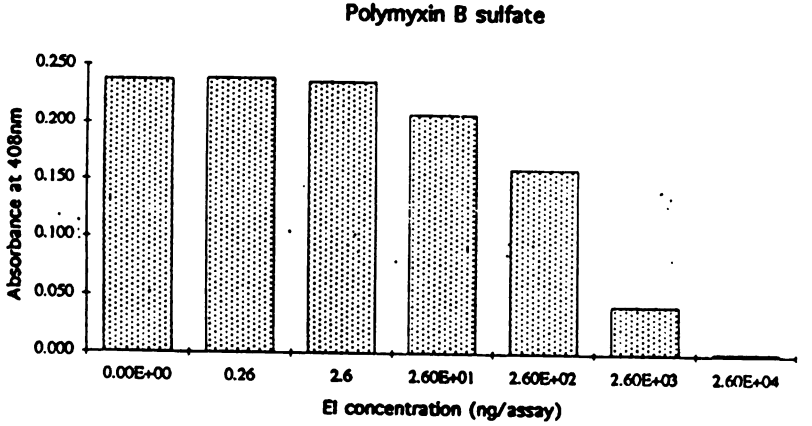
R = 6-methyloctanoic acid

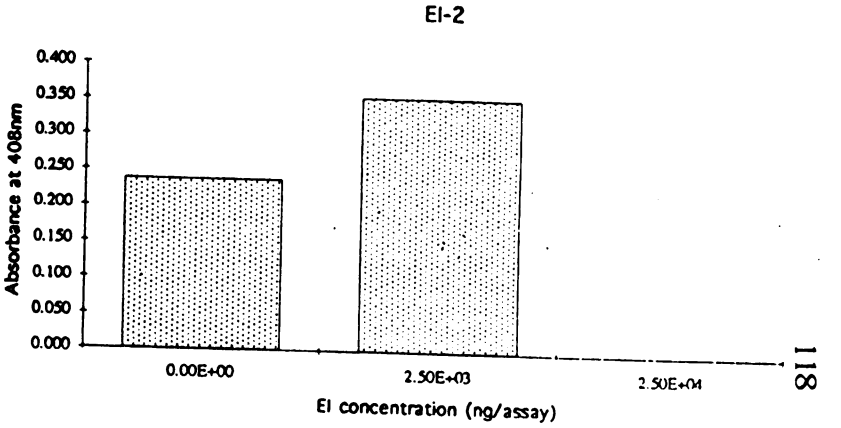
POLYMYXIN B

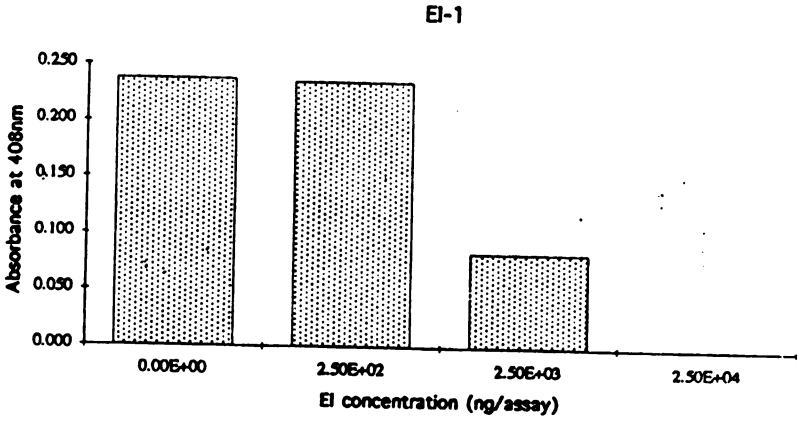
Figure 30

Figure 31

The ability of the EIs to inhibit endotoxin activity was measured by their ability to prevent a positive reaction in the LAL assay.


Polymyxin B sulfate
Absorbance at 408nm
0.250
0.200
0.150
0.100
0.050
0.000
0.00E+00
0.26
2.6
2.60E+01
2.60E+02
2.60E+03
2.60E+04
EI concentration (ng/assay)


EI-2
Absorbance at 408nm
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0.00E+00
2.50E+03
2.50E+04
EI concentration (ng/assay)


EI-1
Absorbance at 408nm
0.250
0.200
0.150
0.100
0.050
0.000
0.00E+00
2.50E+02
2.50E+03
2.50E+04
EI concentration (ng/assay)

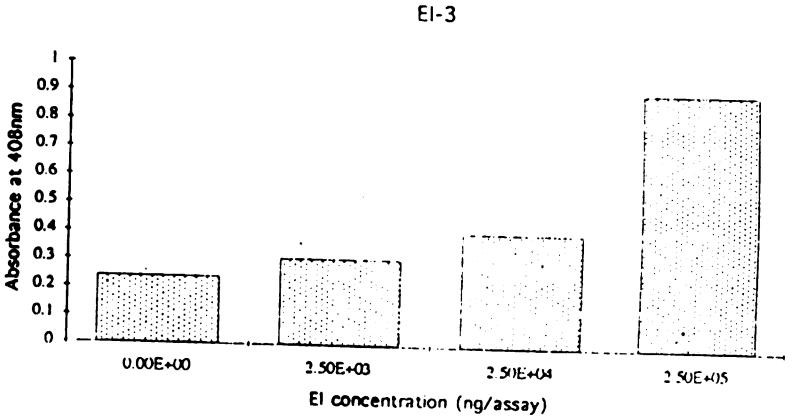

EI-3
Absorbance at 408nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.00E+00
2.50E+03
2.50E+04
2.50E+05
EI concentration (ng/assay)

Figure 31

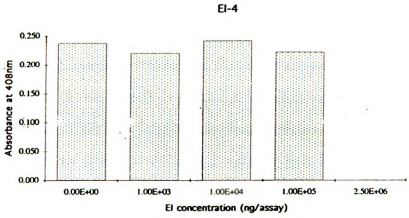


Figure 31 (cont'd)

release by macrophage. They reported that EI-1 killed the cells used in the assay. We tested all EIs and polymyxin B sulfate for their ability to cause destruction of cell line RAW264.7. EI-1 and EI-2 caused cell lysis at concentrations of $9.5x10^{-2}$ mg/mL. This was the same concentration previously used in an attempt to determine if these molecules had bacteriocidal ability. (They did not whereas polymyxin B did.) At concentations of one tenth and one hundredth of this value, EI-1 and EI-2 did not cause lysis of the RAW264.7 cells. EI-3 at $9.5x10^{-2}$ mg/mL did not affect macrophage cell growth. Similarly, polymyxin B (PMB) and EI-4 at concentrations of $8.2x10^{-2}$ mg/mL had no effect on cell growth. It appeared that EI-1 and EI-2 were capable of making the cell membranes of RAW264.7 very fluid, thus resulting in cell destruction.

The Effect of EIs on Labeling with Sal-FITC and R.t.-FITC When Cells are Grown in the Presence of EIs

RAW264.7 cells were treated with EI-1, EI-2, EI-3, and PMB at concentrations of $9.5x10^{-2}$ mg/mL for the EIs and $8.2x10^{-2}$ mg/mL for PMB. The cells remaining and the cellular debris were labeled with Sal-FITC and R.t.-FITC. Fluorescent microscopy indicated the following. With EI-1 and EI-2, most of the cells were destroyed, cellular debris fluoresces, as do the remaining cells (Figure 32). There was no difference between cellular labeling with Sal-FITC and R.t.-FITC. Both fluorescent labels were found in the interior of the cells. For EI-3 and PMB, no cell lysis occurred. In these cases, Sal-FITC moved across the plasma membrane, while R.t.-FITC stayed on the cell surface.

Effect of EIs on Labeling: EIs Used in Binding Assays

We wanted to ascertain the ability of the EIs to prevent the binding of fluorescently labeled LPS to cells from the cell line RAW264.7. The EIs were mixed with the labeled LPSs and left in the cold for 30 min. before having contact with the cells. In the LAL assay system, the inhibitors were used at a concentration of 10^{4} ng per assay, compared to *E. coli* LPS which was used at a concentration of 10^{-3} ng per assay. Due to this large difference in concentration between endotoxin and inhibitor, and the

concentration of LPS-dye conjugate that must be used in order to view it under the microscope, we chose to use EIs at concentrations of 10x, 0.1x, and the same concentration, x, as endotoxin. What we observed can be described as follows.

EI-1: 10x EI-1 caused increased fluorescence in both the *Salmonella* and *R. trifolii* LPS treated cells (Figure 33). *R. trifolii* moved into the cell interior. At x EI-1, there was no change in degree of fluorescence for either LPS type. At 0.1x EI-1 there was a small decrease in fluorescence for *Rhizobium* and no change for *Salmonella* .

EI-2: 10x EI-2 caused increased fluorescence for both LPS types. EI-2 used at the same concentration as LPS had no effect. 0.1x EI-2 appeared to decrease fluorescence in both *Salmonella* and *Rhizobium* LPS-treated cells.

EI-3: At 10x, x, and 0.1x EI-3 appeared to decrease the degree of fluorescence for both *Salmonella* and *Rhizobium* as compared to normally labeled cells. There appeared to be no difference between the degree of fluorescence in all LPS solutions tested.

EI-4: EI-4 did not change the degree of fluorescence for *Salmonella* at any concentration. 10x EI-4 gave a slight increase in rhizobial fluorescence. EI-4 at the other two concentrations for *Rhizobium* had no effect.

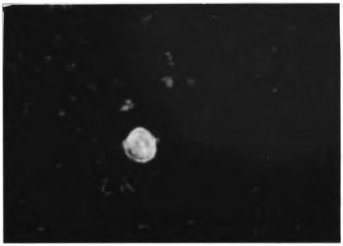
PMB: At 10x and x PMB there was an increase in the degree of fluorescence for *Salmonella* and at 0.1x it decreased. *R. trifolii* fluorescence decreased at x and 0.1x concentrations. 10x showed no difference.

Figure 32(A)

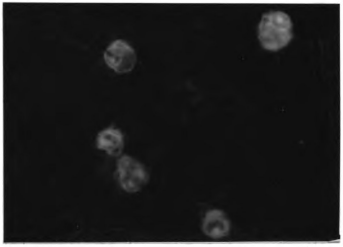
RAW264.7 cells grown in the presence of EI-1, followed by labeling with Sal-FITC.

Figure 32 (B)

RAW264.7 cells grown in the presence of EI-1, followed by labeling with R.t.-FITC. Notice the location of the R.t.-FITC conjugate.



32A



32B

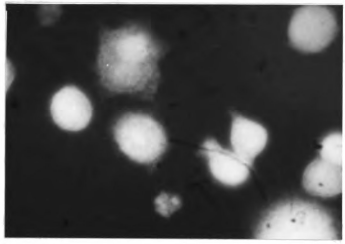
Figure 32

Figure 33(A)

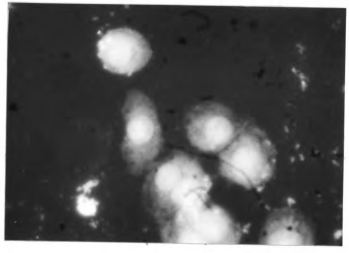
RAW264.7 cells labeled with a mixture of Sal-FITC and 10x EI-1.

Figure 33(B)

RAW264.7 cells labeled with a mixture of R.t.-FITC and 10x EI-1.



33A



33B

Figure 33

Discussion and Conclusions

From the binding of the fluorescent LPS conjugates to various mammalian cells I summarize the following observations. The two LPSs, rhizobial and enteric, bind in different fashions to all cells tested. The *Salmonella* LPS was capable of crossing the plasma membranes of all cell lines studied and labeled the exterior surface as well as being localized in discrete vesicles found throughout the cytosol (Figure 34). These vesicles were localized heavily around the nucleus, and the dye was excluded from the nucleus. Our results agree with those of another group which labeled RAW264.7 cells with an FITC-LPS conjugate (*S. minnesota*) (59). They also report FITC-LPS to be taken up by each individual cell and be localized in the perinuclear regions of the cells. When they focused through their cells they found fluorescence to be associated with intracellular vacuoles and it also did not cover the nuclear regions. LPS from *R. trifolii* behaved differently to the enteric LPS in all cell lines studied. The rhizobial LPS bound to the surface of cells only. Perhaps possession of the 28 carbon, long chain fatty acid by *R. trifolii* LPS is responsible for preventing the LPS from crossing the plasma membrane and getting into the interior of the cell.

The use of unlabeled LPS to prevent binding of the fluorescent conjugates produced two interesting results. Firstly, the *Salmonella* LPS was not disrupting the membrane by making it leaky so that more LPS gets in. This was based on the observation that cells prelabeled with *Salmonella* LPS still label *R. trifolii* LPS only on the cell surface. If *Salmonella* LPS disrupted the membrane, one would expect the follow-up label with R.t.-FITC to find its way into the interior of the cell. Trypan blue exclusion also showed the cells to be intact.

Secondly, unlabeled *Salmonella* and *Rhizobium* LPSs were unable to prevent binding of either labeled conjugate. These results support those of Zimmerman (143), who could not demonstrate saturation of binding on murine leukocytes. Morrison reports of the "lack of a saturable limit" to the total amount of LPS which can be associated with

Figure 34

RAW264.7 cells labeled with Sal-Rho. **Notice the vesicles of LPS-dye conjugate found inside the cell.**

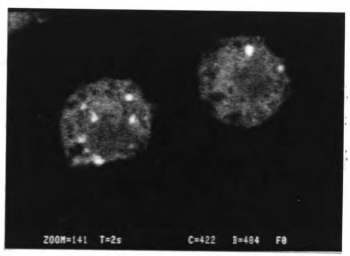


Figure34

a cell. This statement was based upon results of different investigators (55).

These results, and the fact that we could observe LPS binding on different cell types, point to the lack of a specific LPS receptor. It is highly unlikely that all three cell types we tested (plus all cells LPS has been shown to bind in the literature) would possess a receptor specific for LPS and that it would be identical in all cells. Our results suggest that endotoxin will intercalate into the mammalian cell plasma membrane by hydrophobic interaction in a variety of cell lines. Endotoxin is known to interact with phospholipid, protein, glycoprotein, and lipoprotein, all of which are found on the surface of mammalian cells, and all of which can be polar molecules containing net positive charges. With LPS being a charged species, electrostatic interaction between LPS and the surface of the cell is also important in order for LPS to get near the surface of the cell so that lipid A can intercalate. Our results may very well be influenced by the concentrations of LPS which we must use in order to visualize binding. We are working at concentrations which are much higher than would be encountered under physiological conditions. If an LPS receptor were present, it would indeed be saturated and the excess labeling would occur by fusion. Again, we stress that it is unlikely that a receptor for LPS would be found on many cell types and be identical. As was mentioned in the literature review (73,74,77,78,81,82,83,84,88,89,90), many proteins/receptors have been isolated that bind LPS, yet have different protein sequences and molecular weights. For such a molecule to have so many different receptors on different cell types is rather far-fetched. More likely it is the ability of these proteins to adsorb the LPS aggregate onto the cells' surfaces, followed by the disaggregation of LPS monomers and fusion of the lipid A moiety with the plasma membrane.

Binding to the Chinese hamster ovary cell line demonstrated that LPS has the ability to bind many cell types which may not appear to be activated by LPS. This suggests that binding alone is not sufficient to initiate activation, but is only the initial step of the activation process. However, we cannot fairly state that binding to CHO-K1

cells, or any other type of non-immunological cell results in zero activation. It is possible that these cells are activated to proceed with functions that follow once these cells become stimulated. They just are not a part of the endotoxin cascade. More importantly, the results of *R. trifolii* ANU843 LPS binding and the biological activities we tested for demonstrated that binding alone is not sufficient to initiate activation. We will discuss this in more detail later.

What can we conclude about the endotoxin inhibitors and how does this correlate with the mechanism of endotoxin action? The endotoxin literature cites LPS binding as the initial step towards endotoxin activation of cells. Binding is followed by a signal which causes the cell to become activated. There have been suggestions that endotoxin affects the fluidity of target cells and it is this membrane perturbation that activates the cell. Studies have shown that lipid A can increase the order of the lipid bilayer (59,65,67,68).

Our results involving endotoxin inhibitors suggest that some of them act by increasing membrane fluidity. EI-1 and EI-2 act by making the cell membranes more fluid. In other words, they are disruptive to cell membranes. This is supported by the following observations concerning EI-1 and EI-2. EI-1 was sent to XOMA Corp. to determine how well it prevented TNF release by macrophage. During this study it was determined that EI-1 lysed the cells. These experiments were performed at a concentration of EI-1 similar to that used in the LAL assay. Both EI-1 and EI-2 killed cell line RAW264.7 cells when the cells were grown in their presence at concentrations equal to those used in the LAL assay. EI-3, EI-4, and polymyxin B had no effect at similar concentrations. Cells labeled in the presence of EI-1 and EI-2 had increased dye uptake at 10x their concentrations. Normal concentrations of EI-1 and EI-2 had little effect on prevention of binding. At one tenth their concentrations there was decreased binding. This suggests there is some interaction between LPS and the EIs that makes less LPS available for binding, yet at higher concentrations the increase in membrane fluidity

overrides any prevention of binding based on interaction between the inhibitor and LPS. EI-3 lacks a hydrophobic moiety and appears to be the best suited for binding prevention. There was interaction between LPS and EI-3 which prevented LPS interaction with the cell membrane. Since EI-3 lacks hydrophobicity, one does not see the increase in fluorescence seen with the other inhibitors. EI-3 does not inhibit LAL, yet its inherent color is responsible for making it test falsely positive.

The results of the LAL assay involving the EIs shed some light onto the mode of endotoxin action. It is known that membranes must be present within the lysate in order for the LAL assay to function correctly. If membranes are lacking from the lysate, the positive results cannot be had. If EI-1 and EI-2 function by increasing membrane fluidity, then they will be capable of disturbing the integrity of the membrane component of the lysate. If endotoxins function by making the membrane more rigid, then activity will not be detected if the membrane was fluid due to EI effects.

The critical test involved EI-4. EI-4 is similar to EI-3 in structure, except for the octyl chains on EI-4. At low concentrations EI-4 does not inhibit, but if EI-4 concentration was raised to a higher value (10^5 to 10^6), then EI-4 was capable of inhibition. We believe the inhibitors act by making the lysate membrane so fluid that the endotoxin can no longer increase the packing order of the bilayer and hence no activation of the cascade occurs.

The central dogma of the endotoxin literature is the structural requirement necessary for endotoxic activity. Great importance has been placed on the diphosphorylated, glucosamine disaccharide with its fatty acid chains of specific length. Many studies have been devoted to correlating a specific biological activity with a specific structure, yet no one has been able to successfully accomplish this.

R. trifolii ANU843 has now been shown to possess a lipid A structure which deviates from the classical endotoxins. It possesses a lipid A composed of sugars other than glucosamine, lacks phosphate, and contains unusual fatty acids, yet it is still capable

of inducing some of the classical endotoxin effects. Obviously the classical endotoxin structure is not necessary. A structure which has a similar 3-dimensional geometry and charge distribution which can bind a membrane and induce the same effect on that membrane, such as decreasing membrane fluidity, will show the same net result.

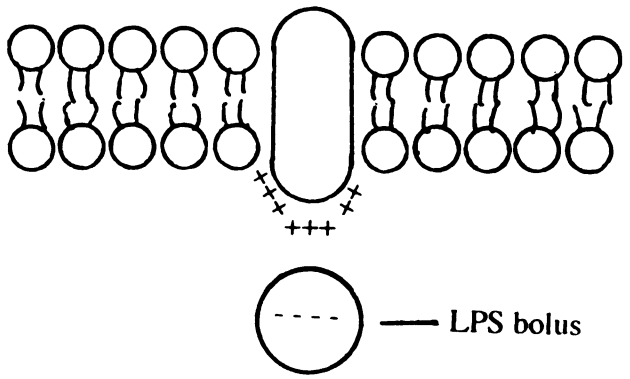
Our results show that *R. trifolii* LPS is not toxic compared to that of the enterics. It was able to induce fever and a local Schwartzmann reaction at concentrations much higher than the classical LPS. It will cause mice to become transiently lethargic, but it will not kill them as *Salmonella* LPS does. We believe these differences in endotoxic response to be attributed to the fact that *R. trifolii* LPS does not induce cytokine production. It has been suggested that TNF and IL-1 are the species responsible for the toxic effects of endotoxins (144,145). Since *R. trifolii* LPS does not readily induce TNF and IL-1, it therefore does not cause the detrimental effects of endotoxin.

Why does *R. trifolii* LPS induce some responses and not others? Could it be that different cell types respond to LPS in different manners and therefore one cannot make comparisons between cell types? This is an example where binding alone is not sufficient to induce a cellular response. We believe that the lipid A must intercalate into the plasma membrane and this results in decreased membrane fluidity. For a mitogenic response to occur, maybe the LPS does not have to insert into the membrane as it does for protein synthesis to occur, and this is why we see proliferation, but not synthesis of IgM, TNF, or interleukins, which are ultimately responsible for the toxic effects of endotoxin such as fever response and lethal toxicity.

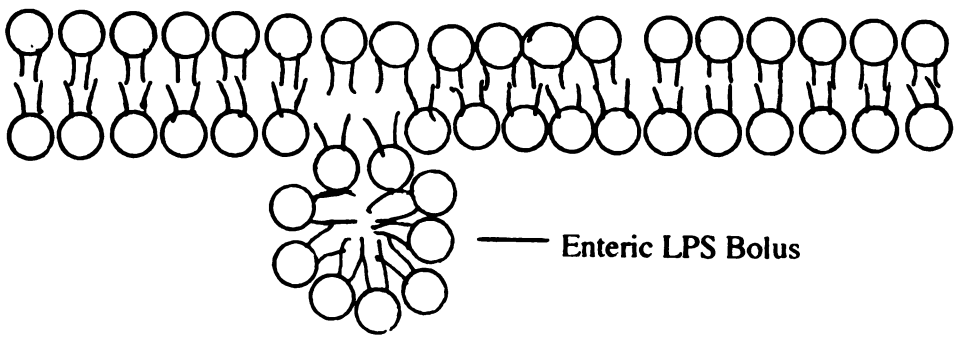
Our proposed mechanism for endotoxic activity is described as follows. In the 1960's, Shands proposed a model for LPS insertion into the lipid bilayer which involved end attachment of the LPS aggregate. This resulted in disaggregation which allowed individual LPS monomers to insert into the bilayer (Figure 35). We propose that a mechanism similar to the Shands model is indeed what occurs. The negative charge of the LPS aggregate is attracted to the cell surface by net positive charges present on

Figure 35

Attachment of an LPS aggregate to a lipid bilayer, adapted from Shands. (Adapted from "Bacterial Toxins and Cell Membranes" 1978, Academic Press.)
35(A) Disaggregation of an LPS bolus, followed by insertion of LPS monomers into the target cell bilayer.
35B) Disaggregation of the *R. trifolii* LPS bolus, followed by monomer insertion. Notice the position of the 27-hydroxyoctacosanoic acid.



35A



35B

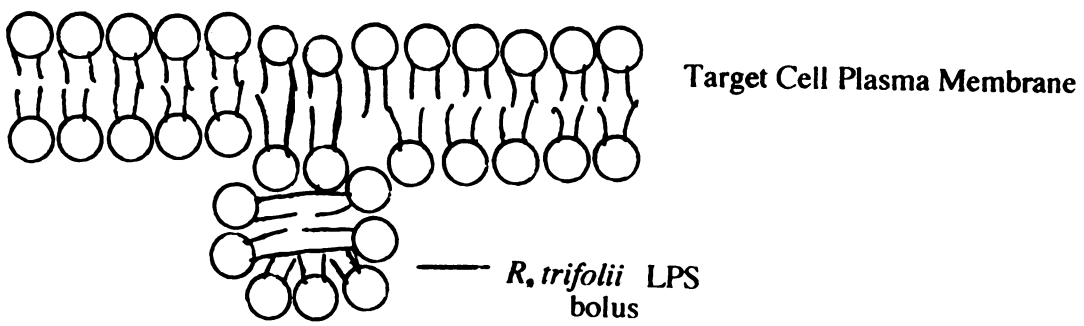


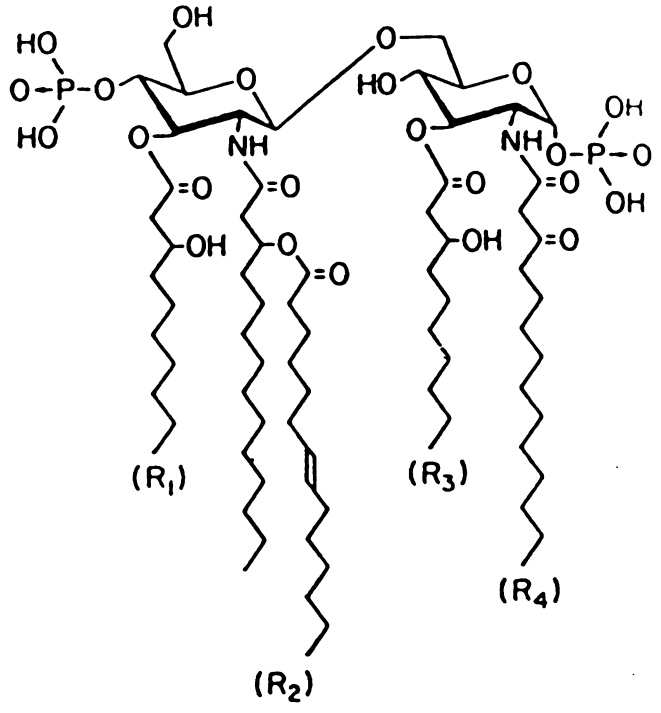
Figure 35

species such as protein. This first step is independent of temperature and can be reversible. The second step involves disruption of the LPS aggregate so that LPS monomers can insert into the lipid bilayer. This second step is irreversible and temperature dependent. Now that the lipid A is in the membrane, a membrane perturbation occurs which results in an increase of the microviscosity of the bilayer and thus causes an increase in the order of the lipid bilayer (59,65,67,68). In other words, that area of the membrane becomes less fluid, more rigid. This is the signal which is triggered causing the cell to become activated.

Other information supporting this model comes from the lipid A structure of the non-toxic, devoid of biological activity, *Rhodopseudomonas sphaeroides* ATCC 17023 LPS (45). This molecule possesses a glucosamine disaccharide backbone, contains phosphate at positions C1 and C4', and possesses amide and ester linked fatty acids with lengths of 10-14 carbon atoms. This molecule possesses all of the classical endotoxin "hardware", but it is totally devoid of biological activity. If you examine its structure in Figure 36, you will notice it has 2 unusual fatty acids : 3-oxotetradecanoic acid and a 7-tetradecenoic acid (50).

R. sphaeroides ATCC 17023 is known to bind to eukaryotic cells (51), but it does not stimulate them. (Here is another example where binding alone is not sufficient to induce biological activity.) *R. sphaeroides* possesses fatty acids containing double bonds. Degrees of unsaturation are known to make membranes more fluid. We propose that when the *R. sphaeroides* lipid A fuses with the plasma membrane, its unsaturated fatty acids make that area of the membrane more fluid and thus the signal for activation does not occur.

Our labeling studies show that a longer time must pass before *R. trifolii* LPS can be detected visually on the surface of target cells. *Salmonella* LPS binding can be visualized at 15 min., whereas *R. trifolii* appears at 2-3 hrs. We know that *R. trifolii* has a C28 fatty acid which has been shown to traverse a cell membrane by results from



R. sphaeroides Lipid A

Figure 36

The lipid A of *R. sphaeroides* ATCC 17023, adapted from Qureshi, et. al. (50).

electron microscopy of freeze fractured membranes. It would be more difficult for an *R. trifolii* LPS molecule to pull out of its LPS aggregate and insert into the target cell membrane, for the C28 fatty acid must be ripped out of the bolus and then inserted all the way through the plasma membrane (Figure 35B). A *Salmonella* lipid A, lacking any transmembrane fatty acids, could more easily move from LPS aggregate to insertion into the plasma membrane bilayer. This would explain why the *R. trifolii* LPS labels more slowly. From our chemical analyses we know that the *R. trifolii* LPS forms very stable aggregates which are difficult to disperse. This idea also points to the difficulty of release of the LPS from the aggregate followed by membrane insertion.

Results for TNF and interleukin production indicate that a higher concentration of rhizobial LPS is needed to induce secretion. Since rhizobial LPS does not label as readily as enteric LPS , it makes sense that by increasing the concentration of rhizobial LPS you increase the chance that more of it will bind and thus cause the biological effects.

Once the LPS has bound, it is possible that the *Salmonella* LPS causes a membrane fluidity change that is different from that of the change caused by *R. trifolii* LPS binding. Possibly the rhizobial LPS does not change membrane fluidity to the extent that enteric LPS does. This would explain the lack of subsequent biological response.

From our labeling studies we see that *Salmonella* LPS is endocytosed and finds its way into the cell interior. Is this important for activation? The concentration of rhizobial LPS necessary to stimulate TNF and interleukin synthesis is much less than the concentration used in labeling. At the higher concentration rhizobial LPS is still present on the surface of the membrane and thus it appears that the ability to transverse the membrane is not important. The exception to this would be if a small amount of *R. trifolii* LPS were able to cross the membrane, but at such a small concentration that its fluorescence could not be detected. This scenario would not fit the membrane fluidity model.

Our data does not allow us to define the intracellular processes which are directly

triggered by the attachment of endotoxin to the target cell. This ultimately stimulates the various metabolic events, such as de novo synthesis and secretion of cytokines, leading to the condition known as an endotoxic crisis. We hope that our studies shed some light onto the mechanism of endotoxin action.

There are aspects of the project that need to be further investigated. We were able to isolate and characterize a minor component of the lipid A of *R. trifolii* ANU843 LPS. As stated earlier, TLC separation of the rhizobial crude lipid A yields several components. The other components need to be isolated and characterized. Most likely a disaccharide will be found. I suggest that the methods used to fractionate the minor component be used again, with the exception of using larger amounts of LPS. It is quite easy to grow cells on a large scale and then isolate the LPS. HPLC can be used to separate the components as it was used before. This would again be followed by further fractionation using preparatory TLC if necessary. We had great difficulty in chemically manipulating *R. trifolii* LPS. Researchers in our lab have since developed a method to deacylate both ester- and amide-linked fatty acids. This method utilizes NaOH and thiophenol and has been successful in removing all acyl groups from the 2011 strain of *Rhizobium meliloti* . *R. meliloti* also contains the 27-hydroxyoctacosanoic acid, which you will remember, was difficult to cleave from *R. trifolii* LPS. This method of deacylation should work on *R. trifolii* LPS and should definitely be tried.

Once the lipid A backbone has been completely freed of acyl groups, then the sugar can be subjected to linkage analysis by using methylation procedures followed by acetylation. Further involved chemistry should be performed, however these ideas are a starting point.

As far as further work on the biological aspects are concerned, several things can be done. I believe that the biological assays should also be performed with other species of *Rhizobium* such as *R. meliloti* , which is also known to possess the 27-OH, C28 fatty acid and uses sulfate as its charged species instead of phosphate, or carboxylate in the

case of *R. trifolii* LPS. It would be interesting to determine if *R. meliloti* LPS behaves the same way in the binding assays as *R. trifolii* LPS. I would suspect that it does knowing about its long chain fatty acid and the fact that preliminary work shows it to have reduced activity like *R. trifolii* LPS in the TNF assay.

The question concerning decrease in membrane fluidity should be addressed. We should determine whether *R. trifolii* LPS has the same effect as *Salmonella* LPS in changing the membrane viscosity of artificial membranes composed of phospholipid. A probe such as the one used in the Jacobs' experiment (68) could be used to measure changes in membrane fluidity. It would also be interesting to get *R. sphaeroides* LPS and test this one in parallel with the other LPSs.

It would be interesting to determine if all "usual" lipid As get incorporated into vesicles and taken into the cell as measured by fluorescence microscopy. What about an LPS like *R. sphaeroides* which has a lipid A similar to the classical lipid A, but lacks its activity? Further work should be done to determine if LPS uptake is important. Must it get into the cell and just how does it get in? Maybe LPS induces the cell to phagocytize it. Studies have shown that *Shigella* is capable of doing this by mobilizing actin filaments (146), and *Shigella*, being a gram-negative organism, has LPS at its surface. Could treating cells with substances that prevent actin from polymerizing, such as cytochalasins, prevent LPS uptake, while still keeping LPS bound at the cell surface?

One could also use radiolabeled LPS more effectively than the fluorescent conjugates. Using a ^{3}H-labeled *Salmonella* LPS, one could label the cells and then try to extract the LPS out again. As we tried with Sal-Rho, one could separate the LPS based on size and compare this with ^{3}H-*Salmonella* LPS that was not used to label. Hopefully this would give information regarding whether or not the LPS needed to be cleaved in order to get inside of the cell.

Since *R. meliloti* LPS contains sulfate, it can be ^{35}S-labeled and used in binding assays at concentrations which are physiological instead of at the higher concentrations

needed to be visualized by the fluorescence microscope. One could perform competition binding assays at more reasonable concentrations to determine if binding is really saturable.

As we said before, much work has gone into studying the structures of endotoxins and their mechanisms of action since Pfeiffer first discovered them. Much effort will go into studying these unique molecules in the future.

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